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Masters Theses

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Identification of the Putative Plant-functional Homolog of Double-stranded RNA-dependent Protein Kinase R

Elaine Chan

Abstract

Viruses constantly challenge plants; in response, plants have evolved a number of defense systems that protect them or reduce the severity of the virus invasion. Translational inhibition, an innate antiviral defense mechanism that is utilised by, and most studied in, mammals, is proposed also to exist in plants. This mechanism is activated by the presence of double-stranded (ds) RNA, a necessary replication intermediate of RNA viruses, and relies on the phosphorylation of the α subunit of the eukaryotic initiation factor (eIF) 2 by the dsRNA-dependent protein kinase R (PKR). Phosphorylation of eIF2α results in the global inhibition of translational initiation in the host cell, and subsequently blocks the infection of the virus.

Initial evidence supports the presence of a plant-functional homolog of PKR (pPKR), but no homologous sequence has so far been detected. The aim of this doctoral research was to identify pPKR via a proteomics approach using Arabidopsis thaliana, whereby the protein was first enriched as determined by the correlation of its activity, and then sequenced. The identification of pPKR would provide evidence for the presence of a novel antiviral defense mechanism in plants.

The immobilised eIF2α peptide assay was developed, refined, and validated to allow the rapid detection and quantification of eIF2α phosphorylation activity in vitro. Flower buds from young Arabidopsis plants following light exposure (4 hr) had the highest eIF2α phosphorylation activity and ds but not single-stranded RNA activated this pPKR activity. Of the six different techniques used to enrich for pPKR from Arabidopsis, size exclusion chromatography was the most consistent. Mass spectrometry analysis of proteins associated with kinase activity identified a number of RNA binding proteins and protein kinases, including calcium-dependent protein kinases (CPKs). The assessment of ions and chelators on putative pPKR activity revealed that eIF2α phosphorylation was activated in the presence of both calcium and the chelator ethylene glycol tetraacetic acid (EGTA).

Arabidopsis T-DNA insertion lines (15 of the 16 Arabidopsis dsRNA binding motif-containing genes, all 34 Arabidopsis CPKs, a kinase of unknown function, the eIF2α kinase GCN2, and the proposed plant-functional homolog of the mammalian PKR inhibitor p58IPK) were imported, and where possible, crossed to homozygosity and tested for eIF2α phosphorylation activity. Attempts were made to generate and test the eIF2α phosphorylation activity in nine transgenic Arabidopsis lines overexpressing either mammalian PKR, an inhibitor of PKR, or a disrupted eIF2α. Of the 20 transgenic plant lines that could be tested, a statistically significant decrease in eIF2α phosphorylation activity was seen in the CPK19 T-DNA insertion line and p58IPK overexpression line. These two plants also had similarly stunted phenotypes. These findings provide evidence for CPK19 as the putative plant-functional homolog of PKR.
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Abbreviations

SI (Le Système International d’Unités) abbreviations are used for units, chemicals, elements and formula. Other abbreviations used in the text are listed below.

~ approximately
αP α phosphate
aa amino acids
A ampere
A
 absorbance at x nm
ABRC Arabidopsis Biological Resource Centre
Arabidopsis Arabidopsis thaliana
ATP adenosine 5’-triphosphate
avr avirulence
BLAST Basic Alignment Search Tool
BN PAGE Blue Native Polyacrylamide Gel Electrophoresis
bp base pair
BSA bovine serum albumin
CaM-LD calmodulin-like domain
cDNA complementary DNA
CPK calcium-dependent protein kinase
CPM counts per minute
ΔCn delta correlation
Da dalton
DEAE diethylaminoethyl
DNA deoxyribonucleic acid
dNTPs 2’-deoxynucleotide 5’-triphosphate
ds double-stranded
dsRBD dsRNA binding domain
dsRBM dsRNA binding motif
dsRBM1 first dsRBM of mPKR
dsRBM2 second dsRBM of mPKR
DTT dithiothreitol
EDTA ethylene diamine tetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>NSSA</td>
<td>Hepatitis C virus-encoded non-structural 5A protein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>ocs3’</td>
<td>octopine synthase gene</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAPIA</td>
<td>Parallel Protein Information Analysis system</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS/0.1% (v/v) Tween 20</td>
</tr>
<tr>
<td>PC2</td>
<td>Physical Containment Level 2</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PERK</td>
<td>RNA-regulated protein kinase-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-1,4-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PKR</td>
<td>dsRNA-dependent protein kinase R</td>
</tr>
<tr>
<td>PKZ</td>
<td>fish PKR</td>
</tr>
<tr>
<td>Plant and Food Research</td>
<td>The New Zealand Institute for Plant and Food Research Ltd</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>pPKR</td>
<td>plant PKR</td>
</tr>
<tr>
<td>p.s.i.</td>
<td>pound-force per square inch</td>
</tr>
<tr>
<td>PSTP</td>
<td>pentasodium triphosphate</td>
</tr>
<tr>
<td>PTGS</td>
<td>post-transcriptional gene silencing</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>Q</td>
<td>quaternary ammonium</td>
</tr>
<tr>
<td>Q HP</td>
<td>HiTrap™ Q Sepharose HP</td>
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<tr>
<td>R</td>
<td>resistance</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>S</td>
<td>methyl sulphonate</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dedocyl sulfate</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>sodium dedocyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SMS</td>
<td>Sequence Manipulation Suite</td>
</tr>
<tr>
<td>Sp</td>
<td>preliminary raw score</td>
</tr>
<tr>
<td>SP</td>
<td>sulphopropyl</td>
</tr>
<tr>
<td>Term</td>
<td>Definition/Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>SP XL</td>
<td>HiTrap™ SP Sepharose XL</td>
</tr>
<tr>
<td>ss</td>
<td>single-stranded</td>
</tr>
<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>first generation line</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;</td>
<td>fourth generation line</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
</tr>
<tr>
<td>TAR</td>
<td>transactivator responsive region</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCV</td>
<td><em>Turnip crinkle virus</em></td>
</tr>
<tr>
<td>T-DNA</td>
<td>transferred DNA</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMV</td>
<td><em>Tobacco mosaic virus</em></td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TuMV</td>
<td><em>Turnip mosaic virus</em></td>
</tr>
<tr>
<td>TYMV</td>
<td><em>Turnip yellow mosaic virus</em></td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>Xcorr</td>
<td>raw cross correlation</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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1

Introduction

1.1 Viral Infection and Host Defense Mechanisms

Viruses are obligate intracellular parasites that invade host cells to replicate and spread their infection. They do not multiply through cell division, but instead, utilise the infected host as a ‘factory’ to produce thousands more copies of themselves. Viruses do not encode their own metabolic machinery, and therefore exploit the energy supply, biochemical compounds, and protein synthesis machinery of the host (Hull, 2002). Accordingly, the host defense system for any virus depends on its replication strategy and in many instances, acts by targeting processes such as the translation mechanism that is commonly used by all viruses.

Virus particles carry only a minimal amount of genetic material within their viral capsid. Viral genomes may comprise either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), and may be double-stranded (ds) or single-stranded (ss) (Hull, 2002). In turn, the form that the encapsidated genetic material takes determines how the virus executes its replication process. DNA viruses replicate in the host cell along with the host DNA, but two different replication processes are seen for viruses with RNA genomes. For retroviruses, the viral-encoded reverse transcriptase enzyme is employed to synthesise a complementary DNA strand of their RNA genome. The DNA molecule,
rather than RNA, is then further replicated using the host resources. The other replication process for RNA viruses involves another virus-derived enzyme known as RNA-dependent RNA polymerase (RdRp) (Thivierge et al., 2005). This enzyme initially makes a direct copy of the RNA genome to form long dsRNA intermediates. Each strand of these dsRNA replicative forms can be used to generate further copies of RNA. The non-coding strand is used as a template by RdRp to generate positive sense RNAs or subgenomic RNAs from which the encoded proteins can be translated. Subsequently, during infection, the RNA genetic material is either repacked into a virion or into a ribonucleoprotein movement complex that moves throughout the host. New virions can also be transmitted to new hosts to initiate the cycle again.

The interactions that occur between viruses and potential hosts are very complex (reviewed in Soosaar, Burch-Smith, & Dinesh-Kumar, 2005). In the plant-virus interaction, not all plants are able to be infected; a given virus may only infect a selection of plant species. If the pathogen fails to infect, then the plant is described as a non-host. On the other hand, when the virus can infect and replicate in the plant species, the plant is said to be a host for that particular virus. If the infecting virus can also produce disease in the host, the virus is termed virulent and the plant is susceptible, and this interaction between the virus and plant is known as compatible (Soosaar et al., 2005). Even in such a compatible interaction, upon infection, the long dsRNA replication intermediates of viruses are recognised by the antiviral system of the plant and a series of signals are activated which prompts the plant to defend itself against the viral infection. Conversely, plant viruses counter this antiviral activity with the expression of suppressors of silencing and other inhibitors of the plant antiviral defense that have been less well characterised (Alvarado & Scholthof, 2009; Bilgin, Liu, Schiff, & Dinesh-Kumar, 2003). The complex interaction and competition between plant or mammalian hosts and viruses, in particular the host defense pathways and their counteraction by viruses, is further discussed in the following sections.

1.1.1 Antiviral Defense Systems in Plants and/or Mammals

Host organisms require a means to recognise the variety and complexity of viruses in order to successfully defend themselves from this pathogen invasion. To date, a number of antiviral systems have been identified in both plants and mammals and these exploit a broad range of mechanisms that ensure the majority of viruses are unable to establish an infection in the organism or to reduce the impact of a successful virus infection. Two main antiviral strategies have thus far been identified in plants and these include the expression of plant disease resistance $R$ genes.
(Section 1.1.1.1) and the post-transcriptional gene silencing (PTGS) mechanism (Section 1.1.1.2).

There is, however, a further antiviral defense strategy that has so far only been well characterised in mammals and is known as translational inhibition (Section 1.1.1.3).

Virus infection can have a significant impact on the value and volume from primary production as plant disease can dramatically reduce crop yield as well as reduce the quality and appearance of fruits and vegetation. This highlights the importance of understanding the mechanism of innate virus resistance or tolerance that exists in plants. In turn, this knowledge will enable us to develop novel tools to protect plants against virus infections or to reduce the impact of viruses on infected plants.

1.1.1.1 Plant Disease Resistance R Genes

Plants do not have the benefit of a circulating antibody system like mammals, but they conduct autonomous detection of pathogen invasion by the expression of a large array of resistance (R) genes. These plant-encoded disease resistance gene products interact directly or indirectly with their pathogen-derived counterpart avirulence (avr) genes and this recognition acts as the main stimulus for the activation of defense responses in plants (reviewed in Hull, 2002; McDowell & Woffenden, 2003). The direct interaction between plant R proteins and pathogen avr proteins depicts the classical receptor-elicitor hypothesis. However, R proteins can alternatively act as a guard to detect the presence of modifications of certain host proteins. This model is known as the guard hypothesis whereby the targeting actions by avr proteins to the guardee notify the R protein to trigger signals within the host in response to pathogen infection (Jones & Dangl, 2006).

Upon recognition of virus infection, R genes activate the hypersensitive response in the host and this is recognised as a rapid and localised induction of cell death in response to pathogen invasion, represented by the presence of brown, necrotic cells at the site of infection (reviewed in Hayward, Tsao, & Dinesh-Kumar, 2009; Heath, 2000; Hull, 2002; Mur, Kenton, Lloyd, Ougham, & Prats, 2008; Pontier, Balague, & Roby, 1998). Effectively, a complex signal transduction cascade is activated, resulting in biochemical and physiological changes in the cell that include protein phosphorylation, production of reactive oxygen species, modification of ion fluxes, and ultimately an induction of programmed cell death in the vicinity of the infection sites. The cellular machinery is hence unavailable to the virus which therefore cannot replicate and is effectively barred from
spreading. This localised induction of apoptosis is one of the key mechanisms that plants utilise to protect themselves from pathogen attack.

Following the induction of the hypersensitive response, plants are observed to acquire a systemic resistance towards that particular pathogen (Pontier et al., 1998). In addition, a secondary systemic resistance mechanism is activated and the plant becomes resistant to a much broader range of pathogens. This widely observed phenomenon with a lack of specificity in resistance is known as systemic acquired resistance (Vlot, Klessig, & Park, 2008).

1.1.1.2 Post-transcriptional Gene Silencing

Another adaptive response, known as PTGS, enables the host to recognise viruses at the RNA level (Lindbo & Dougherty, 1992). This powerful system of protection, discovered initially in plants, involves the post-transcriptional control of gene expression and directs the sequence-specific degradation of target viruses using RNA molecules of 21 – 25 nucleotides (nt) as guides (Baulcombe, 2004).

In plants, the activation of PTGS during viral infection is mediated by the presence of long dsRNA, such as virus replication intermediates or highly structured viral ssRNAs (reviewed in Baulcombe, 2004; Ding, Li, Lu, Li, & Li, 2004). These RNA molecules are recognised by host mechanisms and a Dicer-like enzyme DCL4 or DCL2 (Waterhouse & Fusaro, 2006) cleaves the dsRNA into short interfering RNA (siRNA) of 21 or 22 nt, respectively (Fig. 1.1). These siRNAs are arranged in a staggered duplex and have two unpaired nt at the ends. Of these staggered duplexes, one strand is relatively lower in thermodynamic stability than the other strand and is selectively incorporated into a ribonucleoprotein comprising of RNA helicase and other proteins to form a RNA-induced silencing complex (RISC) (Khvorova, Reynolds, & Jayasena, 2003). The incorporated RNA strand then acts as a ‘guide RNA’ and directs RISC to the cognate messenger RNA (mRNA) transcript, i.e., the viral mRNA or genomic RNA in the case of virus-derived siRNAs, or for cell-encoded microRNA to a cognate mRNA. Subsequently, the Argonaute 1 enzyme within RISC mediates endonucleolytic cleavage in the target RNA and exonucleolytic activity degrades the cleaved RNA. The bound RNA strand complexed to RISC then participates in more cycles of specific RNA degradation and greatly reduces the level of the viral RNA. Consequently, silencing of the targeted genes is achieved and this effectively reduces the amount
of viral RNA that can be translated to produce proteins necessary for replication and/or encapsidation.

**Figure 1.1 Post-transcriptional gene silencing.** The presence of dsRNA is recognised by the Dicer enzyme which cleaves it into short interfering RNAs (siRNAs). The siRNAs then interact with the RNA helicase to form the RNA-induced silencing complex (RISC) and guides the complex to the target RNA for endonucleolytic cleavage and exonucleolytic degradation. (Saleh, Van Rij, & Andino, 2004)

### 1.1.1.3 Translational Inhibition

An efficient antiviral defense system which acts at the translational level has been detected in mammals and relies on the reversible phosphorylation of the heterotrimeric GTPase eukaryotic initiation factor (eIF) 2 at the α subunit. Upon phosphorylation, the functional activity of eIF2 is inhibited and the initiation of translation of nearly all cellular and viral mRNAs is limited (C. de Haro, R. Mendez, & J. Santoyo, 1996).

The process of translation is divided into three phases – initiation, elongation and termination (reviewed in Gebauer & Hentze, 2004; Marintchev & Wagner, 2004; Preiss & M, 2003; Proud, 2005). These events are each controlled by a defined set of factors but the most complex of these events is the initiation phase which is assisted by at least 12 eukaryotic factors. The initiation of translation as well as the function and involvement of eIF2α in the control of this process are discussed in the following sections. The more extensively characterised mammalian translational system is described first (Sections 1.1.1.3.1 and 1.1.1.3.2), followed by a comparison of what is currently known in the plant system (Sections 1.1.1.3.3 and 1.1.1.3.4).
1.1.1.3.1 Translation Initiation in Mammals

As depicted in Figure 1.2, translation initiation in mammals begins with the formation of a ternary complex in which a Met-loaded initiator transfer RNA (tRNA) binds to a guanosine 5'-triphosphate (GTP)-coupled eIF2. In turn, this combines with the small 40S ribosomal subunit and eIFs 1, 1A, 3, and 5 to yield a 43S pre-initiation complex which then binds to cellular mRNA. This binding to the mRNA is assisted through a bridging interaction between eIF3 and the cap-binding

![Image of translation initiation in mammals]

**Figure 1.2 The initiation of translation.** Eukaryotic initiation factors (eIFs) are depicted as coloured, numbered shapes. Briefly, the Met-loaded initiator transfer RNA binds to GTP-coupled eIF2 to form the ternary complex. This interacts with the 40S ribosome subunit and forms the 43S pre-initiation complex with eIFs 1, 1A, 3, and 5. This complex recognises the mRNA (black line) and forms a circularised mRNA via interactions with the eIF4F complex and poly(A)-binding protein (PABP). The 43S pre-initiation complex then scans the mRNA in a 5' to 3' direction and binds to the start codon (AUG) to form the 48S initiation complex. The 60S ribosomal subunit joins and GTP hydrolysis is triggered on eIFs 2 and 5B. Subsequently, the eIFs dissociate off and polypeptide synthesis begins. See text for more details. (Gebauer & Hentze, 2004)
eIF4F complex (comprised of eIFs 4A, 4E, and 4G). It is believed that eIF4G, together with the poly(A)-binding protein and eIF4E, is responsible for the formation of a circularised mRNA structure where the 3’ end of the mRNA is brought into close proximity with the 5’ untranslated region (UTR). Subsequently, 3’ UTR-binding factors can regulate translation initiation through this spatial arrangement.

Once the 43S pre-initiation complex is bound with the cellular mRNA, scanning commences in an orderly 5’ to 3’ direction with the support of eIFs 1 and 1A. These factors search for a suitable start codon, such as the ‘canonical’ AUG, and activates translation through recognition of the codon by the initiator tRNA. However, this is a simplistic view of the initiation process. Often, ‘non-canonical’ codons, such as UUG and GUG, are preferentially selected and in eukaryotes, eIF1 also discriminates start codons via the surrounding nucleotide context known as the ‘Kozak’ consensus element in mammals (Kozak, 1986; Marintchev & Wagner, 2004), or another sequence in plants (Lutcke et al., 1987). To add to the complexity, alternative methods of translation initiation have been identified which mediate the activation of protein synthesis through secondary structures such as hairpins, internal ribosomal entry sites, and upstream open reading frames (ORFs) (Fig. 1.3). These modes of initiation are independent of the cap structure and in the instance of internal ribosomal entry sites, the ribosome is directly recruited to an internal position of the mRNA, rather than relying on the binding of eIFs (reviewed in Gebauer & Hentze, 2004; Hellen & Sarnow, 2001; Jackson, 2005; Kozak, 2002).

![Figure 1.3 Elements that influence the translation of mRNA.](image)

The stable binding of the tRNA initiator to the selected start codon results in the formation of the 48S initiation complex. This signals the hydrolysis of GTP on eIF2 by the GTPase-activating protein eIF5 and a guanosine diphosphate (GDP)-coupled eIF2 is released. Subsequently, the 60S ribosomal subunit is recruited into the 48S complex and an 80S initiation complex is formed.
During this process, most of the initiation factors are released and leave the initiator tRNA, base-paired to the start codon, at the ribosomal P-site. A second hydrolysis of GTP is then triggered, this time on eIF5B, and the resultant 80S complex is finally ready for polypeptide synthesis.

For another round of initiation to occur, the GDP on eIF2 is exchanged for GTP by the guanine nucleotide exchange factor eIF2B and the consequent GTP-coupled eIF2 is available to bind with another Met-loaded initiator tRNA to begin the translation initiation process again. This continued recycling of eIF2 between GTP- and GDP-bound states thus ensure that polypeptide chain synthesis is maintained. On the other hand, the phosphorylation state of eIF2 can significantly affect its ability to recycle GDP to GTP and this is recognised as an important means of global protein synthesis control.

1.1.1.3.2 Translational Control in Mammals via Phosphorylation of eIF2α

In mammals, eIF2 has a crucial role in the control of translation initiation. The initiation factor eIF2 is made up of three subunits – α, β, γ – with the GTP molecule binding to the γ subunit (Fig. 1.4). Typically through initiation, the GTP is hydrolysed to GDP and eIF2B regenerates an active eIF2 by recycling the GDP back to GTP (Gebauer & Hentze, 2004; Proud, 2005). However, in the presence of eIF2α kinases, the α subunit of eIF2 is phosphorylated at residue Ser51. This sterically blocks the GTP-exchange reaction by enhancing the stability of the eIF2-P/GDP/eIF2B complex and the dissociation rate of eIF2 from eIF2B is significantly reduced. In turn, the recycling of GDP

![Figure 1.4 Global control of protein synthesis via eIF2α phosphorylation](COPYRIGHT)

Figure 1.4 Global control of protein synthesis via eIF2α phosphorylation. eIF2 (pink) consists of three subunits – α, β, γ. During translation initiation, the GTP molecule on eIF2γ is hydrolysed to GDP and eIF2B (green) mediates the GDP-GTP exchange to regenerate an active eIF2. Phosphorylation of eIF2α leads to a slower dissociation rate from eIF2B and blocks the GDP-GTP exchange reaction. Subsequently, translation cannot be initiated and protein synthesis is inhibited. (Gebauer & Hentze, 2004)
to GTP is hindered, leading to the global inhibition of translation initiation. However, it is important to note that although phosphorylation of eIF2α results in a general decrease of translation initiation, it also has a role in enhancing translational activation for specific mRNAs containing upstream ORFs as is briefly discussed in Section 1.2.1.4.4.

So far, four eIF2α kinases have been identified in mammals and each is uniquely activated by a variety of stress signals. The hemin-regulated inhibitor kinase HRI phosphorylates eIF2α in response to the availability of heme (Chen & London, 1995); the general control of amino acid (aa) biosynthesis kinase GCN2 responds to aa starvation (Dever et al., 1992); the RNA-regulated protein kinase-like endoplasmic reticulum kinase PERK is activated by endoplasmic reticulum stress (Sood, Porter, Ma, Quilliam, & Wek, 2000); and the dsRNA-dependent protein kinase R PKR is induced by interferons (IFNs) and is activated by the presence of dsRNA (Proud, 1995). It is this last eIF2α kinase that assists the mammalian host in protecting itself from viral invasion. The presence of dsRNA replication intermediates activate PKR to phosphorylate eIF2α, resulting in the general shutdown of cellular translation machinery. As viruses depend on the protein synthesis apparatus of the host for their replication, the PKR-mediated inhibitory effect of eIF2α phosphorylation strongly limits the replication and spread of viral infection. In fact, this translational control allows a much more rapid repression of viral replication compared to defense mechanisms that require a host response at the transcriptional level.

1.1.1.3.3 Translation Initiation in Plants

In general, the initiation of translation is similar between plant and animal cells. However, there are a number of distinct differences, particularly with respect to the types of initiation factors present (reviewed in Browning, 1996, 2004; Futterer & Hohn, 1996; Kawaguchi & Bailey-Serres, 2002).

In plants, there are two isoforms of the cap-binding eIF4F complex. The second form of eIF4F, named eIF(iso)4F, is not found in mammals but is similar to eIF4F in that it has in vitro activities analogous to mammalian eIF4F and comprises a small cap-binding subunit eIF(iso)4E and the large scaffolding subunit eIF(iso)4G (Browning, 1996). The eIF(iso)4E subunit is similar in sequence and molecular mass to eIF4E, but eIF(iso)4G is significantly different in molecular size to eIF4G. In addition, sequences of the N-terminal region normally present in eIF4G are absent in its isoform, suggesting that the two forms might have different roles in the regulation of translation.
initiation. Studies have found that these isoforms may be responsible for distinguishing between different types of mRNAs. Particularly, eIF4F efficiently translates transcripts that have a stem-loop structure near the 5’-cap, mRNAs which lack the 5’-cap, and the downstream ORFs of uncapped dicistronic mRNAs. On the other hand, mRNAs that lack a structured 5’ UTR are preferentially translated by eIF(iso)4F (Gallie & Browning, 2001).

Another initiation factor that is unique to plants is eIF3. This is one of the most complex factors in the initiation of translation and in mammals, it is made up of 11 non-identical subunits. In comparison, plant eIF3 consists of 12 non-identical subunits and of these, two are unique to plants and are not analogous to the mammalian proteins (Browning et al., 2001). However, the roles of these plant-specific subunits are still unclear.

While the composition and aa sequences of plant eIF2 is similar to the mammalian homolog, there is speculation whether plant eIF2 may have the same regulatory role for protein synthesis as in mammals. To date, the gene of one eIF2α kinase has been identified in plants. The homolog of GCN2 has been cloned from Arabidopsis thaliana and its transcript is detected in the roots, leaves, stems, buds, flowers, siliques, and seedlings of Arabidopsis (Y. Zhang, Dickinson, Paul, & Halford, 2003). This RNA profile is supported by data from the Affymetrix Arabidopsis ATH1 microarray (Genevestigator; Hruz, Natora, & Agrawal, 2008) which showed the highest level of GCN2 transcript in Arabidopsis seedlings and matured siliques (Fig. 1.5). In terms of function, Arabidopsis GCN2 is able to complement yeast gcn2 mutants under aa stresses (Y. Zhang et al., 2003) and is essential for plant growth in stress situations, particularly in response to aa and purine starvation, ultraviolet radiation, cold shock, and wounding (Lagix et al., 2008; Y. Zhang et al., 2008). Significantly, this activation of Arabidopsis GCN2 results in the phosphorylation of eIF2α, suggesting a role for Arabidopsis GCN2 as a plant eIF2α kinase and possibly essential as a regulator of diverse stress-response pathways. Although a GCN2 homolog has been identified, an important translational control partner, GCN4, has not been found. GCN2 is the eIF2α kinase which triggers the inhibition of translation initiation in response to aa deficiency. On the other hand, GCN4 is a transcriptional activator of aa biosynthesis genes and the translation of its mRNA increases in the event of eIF2α phosphorylation (Dever, 2002). Thus, GCN4 positively stimulates aa storage while global protein synthesis is negatively regulated. The apparent absence of GCN4 in plants (Halford et al., 2004) suggests that a different regulatory process might be present in the response to aa starvation.
Figure 1.5 RNA profiling of GCN2 as detected by the Affymetrix Arabidopsis ATH1 microarray. The presence of the GCN2 transcript in various tissues of Arabidopsis: A, germinated seed; B, seedling; C, young rosette; D, developed rosette; E, bulting stem; F, unopened flower bud; G, flower bud from young plants; H, flower bud and developing siliques from adult plants; I, matured siliques from old plants. The Y-axis indicates the signal intensity as measured by the microarray. This value is normalised to a target signal value of 1000 and assumes an approximately constant total abundance of mRNA. (Modified from Genevestigator; Hruz et al., 2008)

1.1.1.3.4 Translational Control in Plants via Phosphorylation of eIF2α

Although translational control via plant eIF2α phosphorylation has yet to be demonstrated in planta, studies using a Vaccinia virus recombinant system in monkey kidney BSC-40 cells (Gil, Esteban, & Roth, 2000) and an expression system in yeast (Chang, Yang, & Roth, 2000) have both shown that plant eIF2α possesses similar functional activity to mammalian eIF2α. In the study by Gil et al. (2000), the presence of activated mammalian PKR (mPKR) resulted in the phosphorylation of Vaccinia virus-expressed wheat eIF2α, which in turn induced protein synthesis inhibition as well as cellular apoptosis. As for the study by Chang et al. (2000), wheat eIF2α was shown to participate in the yeast phosphorylation-mediated control pathway, where growth under starvation conditions led to the phosphorylation of wheat eIF2α by GCN2. This in turn resulted in the activation of yeast GCN4 to regulate aa biosynthesis. Wheat eIF2α was also able to interact with the β and γ subunits of yeast eIF2 and the resulting complex was capable of interacting with
yeast eIF2B. These findings give support that a similar pathway exists in plants where protein synthesis is regulated by the phosphorylation of eIF2α. However, further evidence for eIF2α translational control in plants will be necessary to validate these claims.

Another eIF2α kinase which has yet to be identified in plants is the dsRNA-activated PKR. As discussed in Section 1.3.1, there is strong evidence that a plant-functional homolog of PKR (pPKR) exists and the presence of its activity correlates with the phosphorylation of eIF2α.

### 1.2 The Double-stranded RNA-dependent Protein Kinase R

While there is little information existing for PKR in plants, PKR has been extensively studied and characterised in mammals. The structure and functional characteristics, particularly the antiviral role, of mPKR are described in this section. Where appropriate, reference has been made in this section to similar findings from plants or plant viruses. The published evidence for and characteristics of PKR in plants are described in Section 1.3.1.

#### 1.2.1 Characteristics of the Mammalian Protein Kinase R

In mammals, PKR is strongly induced at the transcriptional level within a few hours of IFN activation and the intracellular concentrations of PKR protein can rise by a factor of five- to ten-fold (Balachandran & Barber, 2007). The predominant molecule that activates PKR activity is dsRNA, either cellular- or viral-derived, but PKR also has affinity for oligonucleotide aptamers that include secondary structural imperfections such as bulges, internal loops, hairpin loops, multi-stem junctions and pseudoknots (Ben-Asouli, Banai, Pel-Or, Shir, & Kaempfer, 2002; Tian, Bevilacqua, Diegelman-Parente, & Mathews, 2004; Zheng & Bevilacqua, 2004). This interaction between PKR and dsRNA induces a conformational change in the protein and PKR is subsequently activated to autophosphorylate and trans-phosphorylate a number of substrates. In addition, PKR binds to and is activated intramolecularly by the polyanionic agent heparin (Fasciano, Hutchins, Handy, & Patel, 2005) as well as by the stress-modulated cellular protein PKR-activating protein PACT (C. V. Patel, Handy, Goldsmith, & Patel, 2000; Ruvolo, Gao, Blalock, Deng, & May, 2001).

PKR is expressed in a wide range of cell types in mammals and is constitutively active at low concentrations under normal physiological conditions. It is normally expressed in cells at low
concentrations and 80% of the protein is found in the cytoplasm where it is mostly associated with ribosomes (Jeffrey et al., 1995). A fraction of PKR is also found distributed to the nucleolus. However, nucleolar PKR is not responsive to IFN treatment and no significant induction of protein levels is seen in this subnuclear fraction (Jeffrey et al., 1995). Moreover, it is suggested that this nucleolar portion of PKR might be involved in chromatin remodelling.

1.2.1.1 The Structure of Mammalian Protein Kinase R

PKR was first cloned from human where it is synthesised as a 551 aa protein with an apparent mass of 68 kDaltons (Da) (E. Meurs et al., 1990). It has since been identified in other mammalian species such as mouse (Feng, Chong, Kumar, & Williams, 1992), rat (Mellor, Flowers, Kimball, & Jefferson, 1994), monkey, cow and pig (Asano, Kon, & Agui, 2004), as well as in chicken and fish (C. Y. Hu, Zhang, Huang, Zhang, & Gui, 2004; Rothenburg et al., 2005). The aa lengths of PKR and its homologs range from 513 to 551 aa. They all contain a C-terminal catalytic domain and N-terminal regulatory domain, although in fish PKR (PKZ), a different regulatory domain which has the ability to bind left-handed Z-RNAs is utilised as compared to its mammalian counterparts (Rothenburg et al., 2005).

The C-terminal catalytic domain of PKR comprises of a Ser/Thr kinase domain (Fig. 1.6). This domain contains the 12 characteristic subdomains that typically fold into a conserved catalytic core structure with two separate lobes and this topology defines these enzymes as protein kinases (C de Haro, R Mendez, & J Santoyo, 1996; S K Hanks & Hunter, 1995; Huse & Kuriyan, 2002). The

![Figure 1.6 The functional domain structure of human PKR.](COPYRIGHT)
small N-most region of the kinase domain is comprised of an α-helix with a five-stranded β-sheet while the C-terminal lobe is generally larger with a predominantly helical structure. The subdomains I, VIB and VII are responsible for adenosine 5′-triphosphate (ATP) binding and hydroxyl aa recognition, while an invariant lysine residue in subdomain II is essential for the phosphate transfer reaction (S. K. Hanks & Quinn, 1991). The kinase domain of PKR also possesses a short 24 aa spacer sequence between catalytic subdomains IV and V (Fig. 1.7). The presence of a spacer sequence is characteristic of eukaryotic eIF2α kinases, with the length of space in the other kinases being relatively longer than 24 aa (Samuel, 1993). Furthermore, a highly conserved region that is shared among eIF2α kinases is found in subdomain V (Fig. 1.7). This motif contains the sequence LFIQMEFCD and is recognised to have a role in both PKR autophosphorylation and trans-phosphorylation activity as well as eIF2α interaction (Cai & Williams, 1998). A similar sequence motif is also seen in the kinase domain of Arabidopsis GCN2, with only a single aa difference to the mammalian motif where a Tyr is substituted in place of Phe. This resemblance suggests that this motif may be important for the catalytic function of GCN2 as an eIF2α kinase in plants.

At the N-terminus of PKR, the regulatory region encompasses a consensus dsRNA binding domain (dsRBD) which contains two partially repeated dsRNA binding motifs (dsRBMs) at residues 10 – 78 and 101 – 168 (in human PKR), each comprised of approximately 70 aa residues (Kaufman, 2002) (Fig. 1.6). The dsRBM is a common dsRNA binding motif and is found in more than 544 eukaryotic proteins, of which 30 are from human and 16 are from Arabidopsis (data taken from SMART database, developed by Letunic et al., 2006). Moreover, dsRBM-containing proteins are mostly associated with roles in RNA interference, RNA processing, RNA localisation, RNA editing and translational repression (Stefl, Skrisovska, & Allain, 2005).
The dsRBM commonly spans around 70 – 75 aa and is folded into an α/β sandwich topology where two α-helices are stacked against a three-stranded antiparallel β-sheet in an α1-β1-β2-β3-α2 arrangement (Fig. 1.8). These domains are found either as a single copy motif or in multiple copies and as in the case of PKR, the presence of additional motifs allow cooperative binding to the substrate (S. Nanduri, Carpick, Yang, Williams, & Qin, 1998). The substrate selectivity of dsRBMs is very specific where it binds only dsRNA and not dsDNA or DNA-RNA hybrids. This interaction is also sequence-independent and dsRBMs recognise dsRNAs on a structure and shape basis.

Figure 1.8 The sequence-independent interaction of a single dsRBM with dsRNA. The topology of a single dsRBM (blue) comprising of two α-helices stacked against a three-stranded antiparallel β-sheet. (a) Side-on view shows the contact between dsRBM and dsRNA (grey) at the three regions in the order of 1-3-2. Specific interactions occur at the 2’-hydroxyls (red) and phosphates (green). (b) End-on view illustrates that contacts between dsRBM and dsRNA occur on one face of the RNA duplex. (Tian et al., 2004)

To date, three dsRBM-dsRNA complexes have been determined (Ramos et al., 2000; Ryter & Schultz, 1998; H. Wu, Henras, Chanfreau, & Feigon, 2004) and these structures illustrate the importance of three regions in a single dsRBM for the binding to dsRNA; α1 (region 1), two highly conserved basic loops between β1-β2 (region 2) and β3-α2 (region 3) (Fig. 1.8). The dsRBM interact with only one face of the dsRNA, spanning 16 base pairs (bp) or 1.5 turns of the helix which is achieved by the interactions between positively charged residues on the face of the dsRBM and the negatively charged phosphate backbone (reviewed in Perez-Canadillas & Varani, 2001; Stefl et al., 2005; Tian et al., 2004). The regions make contacts with the dsRNA in the order of 1-3-2 and bind to the minor-major-minor groove of the dsRNA, respectively. The interactions of regions 1 and 2 occur through the 2’-hydroxyls of the ribose sugar and the phosphodiester backbone of the helix, whereas region 3 binds dsRNA via six direct or water-mediated interactions.
with non-bridging oxygen residues of the phosphodiester backbone. Together, these observations illustrate a model where conserved residues on the surface of the dsRBM interact non-specifically with dsRNA via hydrogen bond contacts and electrostatic interactions. This in turn facilitates the dsRBM to discriminate between different forms of nucleic acids.

PKR is capable of binding RNA duplexes as short as 16 bp and the binding affinity and activation of the protein increases with lengths up to 85 bp (Spanggord & Beal, 2001; Manche & Green, 1992). However, dsRNA molecules longer than 85 bp have no greater effect on PKR activation. It is also interesting to note that the activation curve for dsRNA concentration is bell-shaped whereby low and high concentrations of dsRNA both inhibit the activation of PKR kinase activity. This finding may be explained by the fact that the two dsRBMs require cooperative binding to the same dsRNA to achieve activation of PKR (Fig. 1.9). Furthermore, the two motifs are joined by a 22 aa linker sequence which is comprised of random coil conformations (Fig. 1.10). This increases the flexibility of the dsRBMs and hence, in the presence of high dsRNA concentrations, there is a greater chance of each dsRBM binding to different dsRNA molecules. On the other hand, when the level of RNA duplexes is appropriate, the flexible linker acts to enhance the binding of dsRNA by PKR. They allow the dsRBMs to form a dumb-bell shape which then permits the dsRBD region to wrap around the single dsRNA molecule, optimising the contact interactions between each dsRBM and the same RNA helix (S. Nanduri et al., 1998).

Figure 1.9 The two dsRBMs of PKR wrap around the RNA helix to form a dumb-bell interaction. Model of the dsRNA-PKR complex that is formed when the two dsRBMs (light blue) interact with the RNA helix (grey). In appropriate dsRNA concentrations, the flexible linker allows the dsRBMs to wrap around a single dsRNA molecule and optimise contact interactions (depicted by green, red, and dark blue) between PKR and the dsRNA. dsRBM, dsRNA binding motif. (S. Nanduri et al., 1998)
Although the two dsRBMs act synergistically to promote a stable contact with the RNA duplex, there are significant differences between the two sequences. In fact, they show limited homology to each other at the aa level (Fig. 1.11) and it is postulated that these differences give the two dsRBMs distinct properties in RNA binding (Green, Manche, & Mathews, 1995). In comparison, the first motif (dsRBM1) displays a better alignment to the consensus sequence of dsRBMs than the second dsRBM of PKR (dsRBM2) and exhibits a stronger binding to the dsRNA substrate. Likewise, mutations in the N-terminal and central portions of dsRBM2 had moderate effects to its dsRNA binding activity whereas the same defects in dsRBM1 had drastic effects on its activity. These observations suggest that dsRBM2 may be structurally more flexible than dsRBM1 and may function distinctly with respect to the process of RNA binding by PKR.

**Figure 1.10 The two dsRBMs of PKR are joined by a 22 amino acid linker.** The 22 amino acid (aa) linker between the two dsRNA binding motifs (dsRBMs) gives flexibility to the motifs and, in the presence of high dsRNA concentrations, the two dsRBMs are more likely to bind to different dsRNA molecules. (modified from S. Nanduri et al., 1998)
DsRBM2 has been shown to have an autoinhibitory role in the regulation of PKR activity. In the uninduced form, the kinase domain of PKR is ‘locked’ in conformation through intermolecular interactions with dsRBM2 (Kaufman, 2002; S Nanduri, Rahman, Williams, & Qin, 2000). However, with the binding of dsRNA to the dsRBD, PKR undergoes a conformational change as evidenced by gel analyses of protein-RNA complexes and by biophysical techniques using tryptophan fluorescence quenching and neutron scattering (Carpick et al., 1997). This conformational change transforms PKR to an elongated form where the dsRBD swings away from the rest of the protein and the catalytic site for phosphorylation activity is exposed for activation. Further evidence for this model is also seen in yeast two-hybrid assays where the dsRBD is shown to interact with the C-terminal kinase domain (Sharp et al., 1998). In addition, deletion studies confirm that the removal of dsRBD creates a constitutively active protein kinase (S. Wu & Kaufman, 1997).

A third basic aa cluster (residues 232 – 261 in human PKR) is found in PKR in addition to the two basic aa clusters which forms the two dsRBMs (residues 10 – 78 and 101 – 168 in human PKR) (Fig. 1.6). The role for the third basic aa cluster in PKR is not yet fully understood. Deletion of this basic region in full-length PKR is observed to abolish its kinase activity in vivo, while a λ repressor fusion system shows that direct protein-protein interaction can occur via this domain (Tan, Gale, & Katze, 1998). Additionally, regulators of PKR, such as the cellular inhibitor p58<sup>IPK</sup>, are observed to bind to this region, suggesting that the third aa cluster may have functional importance in the antiviral activity of PKR. Hence, the third basic region may define a second regulatory domain in addition to the dsRBMs.
1.2.1.2 The Activation of Mammalian Protein Kinase R

Following the conformational change induced by the binding of dsRNA, PKR dimerises and undergoes autophosphorylation at a number of sites which results in its activation. The autophosphorylation of PKR follows similar observations in other protein kinases where residues between kinase subdomains VII and VIII are phosphorylated for their activation (Johnson, Noble, & Owen, 1996). These central subdomains of the kinase constitute the conserved Ala-Pro-Glu motif and encompass the activation loop which facilitates the binding and orientation of ATP and protein substrate as well as the transfer of γ phosphate (γP) from ATP to the acceptor hydroxyl residue of the protein substrate. On PKR, two important activation sites are mapped to Thr residues at 446 and 451. The significance of these residues is illustrated with mutagenesis studies where the activity of expressed mutant PKR was determined in yeast and mammalian cells (Romano et al., 1998). With the substitution of Thr446 to Ala, a partially active PKR was derived. In contrast, the mutation of Thr451 led to a complete abolishment of PKR activity. However, it is interesting to note that mass spectrometry (MS) analysis fails to detect phosphorylation at Thr451. Hence, it is currently unknown whether Thr451 is phosphorylated in vivo and whether the Thr residue is functionally important for PKR activity.

Several other phosphorylated residues on PKR have also been tested to determine their potential roles in activating the protein kinase (Taylor et al., 1996). These sites – Thr258, Ser242, and Thr255 – are located outside the activation loop, situated between the dsRBD and kinase domains, and have each been substituted with an Ala. The first substitution, Thr258, is found in the third cluster of basic aa in PKR and has been observed to cause a slight reduction in the efficiency of autophosphorylation and substrate phosphorylation by PKR as well as partial inhibition of kinase function. On the other hand, substitutions of the latter two sites (Ser242 and Thr255) had little effect on the kinase function of PKR. The combined triple PKR mutant of these sites also had insignificant effect on phosphorylation activity, suggesting that these residues may not contribute to the full activation of PKR.

It is recognised that the phosphorylation of Tyr residues on PKR may also be important for the activation of PKR. Although PKR is generally recognised as a Ser/Thr kinase, it is suggested that PKR may regulate its own function via Tyr phosphorylation as well. In fact, there is clear evidence that PKR is a dual specificity kinase (Icely et al., 1991; J. Lu, O'Hara, Trieselmann, Romano, & Dever, 1999). In the activation of PKR, three Tyr residues at Tyr101, Tyr162, and Tyr293 have
been shown to be phosphorylated *in vitro* and *in vivo*. The first two Tyr are found flanking the sequence of dsRBM2, while the third phosphorylated Tyr is located in the kinase subdomain II of PKR. Moreover, Tyr293 is a conserved residue present in the orthologs and homologs of PKR (Su et al., 2006). Functionally, it is observed that these sites have essential roles in dsRNA binding, kinase dimerisation and activation, as well as PKR autophosphorylation and eIF2α phosphorylation.

A simple model of PKR activation can be depicted where the binding of dsRNA at the dsRBD induces a conformational change in PKR. This initiates homodimerisation and leads to PKR autophosphorylation at a number of sites, including Tyr101, Tyr162, Ser242, Thr255, Thr258, Tyr293, Thr446, and Thr451. Subsequently, the dimerised PKR is activated and carries out its phosphorylation activities on the recognised targets. However, additional studies demonstrate that the activation of PKR might not be as straightforward this.

It is realised that the dimerisation of PKR may not be dependent on dsRNA binding (R. C. Patel, Stanton, McMillan, Williams, & Sen, 1995; R. C. Patel, Stanton, & Sen, 1996). Direct evidence supports the self-association of PKR *in vitro* and *in vivo* and this interaction is found to occur at the N-terminal region of the protein, specifically at residues 244 – 296 (in human PKR), a region that overlaps the third cluster of basic aa in PKR. This region has also been observed to have affinity for both matrix-bound PKR and matrix-bound dsRBD. Moreover, mutants that lack the ability to bind dsRNA were still able to exhibit protein-protein interaction with wildtype PKR. Therefore, although the same domain of the protein contributes to its dsRNA binding and dimerisation properties, it is possible that specific residues within this domain comprise distinct roles for these two activities. By using affinity matrix binding assays of *in vitro* translated PKR, more than one self-association domain of PKR has been identified. These results suggest that the dsRBD exists primarily in solution as dimeric molecules.

Although the two dimerised PKR molecules are in close proximity, they cannot *trans-*phosphorylate the bound partner until a dsRNA binds and induces a conformational change of the PKR molecules into their active states. In support of this model, observations show that ATP can only be photo-cross-linked to PKR in the presence of dsRNA (R. C. Patel et al., 1996). In the absence of the dsRNA activator, the ATP-binding site of PKR seems to be masked by protein folding. Conversely, although homodimerisation might not be the propelling force for PKR activation, its formation may still have catalytic functions. With respect to enzymes, dimerisation is known to portray a number of unique properties (Tan et al., 1998). Dimerisation may regulate
the level of enzyme activity or modify enzyme specificity for substrate selection. Importantly, dimerisation may also stabilise the protein or prevent it from protease degradation or phosphatase action. Finally, dimerisation may also play a role in the subcellular localisation of the protein.

It is clear that a combination of dsRNA binding, homodimerisation and autophosphorylation results in the activation of PKR. Subsequently, the activated dsRNA-dependent protein kinase proceeds to phosphorylate a number of substrate targets. These substrates include eIF2α, IκB and histones and they all play a role in contributing to the antiviral and physiological functions of PKR in the mammalian cell.

1.2.1.3 The Antiviral Role of Mammalian Protein Kinase R

In mammals, the antiviral property of PKR is preceded by the innate immune response of IFNs. IFNs are the first line of defense against viral infection and their expression is highly induced at the transcriptional level in response to the presence of viruses. This reaction is shown to be induced immediately after virus infection and it is postulated that the synthesis of IFNs is stimulated by the presence of viral dsRNA replication intermediates (Haller, Kochs, & Weber, 2006). More than 300 genes are induced by the mammalian cell in response to IFNs (Caraglia et al., 2004). One of the main antiviral proteins that is activated by IFN signalling is PKR and this protein kinase rapidly halts the spread of viral infection through the phosphorylation of eIF2α (Langland, Cameron, Heck, Jancovich, & Jacobs, 2006).

As well as the phosphorylation of eIF2α, PKR in mammals has a role in the phosphorylation of IκB, an inhibitor of the nuclear factor κ light-chain-enhancer of activated B cells (NFκB) (Cheshire, Williams, & Baldwin, 1999; Chu et al., 1999; Kumar et al., 1997). The phosphorylation of IκB leads to the release of its inhibitory effect on NFκB and enables NFκB to translocate to the nucleus to induce the transcription of different sets of genes. These include genes involved in immune and inflammatory responses (such as IFNβ) (Ghosh, May, & Kopp, 1998), as well as cell survival genes such as c-IAP and A20 (Lee et al., 2000; Wang, Mayo, Korneluk, Goeddel, & Baldwin, 1998). In fact, it has been demonstrated that this survival pathway is induced by the proapoptotic PKR (Donze et al., 2004). These observations demonstrate that PKR has roles in both the translation inhibition effect of eIF2α phosphorylation and the cell survival signal induced by NFκB – two pathways that execute opposing effects in the cell. A postulated theory, supported by evidence, suggests that these two conflicting programs might contribute hand-in-hand to the
antiviral defense system induced by PKR (Donze et al., 2004). In these studies, the expression and activation of NFκB is seen prior to eIF2α activation, illustrating that the PKR-induced cell death is delayed by a PKR-triggered survival response. It is proposed that in the event of a viral infection, cell survival signals first pause cell death in order for neighbouring naïve cells to become alerted of the invasion of foreign pathogens and produce antiviral cytokines. After a certain temporal delay, the apoptosis signal is activated and infected cells are eliminated thus halting the progress of infection. Hence, PKR might behave as a molecular timer for its antiviral activities, chronologically activating two opposing effects in which cell survival is signalled through the NFκB pathway followed by apoptosis via eIF2α phosphorylation. Together with the temporal delay of cell apoptosis, this suggests that the two influential processes may be regulated differently to prevent competition for the same stimulus, while still achieving antiviral defense activities.

1.2.1.3.1 How Viruses Counteract the Antiviral Activity of Protein Kinase R

Because nearly all viruses synthesise dsRNAs or RNAs that have extensive secondary structures during their replicative cycle, all viruses can potentially activate the antiviral activities of PKR (Balachandran & Barber, 2007; MacDiarmid, 2005). To avoid the deleterious effects of eIF2α phosphorylation on their survival, viruses have developed a number of successful strategies that either block or avoid the antiviral activity of PKR. The mechanisms for counteracting the antiviral activity of PKR, that have thus far been characterised, can be separated into distinct groups (reviewed in Jacobs & Langland, 1996; Kaufman, 2002) for which representative inhibiting responses of animal viruses are described in the following sections. The counteracting effects of these inhibitors on pPKR are as yet unknown, although a cellular inhibitor of PKR, p58IPK, has been identified in plants. The possible role of p58IPK on the activity of pPKR and the ability of plant viruses to encode or recruit proteins which act to inhibit the translational control of pPKR are discussed in Section 1.3.1.5.

1.2.1.3.1.1 Inhibitors of the Double-stranded RNA-mediated Activation of Protein Kinase R

Some animal viruses synthesise RNAs and/or proteins that interfere with the dsRNA-mediated activation of PKR by binding to the conserved dsRNA binding domains of PKR and/or sequestering the RNA that activates PKR.
Examples of these virus-synthesised products include virus-associated RNAs from *Adenovirus* (Jimenez-Garcia, Green, Mathews, & Spector, 1993) and *Epstein-Barr virus*-encoded RNAs (Vuyisich, Spanggord, & Beal, 2002). These viral RNAs act as competitive inhibitors of the dsRNA-PKR interaction by binding at the same sites as the dsRNA activators but without activating the kinase activity. *Human immunodeficiency virus-1* (HIV-1) also encodes for RNAs that affect PKR activation via dsRNA binding (Park et al., 1994). At high concentrations, HIV-1-encoded transactivator responsive region (TAR) RNAs physically bind PKR and inhibit its kinase function. However, low levels of TAR RNAs actually activate PKR, thus HIV-1 has an additional anti-PKR system where it recruits a cellular TAR RNA binding protein to sequester low levels of TAR RNAs and block them from activating PKR. This cellular TAR RNA binding protein also heterodimerises with PKR and promotes direct protein-protein interactions that disrupts the function of PKR.

A number of viruses encode proteins that mask the dsRNAs from activating PKR. The *Vaccinia virus*-encoded E3L protein and *Reovirus*-encoded σ3 protein both have homology to the dsRNA binding protein family and have high affinity binding for dsRNAs. Upon sequestering the dsRNAs, PKR activation as well as substrate phosphorylation by PKR is inhibited (Davies, Chang, Jacobs, & Kaufman, 1993; Langland & Jacobs, 2004; Yue & Shatkin, 1997). *Influenza A virus*-encoded non-structural 1 protein (NS1A) is also a known inhibitor of PKR and has binding affinity for dsRNAs (Tan & Katze, 1998). It was originally postulated that NS1A might influence PKR activity via dsRNA sequestering, however, when a NS1A protein deficient for dsRNA binding activity was expressed in human cells, its inhibitory activity of PKR was not affected (Li, Min, Krug, & Sen, 2006). Further research has shown that the PKR inhibitory effect of NS1A is not due to the sequestration of dsRNAs. Instead, NS1A binds to dsRNAs to block the activation of the 2’-5’-oligoadenylate synthetase/RNase L pathway (Min & Krug, 2006) and this protects *Influenza A virus* from the IFN α/β-induced antiviral mechanism. As such, it is now recognised that the inhibition of PKR activity by NS1A is achieved through preventing IFNβ mRNA production and NS1A directs this inhibition by interacting with the cellular cleavage and polyadenylation specificity protein CPSF30 (Twu, Noah, Rao, Kuo, & Krug, 2006).

### 1.2.1.3.1.2 Inhibitors of the Homodimerisation of Protein Kinase R

A second group of anti-PKR proteins inhibit PKR activation by interfering with PKR dimerisation. *Influenza virus* activates a cellular gene p58IPK which blocks PKR dimerisation by interacting with
the third basic aa region of PKR (Melville, Katze, & Tan, 2000; Tan et al., 1998). P58IPK is a member of the tetratricopeptide repeat family of proteins and contains nine tandemly arranged tetratricopeptide repeats that mediate both homotypic and heterotypic protein-protein interactions. P58IPK also contains a C-terminal DnaJ motif which has protein binding properties, but this region has been determined unimportant in PKR-inhibitory function (Melville et al., 2000). As a cellular protein, p58IPK is constitutively expressed but is kept inactive by specific inhibitory molecules known as inhibitors of p58IPK (Gale et al., 1998). The infection of Influenza virus disrupts this inhibitory complex and enables p58IPK to bind to PKR. Yeast two-hybrid systems demonstrate that p58IPK forms a specific and stable in vivo complex with PKR (Tan et al., 1998).

A viral protein expressed by Hepatitis C virus (HCV) also prevents the homodimerisation of PKR. The non-structural 5A protein (NS5A) is encoded within a discrete region of the HCV genome, known as the IFN sensitivity determining region (ISDR), and it has been suggested that NS5A acts via an ISDR-directed mechanism to block the IFN-mediated response to viral infection (Pawlotsky & Germanidis, 1999; Tan & Katze, 2001). Studies confirm this theory by demonstrating that NS5A has the ability to bind to PKR and inactivate the protein kinase by blocking its dimerisation. It should be noted though that as no tissue culture system is currently available to study HCV replication, the regulation of PKR by NS5A has yet to be examined in HCV-infected cells.

1.2.1.3.1.3 Inhibitors of the Kinase Activity of Protein Kinase R

The function of PKR is inhibited by a group of proteins which block both the kinase catalytic site and PKR-substrate interactions. One example is the Vaccinia virus-encoded K3L protein (Davies et al., 1993; Kawagishi-Kobayashi, Silverman, Ung, & Dever, 1997). K3L displays homology to the phosphorylation site on eIF2α but lacks the phosphorylatable residue that corresponds to the Ser51 found in the initiation factor. K3L thus inhibits PKR function by acting as a pseudosubstrate that buries deep into the catalytic cleft of PKR. PKR becomes stably bound to this unphosphorylatable protein and subsequently loses the ability to activate itself or phosphorylate eIF2α.

HIV-1 also encodes an inhibitor that interferes with the catalytic activity of PKR. The HIV-1-encoded transactivator protein is reported to interact stably with PKR through a dsRNA-mediated bridge and in turn inhibits the role of PKR in auto-activation (McMillan et al., 1995). It is also
suggested that the transactivator protein may function as a pseudosubstrate like K3L, but evidence to support this speculation has yet to be established.

1.2.1.3.1.4 Inhibitors of the Physical Levels of Protein Kinase R

A less common group of PKR inhibitors act by altering the physical levels of PKR. An example is *Poliovirus*, which activates a cellular pathway to degrade PKR. A latent heat-stable cellular protease is activated in the event of *Poliovirus* infection and acts together with viral and possibly cellular dsRNA to target PKR for degradation (McMillan et al., 1995).

1.2.1.3.1.5 Inhibitors of the Regulation of Eukaryotic Initiation Factor 2α by Protein Kinase R

A group of viral inhibitors affect PKR function by either directly targeting eIF2α phosphorylation or regulating components downstream of eIF2α. The *Herpes simplex virus*-encoded protein γ134.5 interacts with a Type 1a protein phosphatase and acts by directing the protein phosphatase to eIF2α. Although PKR is activated, phosphorylation of eIF2α is downregulated by the protein phosphatase and protein synthesis is able to take place (He, Gross, & Roizman, 1998).

The *Simian virus 40*-encoded T antigen inhibits the effects of PKR activation by acting downstream of eIF2α. Despite an elevated level of eIF2α phosphorylation via PKR activation, the T antigen is able to restore efficient translation in infected cells. However, the full mechanism has not been elucidated and it is therefore unclear exactly where the point of action is for the T antigen (Swaminathan, Rajan, Savinova, Jagus, & Thimmapaya, 1996).

1.2.1.3.1.6 Evasion of the Translational Control of Protein Kinase R via RNA Structure

Recent findings have identified viruses which have adapted structures in their mRNA to counteract the translational inhibition effect induced by PKR. In the infection of *Sindbis virus*, PKR is highly activated and results in an almost complete phosphorylation of eIF2α. However, the translation of the *Sindbis virus* 26S mRNA is not restricted by this modification of eIF2α as its mRNA contains a highly stable RNA hairpin loop located downstream of the AUG initiator codon. The RNA structure transiently stalls the scanning ribosomes on the 26S mRNA and allows translation to initiate at a downstream internal AUG that is not normally used as an initiation codon. The RNA
structure also acts as a translational enhancer and has been shown to initiate translation in the absence of a functional eIF2 (Ventoso et al., 2006).

1.2.1.4 The Physiological Roles of Mammalian Protein Kinase R

In view of the fact that mPKR is constitutively expressed at low levels in most cells, it has been postulated that the dsRNA-activated protein kinase may in fact be involved in cellular processes in addition to its antiviral properties. Several studies have demonstrated the involvement of PKR in a range of processes including growth control, cell differentiation, histone phosphorylation and the regulation of preferentially translated mRNAs. The multi-functional roles of the dsRNA-dependent protein kinase are described in the following sections.

1.2.1.4.1 Regulation of Growth Control by Mammalian Protein Kinase R

It is recognised that PKR functions in the regulation of growth control in mammals, chiefly, as a suppressor of cell proliferation and tumourigenesis. In studies where wildtype PKR was overexpressed in yeast or mouse cells, a growth suppressing phenotype was observed whereby translation initiation was inhibited and cell cytotoxicity was induced (Chong et al., 1992; Thomis & Samuel, 1992). Conversely, the expression of catalytically inactive mutants of PKR in healthy mouse NIH 3T3 cells led to malignant transformation of the cells and growth of tumours in nude mice (Barber, Wambach, Thompson, Jagus, & Katze, 1995; Koromilas, Roy, Barber, Katze, & Sonenberg, 1992; E. F. Meurs et al., 1992). In addition, the transfection into NIH 3T3 cells of a PKR mutant which had trans-dominant inhibitor activity on the cellular levels of phosphorylated eIF2α resulted in tumourigenic transformation of the cells (Donze, Jagus, Koromilas, Hershey, & Sonenberg, 1995). In contrast, the generation of PKR knockout mice failed to verify the tumour suppressing functions of PKR (Abraham et al., 1999; Yang et al., 1995). These PKR-null mice developed normally with no significant phenotype or any increase in tumour incidence and the phosphorylation of eIF2α was not impaired in the mice. These observations suggest that PKR might not be essential for growth control and its translation inhibition activity can be rescued by another eIF2α kinase.
1.2.1.4.2 Regulation of Cell Differentiation by Mammalian Protein Kinase R

PKR activation has been demonstrated to function in the regulation of cell cycle and cellular differentiation in mammals. A study reports that transient or inducible overexpression of PKR initiated apoptosis and induced cell arrest (Zamanian-Daryoush, Der, & Williams, 1999). Cell cycle arrest may be related to the need for eIF2α phosphorylation to translate the cell cycle regulator cyclin D1. PKR activation resulted in the accumulation of cells in the G1 phase of the cell cycle and increased the expression of effector molecules that formed death-inducing signalling complexes such as Fas, Fas-associated protein with Death Domain, tumour necrosis factor (TNF) receptor-1 and caspase 8 (Balachandran et al., 1998; Donze, Dostie, & Sonenberg, 1999). Recently, PKR activation has been associated with the differentiation of rat myogenic cell line L8 (Salzberg, Mandelboim, Zalcberg, Shainberg, & Mandelbaum, 1995) and the expression of a dominant-negative PKR mutant was found to disrupt the myogenic process (Salzberg, Vilchik, Cohen, Heller, & Kronfeld-Kinar, 2000).

1.2.1.4.3 Phosphorylation of Histones by Mammalian Protein Kinase R

In addition to phosphorylating eIF2α and IκB, activated PKR is postulated to have a role in the phosphorylation of histones in mammals. It is observed that histone H1 can be phosphorylated by PKR in a dsRNA-dependent manner (Tan, Tareen, Melville, Blakely, & Katze, 2002). However, other reports find that H1, as well as the other histones H2, H3, and H4, are poor substrates for PKR activity (Berry, Knutson, Lasky, Munemitsu, & Samuel, 1985; Pestka, Langer, Zoon, & Samuel, 1987). These discrepancies may be related to differences in assay protocols for the analysis of kinase activities or even the technique for the purification of PKR. The role of PKR in histone phosphorylation should not be overlooked but further investigation of this activity remains to be conducted. A similar phosphorylation profile of histones in the presence of increasing amounts of dsRNA has been demonstrated in plant extracts. Evidence for this activity is described in Section 1.3.1.3.

1.2.1.4.4 Regulation of Preferentially Translated Messenger RNAs by Mammalian Protein Kinase R

PKR may function through translational control to alleviate cellular injury in response to environmental and cellular stress. In the early events of the stress response, eIF2α kinases recognise distinct stress signals and are activated to modulate downstream response pathways via
eIF2α phosphorylation. Although the phosphorylation of eIF2α generally results in the suppression of translation initiation, it can also enhance the translational activation of specific mRNAs which have a role in relieving stress damage in cells. The molecular mechanism by which this occurs involves the presence of upstream ORFs and the control of ribosome scanning and re-initiation at downstream codons via the low availability of eIF2-GTP. Examples of these preferentially translated mRNAs include the transcription activator GCN4 (Hinnebusch, 2005), cationic aa transporter Cat-1 (Fernandez et al., 2002), and transcription regulator ATF4 (Vattem & Wek, 2004).

The induced translation of the basic leucine zipper transcriptional activator ATF4 is a recently discovered example of preferential mRNA translation in the event of stress-activated eIF2α phosphorylation (Vattem & Wek, 2004). Two upstream ORFs are located in the 5’ noncoding portion of the ATF4 mRNA (Fig. 1.12). The 5’-proximal upstream ORF (uORF1) encodes for a three aa polypeptide and the second upstream ORF (uORF2) produces a 59 aa polypeptide. Translation is primarily initiated at uORF1 and in non-stressed cells where eIF2-GTP is plentiful, the ribosome continues to scan downstream of uORF1 and re-initiates at the next coding region, uORF2. ATF4 translation is suppressed in this non-stressed state as the coding region of uORF2 overlaps with the ATF4 coding region and thus ATF4 translation is not initiated. However, under stressed conditions, phosphorylation of eIF2α results in a reduced level of eIF2-GTP and the scanning ribosome requires a longer time to become competent to re-initiate translation at a downstream codon. Hence, the delayed re-initiation allows the ribosome to scan through the inhibitory uORF2 and instead, re-initiate at the ATF4 start codon, leading to ATF4 translation.

Figure 1.12 ATF4 expression is enhanced in response to stress-induced eIF2α phosphorylation. In non-stressed cells, ATF4 expression is suppressed. In stressed cells, eIF2α phosphorylation results in low levels of eIF2-GTP and the ribosome bypasses the inhibitory uORF2 and re-initiates translation at ATF4. The black line depicts ATF4 mRNA. Shaded ribosome cartoons indicate ribosomal subunits that are associated with eIF2-GTP. 1, uORF1; 2, uORF2. See text for more details. (Vattem & Wek, 2004)
Elevated levels of ATF4 are invaluable to the stressed cell as this protein induces the activation of additional basic leucine zipper transcriptional regulators, ATF3 and CHOP, and these together activate the expression of a series of genes that are important in alleviating cellular damage. Similarly, the preferential expression of GCN4 and Cat-1 result in the activation of genes that protect the cell from injury induced by stress.

Currently, it is estimated that upstream ORFs are present in around 20 – 30% of plant mRNAs, with 26 upstream ORF homology groups identified (Hayden & Jorgensen, 2007). However, only a few of these upstream ORFs have so far been identified as having functional activity. The roles of the other upstream ORFs are as yet unknown, but it is plausible that they are regulated by eIF2α kinases such as PKR.

### 1.3 Plant-functional Equivalent of Protein Kinase R

#### 1.3.1 Evidence for Putative Plant-functional Equivalent of Protein Kinase R

As well as PKR identification and characterisation in mammals, chicken and fish, there is mounting evidence that a putative functional equivalent of PKR may also be present in plants. This pPKR research has primarily been performed in the laboratory of Professor Donald Roth (University of Wyoming). The earliest observation of virus-induced phosphorylation of specific proteins included a 68 – 70 kDa protein in the infection of tobacco with *Tobacco mosaic virus* (TMV) (Evans, Haley, & Roth, 1985; Roth & He, 1994). Viral infection caused significant changes in the phosphorylation and nucleotide binding properties of a number of different sized proteins and the phosphorylation of a particular 68 kDa protein increased eight- to ten-fold as compared to uninfected plants. A 68 – 70 kDa potential pPKR protein was subsequently partially purified from plants via affinity with dsRNA covalently bound to agarose resin and its activity examined in tobacco, tomato, barley, and wheat (Crum, Hu, Hiddinga, & Roth, 1988; Hiddinga, Crum, Hu, & Roth, 1988; Langland, Jin, Jacobs, & Roth, 1995). The characteristics and activities of the partially purified putative pPKR identified by Roth and co-workers are discussed in the following sections.

Although the activity of a potential pPKR protein has been identified, no major shutdown of protein synthesis upon virus infection has so far been detected in plants. In TMV-infected tissues, host protein synthesis at 15 days after inoculation appeared to reduce by up to 75%, but recovers
by 30 days after inoculation. However, this decrease in host protein synthesis was found to be caused by a diversion of protein metabolisms by TMV to the production of viral proteins (Fraser & Gerwitz, 1980). Hence, there may be evidence for the presence of pPKR activity in plants, but its role and function remains unclear.

1.3.1.1 Subcellular localisation of Putative Plant-functional Equivalent of Protein Kinase R

In mammals, PKR is found both free in the cytosol and in association with ribosomes (Jeffrey et al., 1995). Similarly, pPKR activity is detected in both of these fractions (Fig. 1.13). The localisation of the plant kinase differs to that observed for mPKR as the level of plant kinase activity is generally higher in the ribosome-associated (Fig. 1.13, RSW) than the cytosolic fraction (Fig. 1.13, S-100) of healthy tissues (Langland et al., 1995). The expression of the putative pPKR is induced in the event of virus or viroid infection (Crum et al., 1988; Hiddinga et al., 1988) but this increased level is associated with susceptible, disease-producing reactions rather than an antiviral response as seen for the IFN-induced mPKR phosphorylation. This finding suggests that the role of pPKR may be functionally different to the characterised mPKR.

![Figure 1.13 Subcellular localisation of the putative plant PKR. Phosphorimage analysis of poly(rI).poly(rC)-agarose affinity purified proteins from barley and mouse L cells, separated into their respective cytosolic (S-100; lanes A and C) and ribosomal (RSW; lanes B and D) fractions, and phosphorylated with $\gamma^{32}$P-ATP. Molecular mass markers (kDa) are shown to the left. (Langland et al., 1995)](COPYRIGHT)
1.3.1.2 Double-stranded RNA Binding and Autophosphorylation Activity of Putative Plant-functional Equivalent of Protein Kinase R

It has been shown that the putative pPKR possesses dsRNA-specific binding activity and that this interaction is required for its autophosphorylation (Langland et al., 1995; Langland, Langland, Browning, & Roth, 1996). Similar to the phenomenon observed for mPKR, low and high concentrations of dsRNA resulted in the inhibition of pPKR activation and autophosphorylation (Crum et al., 1988), however, pPKR requires at least 25 µg/mL dsRNA for its activation as compared to 1 µg/mL dsRNA for the activation of mPKR (Fig. 1.14).

The substrate specificity of the putative pPKR was also determined using competition assays and the 68 – 70 kDa protein displayed binding affinity and phosphorylation activity for only dsRNA and not ssRNA (Fig. 1.15). Furthermore, although the basal amount of pPKR phosphorylation is relatively low in healthy plants, this level of phosphorylation was induced four-fold in tissues that were infected with TMV (Bilgin et al., 2003). This suggests that pPKR may respond similarly to mPKR to defend the plant cell against the infection and propagation of viruses. However, virus-activated inhibition may override such activation (Bilgin et al., 2003).

![Figure 1.14 DsRNA-dependent phosphorylation of the putative plant PKR.](image)

**Figure 1.14 DsRNA-dependent phosphorylation of the putative plant PKR.** Phosphorimage analyses of ribosomal fractions from barley (a) and mouse L cells (b), incubated in the presence of γ^32P-ATP and an increasing concentration of soluble poly(rI).poly(rC) (lanes B – D). Molecular mass markers (kDa) are shown on the left. pPKR, plant PKR; mPKR, mouse PKR. (Langland et al., 1995)
1.3.1.3 Phosphorylation Activities of Putative Plant-functional Equivalent of Protein Kinase R

In mammals, PKR is known to phosphorylate three main substrates – eIF2α, IκB, and histones. Accordingly, the trans-phosphorylation activity of pPKR has been investigated.

It is shown that partially purified pPKR is capable of phosphorylating both mammalian and plant eIF2α in the presence of dsRNA (Langland et al., 1996) (Fig. 1.16). This observation is further supported by immunoclearing experiments, PKR inhibitor analyses and dsRNA competition assays (Langland et al., 1995). Additionally, synthetic eIF2α peptides which comprise the known interacting sequences between PKR and the mammalian or plant eIF2α have been used effectively as pPKR substrates to determine that pPKR also phosphorylates eIF2α at Ser51 as observed for mPKR (Langland et al., 1996; Prakash, 2006). The presence of viral infection resulted in the in vitro phosphorylation of eIF2α which correlated with the inhibition of protein synthesis (Roth & He, 1994). Collectively, these findings suggest that pPKR may function as a plant eIF2α kinase.

Although mPKR is known to participate in cell survival and apoptosis by activating NFκB via the phosphorylation of IκB, this effect is not seen in plants. In fact, it is reported that even though the activation of pPKR results in the inhibition of translation, the cell does not undergo virus-induced apoptosis (Langland et al., 1996). As the NFκB pathway involves IFN-induced mechanisms, a system which is not present in plants, these observations fuel speculations that PKR may actually act via distinct pathways in mammals and plants.
Histone proteins are another phosphorylation substrate for mPKR and a similar phosphorylation activity is exhibited for the putative pPKR (Langland et al., 1995). There are also observations that the phosphorylation of histones can occur in the absence of dsRNA. However, the addition of dsRNA did lead to a significant increase in the activity of histone phosphorylation (Fig. 1.17). These findings once again strengthen the hypothesis that regulation of pPKR activity is dependent on the presence of dsRNA.

**1.3.1.4 Immunological and Molecular Characterisation of Putative Plant-functional Equivalent of Protein Kinase R**

Using immunological and molecular assays, significant similarities between the mammalian and putative plant PKR have been demonstrated (Langland et al., 1995). Firstly, immunoprecipitation experiments identified antigenic characteristics on pPKR which are also found in mPKR (Fig. 1.18). Two antisera were used – a monoclonal antiserum raised against sequences that span the second dsRBM and third basic aa domain of human PKR (from residues 117 to 250) and a rabbit polyclonal antiserum which recognises the 69 aa dsRBM of *Rotavirus* NSP3 protein, a sequence similar to the dsRBM located in mPKR. Both antisera were immunoreactive with the 68 – 70 kDa plant protein, suggesting that pPKR may comprise of sequences analogous to human PKR and may contain at least one dsRBM.
Figure 1.17 DsRNA-dependent phosphorylation of histones by the putative plant PKR. Phosphorimage analysis of barley extract ribosomal fraction incubated in the presence of $\gamma^{32}$P-ATP, 300 $\mu$g/mL histone proteins (type IIA from calf thymus) and an increasing concentration of soluble poly(rI).poly(rC), followed by incubation with dsRNA agarose. Molecular mass markers (kDa) are shown on the left. pPKR, plant PKR; H, histone proteins (Langland et al., 1995)

Figure 1.18 Immunoreactivity of the putative plant PKR with antisera to the conserved dsRNA-binding motif and to human PKR. Phosphorimage analysis of poly(rI).poly(rC)-agarose affinity purified ribosomal fractions from barley (lanes A and C) and human HeLa cells (lanes B and D), incubated in the presence of $\gamma^{32}$P-ATP and immunoprecipitated with either antiserum against a conserved dsRNA-binding motif (lanes A and B) or monoclonal antiserum against human PKR (lanes C and D). Molecular mass markers (kDa) are shown on the left. pPKR, plant PKR. (Langland et al., 1995)
On the molecular level, radiolabelled probes created via random-priming reactions of mouse or human PKR complementary DNA (cDNA) clones were used for northern blot analyses against tobacco leaf and wheat germ tissues. These probes cross-reacted with a 2500 bp poly(A)$^+$ RNA transcript in both of the plant tissues and this transcript size corresponds to the size of the human PKR transcript. Together, these findings support the hypothesis that the partially purified 68 – 70 kDa protein is representative of the plant-functional equivalent of PKR and it is possible that the identified 2500 bp transcript may encompass the mRNA of the putative pPKR.

1.3.1.5 Identification of Plant-functional Homolog of p58$^{IPK}$

The plant-functional homolog of the cellular PKR inhibitor p58$^{IPK}$ has been identified in Arabidopsis, Nicotiana benthamiana and tomato (Bilgin et al., 2003). In mammals, p58$^{IPK}$ acts by interfering with PKR dimerisation and prevents the translational inhibition effect of PKR on viral infection (described in Section 1.2.1.3.1.2). The identification of this cellular inhibitor in plants provides indirect evidence of the presence of a plant-functional equivalent of PKR and its interaction with virus-encoded proteins (described below) gives support to the hypothesis that the plant-functional equivalent may have a role in the defense of virus infection in plants.

Plant p58$^{IPK}$ was first discovered by its interaction with the 50 kDa helicase domain of TMV (Bilgin et al., 2003), a protein which is known to induce defense responses in plants via the $R$ gene $N$ (Erickson et al., 1999). The biological function of p58$^{IPK}$ in plants, as well as its role in viral pathogenesis, was assessed by producing p58$^{IPK}$-silenced N. benthamiana using virus-induced gene silencing (Bilgin et al., 2003). By comparing to observations in mammals, the loss of plant p58$^{IPK}$ was postulated to allow an increase in eIF2$\alpha$ phosphorylation and also decrease or halt viral infection in plants. No visible effect on plant growth and development was observed in these silenced plants. However, when the p58$^{IPK}$-silenced plants were challenged with TMV or Tobacco etch virus, the accumulation of the virus was significantly reduced in the mutant plants and the viral infection also resulted in the subsequent death of the plant. Moreover, increased phosphorylation of Arabidopsis eIF2$\alpha$ was detected in the virus-infected p58$^{IPK}$-silenced plants, suggesting a correlation of the activation of eIF2$\alpha$ phosphorylation with the loss of p58$^{IPK}$, and as a result, tissue death. The virus-induced death was rescued by the expression of the nonphosphorylatable Arabidopsis eIF2$\alpha$ mutant S51A. These experiments provide evidence that links p58$^{IPK}$, eIF2$\alpha$ phosphorylation, virus infection, and cell death in plants. Additionally, as p58$^{IPK}$-silenced NN transgenic plants were found to be completely resistant to TMV infection, it is
proposed that the antiviral pathway of p58\textsuperscript{IPK} operates independently from the N-mediated pathway,

It has been observed that some viral infections in mammals can bring about the recruitment of p58\textsuperscript{IPK} to combat the IFN-induced PKR-mediated cell death (Tan et al., 1998). This prevents the release of proinflammatory factors that slowly clear the virus from the cell and allows the viral proteins to be synthesised and propagated throughout the host. Thus, p58\textsuperscript{IPK} plays an important role in mammals in regulating the innate immune response of the host. Similarly, activated plant p58\textsuperscript{IPK} limits cell death and promotes infection of the virus (Bilgin et al., 2003). This suggests that plant p58\textsuperscript{IPK}, like its mammalian-functional homolog, can act as a virulence factor needed for successful infection without the event of host cell death.

1.3.2 Calcium-regulated Protein Kinases

During the course of this thesis, it was recognised that a group of protein kinases, found specifically in plants and some protists, and which respond to calcium as a messenger for signal transduction, was relevant for the doctoral research. It was of interest to study this group of protein kinases as they are possible candidates for the plant-functional equivalent of PKR.

1.3.2.1 The Role of Calcium in Plants

Calcium is an important ubiquitous secondary messenger in eukaryotes that is involved in numerous cell-signalling cascades in response to abiotic and biotic stimuli (reviewed in Cheng, Willmann, Chen, & Sheen, 2002; Harper & Harmon, 2005; W. Ma & Berkowitz, 2007; Romeis, Ludwig, Martin, & Jones, 2001). In plants, the modulation of intracellular calcium levels has functions in most aspects of growth and development including responses to drought, cold and salt stresses, mechanical wounding, symbionts and pathogens. In particular, perturbations in cytosolic calcium are detected as an essential early step in the perception of pathogens and are responsible for the activation of subsequent innate immune responses by plant cells to the pathogen attack. Thus, calcium acts as a messenger for a variety of biological signals, and it has been identified that these different stimuli are able to elicit specific calcium signatures which are generated by altering the kinetics, magnitude, and cellular source of the ion influx. The specific calcium signatures are recognised by different calcium sensors and result in the transduction of specific signals downstream in response to the specific stimuli.
1.3.2.2 Characteristics of Calcium-regulated Protein Kinases

Although the use of calcium is common in both animals and plants, their calcium sensors and signalling components have clearly diverged from each other. Protein kinases which are essential for calcium signalling in animals are absent in plants, and conversely, the 67 potential calcium-regulated kinases that have been identified in Arabidopsis, are not found in animals. The plant calcium sensors can be divided into three distinct groups of closely related kinases that form the calcium-regulated protein kinase superfamily (reviewed in Cheng et al., 2002; Harper & Harmon, 2005).

1.3.2.2.1 Structure and Activation of Calcium-regulated Protein Kinases

Calcium-regulated protein kinases are characterised by four distinct domains – a variable N-terminal domain that contains determinants for subcellular localisation, a highly conserved Ser/Thr protein kinase domain, an autoinhibitory domain, and a calmodulin-like domain (CaM-LD). These features are shared among the group of kinases in this superfamily but each group can be distinguished predominantly by their C-terminal regulatory CaM-LD (Fig. 1.19).

![Figure 1.19](image_url)

**Figure 1.19 Structural differences between the calcium-regulated protein kinases.** The calcium-regulated protein kinases are characterised by four distinct domains – a variable N-terminal domain (N), a Ser/Thr kinase domain (Kinase), an autoinhibitory domain (J) and a distinct C-terminal regulatory domain (CaM-LD). These regulatory domains are defined as calmodulin-like EF hands for CPK, visinin-like EF hands for CCamK, and degenerate EF hands for CRK. (modified from Harper & Harmon, 2005)
Chapter One - Introduction

The archetypal calcium-dependent protein kinase (CPK) is defined by a CaM-LD that contains four functional calcium binding EF hands with sequences similar to calmodulin. EF hands are structural domains that are found in a large family of calcium-binding proteins. These hands form a helix-loop-helix topology which bind the calcium ion and coordinate the ion via ligands within the loop. There are 34 CPKs in Arabidopsis and these are activated by binding to calcium via the EF hands. Another group of calcium-regulated protein kinases, known as CCamK, are similar in structure to CPK and have EF hands in their CaM-LD. However, CCamKs have only three EF hands which are more similar to the EF hand protein visinin than calmodulin. The CRK group of CPK-related kinases are characterised by a regulatory domain with high sequence similarity to the CaM-LD of CPKs, but their EF hands have, for the most part, evolved to no longer bind to calcium, although some isoforms are still able to be activated by exogenous calcium (Hua, Zhang, Liang, Jones, & Lu, 2004).

Conventional calcium-regulated kinases are activated by the binding of calcium to their EF hands. In the basal state, the autoinhibitor functions as a pseudosubstrate and interacts with the CaM-LD (Fig. 1.20). This intramolecular interaction blocks the active site of the enzyme and holds the kinase in an inactive state. However, in the presence of calcium, the EF hands bind to the calcium ions and a conformational change occurs whereby the autoinhibitor is disengaged from the catalytic site and the protein kinase is activated. The activation of CPKs can occur with calcium concentrations as low as 1–10 μM (Harper & Harmon, 2005).

1.3.2.2.2 Role of Calcium-regulated Protein Kinases

Even though 34 CPKs have been identified in Arabidopsis, only a few calcium-regulated protein kinases have so far been characterised, with most of the evidence suggesting that CPKs are involved in environmental stress signalling. CPK transcript levels have been reported to increase in response to cold, salt, drought, or virus infection (Babu, Griffiths, Huang, & Wang, 2008; Marathe, Guan, Anandalakshmi, Zhao, & Dinesh-Kumar, 2004; Tahtiharju, Sangwan, Monroy, Dhindsa, & Borg, 1997; Urao et al., 1994) and transcript levels also elevate when exposed to non-specific elicitors and mechanical wounding (Yoon, Cho, Ha, Liu, & Lee, 1999). Furthermore, CPK enzymatic activity has been correlated with osmotic stress (Takahashi, Isobe, & Muto, 1997) and the regulation of the phenylpropanoid pathway (Allwood, Davies, Gerrish, Ellis, & Bolwell, 1999). A specific CPK isoform in Arabidopsis has been linked with the induction of environmental stress-related promoters after abscisic acid treatment (Sheen, 1996).
Figure 1.20 Activation of calcium-regulated protein kinases through binding of calcium. In the basal state, the autoinhibitor (red rectangle) interacts with the regulatory calmodulin-like domain (orange round circles) and this interaction blocks the catalytic site of the kinase domain (K). In the presence of a calcium signal, the calcium ions (pink circles) bind to the regulatory calmodulin-like domain and this interaction alters the binding of the autoinhibitor to the kinase domain. The inhibition by the autoinhibitor on the catalytic site is released (green dotted lines) and the calcium-regulated protein kinase is activated. (Harper & Harmon, 2005)

Recently, it has been suggested that CPKs may play an essential role in the plant response to pathogen attack (W. Ma & Berkowitz, 2007; Romeis et al., 2001). As mentioned previously, an early step in pathogen perception is the elevation of cytosolic calcium. This in turn results in the activation of plant innate immune responses through a cascade of signalling by protein kinase-dependent phosphorylation which may involve the activity of CPKs. Two CPKs in potato (Solanum tuberosum CPK4 and CPK5) respond to pathogen attack by upregulating the production of reactive oxygen species through phosphorylating NADPH oxidase (Kobayashi et al., 2007) and four Arabidopsis CPKs (CPK14, CPK28, CPK29, and CPK32) are upregulated in the virus-infected transcriptome (Babu et al., 2008; Marathe et al., 2004). However, a CPK with activity that increases in response to virus infection has yet to be recognised. So far, only a few of the identified Arabidopsis CPKs have been assigned a function (CPK1 is stimulated by phospholipids; CPK4 and CPK11 are regulators of abscisic acid signal transduction; and CPK10, CPK11, and CPK30 are involved in drought and salt stress response). Thus, it is possible that more of these calcium-regulated protein kinases may be involved in the response of pathogen invasion in plants.
1.3.3 Summary for Identification of a Plant-functional Equivalent of Protein Kinase R

There is mounting evidence that supports the presence of a plant-functional equivalent of PKR. Although PKR has been extensively studied and characterised in the mammalian system, the gene sequence of pPKR has not been identified. Previous studies have used the mouse and human PKR sequences to search the Arabidopsis, rice, and tomato databanks for mPKR-like proteins, but have so far been unsuccessful. In fact, there are no genes in the Arabidopsis genome that encode for a protein containing both a dsRBM and a Ser/Thr kinase domain in the same ORF. On the other hand, there are a number of genes which show significant resemblance to each of these domains. One hypothesis is that pPKR might exist in plants as two proteins, one possessing the dsRBD and the other containing the kinase domain. These two proteins might then interact with each other to cooperatively perform the functions characteristic of mPKR. However, a 68 – 70 kDa plant protein has been identified in denaturing conditions as being phosphorylated in the presence of dsRNA and is immunoreactive with the mPKR antisera. Thus, the ‘two protein’ model is less likely to be able to explain this observation. Activity akin to PKR has been demonstrated to be present in plants, thus inspiring further research to identify the sequence of the plant-functional equivalent of this dsRNA-activated protein kinase.

1.4 Aims of the Research

The aim of this research was to investigate and identify potential candidates in Arabidopsis for the plant-functional equivalent of PKR. The specific objectives for this work were:

a. To develop an assay which would allow the rapid detection and analysis of eIF2α kinase activity in plants (Chapter 3)
b. To partially purify the putative pPKR using various purification methods and to identify candidates for the plant-functional equivalent of PKR (Chapter 4)
c. To assess the influence of ions on the phosphorylating activity of eIF2α kinases in planta (Chapter 5)
d. To determine the activity of pPKR candidates by identifying transgenic Arabidopsis plant lines containing knockouts or generating plant lines that overexpress specific genes of interest (Chapter 6)
e. To assess the protein structure and sequence of pPKR candidates for the likelihood of PKR-like activities (Chapter 7)
All chemicals used were of analytical grade unless stated otherwise. Commercial supplies of reagents were prepared and used as advised by the supplier. Radioactive nucleotide triphosphates were purchased from Amersham Biosciences or Perkin Elmer.

*freshly added to buffer solution or growth medium before use

*Arabidopsis thaliana* ecotype Columbia plants are termed *Arabidopsis*.

Transgenic *Arabidopsis* transferred DNA (T-DNA) insertion lines were imported under and grown according to the Environmental and Resource Management Authority (ERMA) decision GMO04/HRA083 in accordance to the Hazardous Substances and New Organisms (HSNO) Act 1996.

Genetically modified organisms including *Escherichia coli*, *Agrobacterium tumefaciens*, and *Arabidopsis* generated during this doctoral research were developed under and grown according to the ERMA decision GMD02088 in accordance to the HSNO Act 1996.
### 2.1 Buffers and Solutions

**Agarose gel loading buffer (10x)**
- 0.25% (w/v) bromophenol blue
- 0.25% (w/v) xylene cyanol
- 30% (v/v) glycerol

**Blocking buffer**
- 2% (w/v) BSA
  
  *Made up in 1x TBS*

**Buffer A**
- 20 mM HEPES, pH 7.5
- 100 mM KCl
- 10% (v/v) glycerol
- 5 mM MgCl\(_2\)
- 5 mM MgSO\(_4\)
- 14 mM β-mercaptoethanol*
- 0.1% (v/v) 100x Protease Inhibitor Cocktail (Sigma)*

**Buffer A/DTT**
- 20 mM HEPES, pH 7.5
- 100 mM KCl
- 10% (v/v) glycerol
- 5 mM MgCl\(_2\)
- 5 mM MgSO\(_4\)
- 10 mM DTT*
- 0.1% (v/v) 100x Protease Inhibitor Cocktail (Sigma)*

**Buffer A/DTT/20 mM KCl**
- 20 mM HEPES, pH 7.5
- 20 mM KCl
- 10% (v/v) glycerol
- 5 mM MgCl\(_2\)
- 5 mM MgSO\(_4\)
- 10 mM DTT*
- 0.1% (v/v) 100x Protease Inhibitor Cocktail (Sigma)*
<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Composition Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A/DTT (-salt)</td>
<td>20 mM HEPES, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>5 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>5 mM MgSO₄</td>
</tr>
<tr>
<td></td>
<td>10 mM DTT*</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) 100x Protease Inhibitor Cocktail (Sigma)*</td>
</tr>
<tr>
<td>Colloidal Coomassie G-250</td>
<td>17% (w/v) ammonium sulfate</td>
</tr>
<tr>
<td></td>
<td>3% (v/v) phosphoric acid</td>
</tr>
<tr>
<td></td>
<td>34% (v/v) methanol</td>
</tr>
<tr>
<td></td>
<td>0.1% (w/v) Coomassie G-250</td>
</tr>
<tr>
<td>Gel fixation solution</td>
<td>7.5% (v/v) acetic acid</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) methanol</td>
</tr>
<tr>
<td>hrCNE anode buffer</td>
<td>25 mM imidazole, pH 7.0</td>
</tr>
<tr>
<td>hrCNE cathode buffer</td>
<td>50 mM tricine</td>
</tr>
<tr>
<td></td>
<td>7.5 mM imidazole, pH 7.0</td>
</tr>
<tr>
<td></td>
<td>0.05% (w/v) sodium deoxycholate</td>
</tr>
<tr>
<td>hrCNE sample buffer</td>
<td>50% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) Ponseau S</td>
</tr>
<tr>
<td></td>
<td>0.02% (v/v) dodecylmaltoside</td>
</tr>
<tr>
<td>IMPACT Column buffer</td>
<td>20 mM HEPES, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>500 mM NaCl</td>
</tr>
<tr>
<td>IMPACT Cleavage buffer</td>
<td>20 mM HEPES, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>500 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>50 mM DTT</td>
</tr>
<tr>
<td>Buffer Type</td>
<td>Composition</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Lysis buffer</strong></td>
<td>150 mM PIPES, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>50 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>100 mM KCl</td>
</tr>
<tr>
<td></td>
<td>14 mM β-mercaptoethanol*</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) 100x Protease Inhibitor Cocktail (Sigma)*</td>
</tr>
<tr>
<td></td>
<td>3 mM PMSF*</td>
</tr>
<tr>
<td><strong>Methanol destain</strong></td>
<td>50% (v/v) methanol</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) glacial acetic acid</td>
</tr>
<tr>
<td><strong>10x phosphate buffered saline (PBS)</strong></td>
<td>1.5 M NaCl</td>
</tr>
<tr>
<td></td>
<td>15 mM KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>80 mM Na₂HPO₄</td>
</tr>
<tr>
<td></td>
<td>25 mM KCl</td>
</tr>
<tr>
<td></td>
<td><em>The stock solution was diluted ten-fold with distilled H₂O before use</em></td>
</tr>
<tr>
<td><strong>Ribosomal Salt Wash buffer</strong></td>
<td>20 mM HEPES, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>1.5 M KCl</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>5 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>5 mM MgSO₄</td>
</tr>
<tr>
<td></td>
<td>14 mM β-mercaptoethanol*</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) 100x Protease Inhibitor Cocktail (Sigma)*</td>
</tr>
<tr>
<td><strong>4x SDS PAGE Running buffer</strong></td>
<td>12% (w/v) Tris</td>
</tr>
<tr>
<td></td>
<td>57.6% (w/v) glycine</td>
</tr>
<tr>
<td></td>
<td>0.1% (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td><em>The stock solution was diluted four-fold with distilled H₂O before use</em></td>
</tr>
</tbody>
</table>
2x gel loading buffer  
100 mM Tris, pH 6.8  
4% (w/v) SDS  
0.2% (w/v) bromophenol blue  
20% (v/v) glycerol  
14 mM β-mercaptoethanol*

50x TAE buffer  
40 mM Tris, pH 8.0  
20 mM acetic acid  
50 mM EDTA  
The stock solution was diluted 50-fold with distilled H₂O before use

10x tris buffered saline (TBS)  
20 mM Tris, pH 7.5  
150 mM NaCl  
The stock solution was diluted ten-fold with distilled H₂O before use

Western transfer buffer  
48 mM Tris  
39 mM glycine  
20% (v/v) methanol  
0.0375% (w/v) SDS

2.2 Enzymes and Antibiotics

Enzymes were purchased from New England Biolabs, Invitrogen, and Roche Diagnostics, with all necessary buffers, and were used as advised by the supplier.

Stock solutions of all antibiotics were prepared using the appropriate solvent at the concentration listed in Table 2.1 and filter-sterilised with a 0.2 µm filter (Minisart, Satorius).
Table 2.1 Antibiotics used to select bacterial recombinants and transgenic plant lines

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Solvent</th>
<th>Stock concentration (g/L)</th>
<th>Final concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Distilled H₂O</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Ethanol</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Distilled H₂O</td>
<td>100</td>
<td>50 or 100</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>DMSO</td>
<td>33</td>
<td>100</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Distilled H₂O</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*a* solvent with which the antibiotic was dissolved in; *b* final concentrations used for different experiments; DMSO, dimethyl sulfoxide

2.3 Plasmids

The pCR®2.1-TOPO® vector (Invitrogen) was used for the subcloning of polymerase chain reaction (PCR) amplification products (Fig. 2.1). This linearised plasmid vector contains single 3’deoxythymidine overhangs that facilitate TA Cloning®. Topoisomerase I is also covalently bound to the vector which binds to duplex DNA at specific sites and catalyses the ligation of the DNA insert into the plasmid vector via their respective adenosine and thymidine overhangs.

A binary vector system developed by Gleave (1992) was used for the subcloning of gene constructs for in planta gene expression. This system consists of two plasmids, pART7 (Fig. 2.2) and pART27 (Fig. 2.3). The primary cloning vector, pART7, contains a multiple cloning site where the gene of interest is inserted between the Cauliflower mosaic virus 35S promoter and the transcriptional termination region of the octopine synthase gene (ocs3’). The 35S-gene-ocs3’ sequence can then be removed as a NotI fragment and introduced into the binary vector, pART27. This NotI fragment is inserted between the left and right border sequences of the T-DNA of A. tumefaciens, and allows for A. tumefaciens-mediated T-DNA transformation to occur.

The pTYB2 vector (New England Biolabs) was used for the cloning and expression of recombinant proteins in E. coli (Fig. 2.4). This C-terminal fusion vector contains a multiple cloning site where the gene of interest is inserted adjacent to an intein tag with a chitin binding domain. The intein tag is a protein splicing element which has thiol-inducible self-cleavage activity and allows the expressed protein to be separated from the affinity tag. A T7/lac promoter is also present which allows the induction of gene expression with isopropylthio-β-galactoside (IPTG).
Figure 2.1 Vector sequence map of pCR®2.1-TOPO® showing some of the restriction enzymes sites and features of interest.

Figure 2.2 Vector sequence map of pART7 showing some of the restriction enzymes sites and features of interest.
Figure 2.3 Vector sequence map of pART27 showing some of the restriction enzymes sites and features of interest.

Figure 2.4 Vector sequence map of pTYB2 showing some of the restriction enzymes sites and features of interest. (modified from https://www.lablife.org/ct?a=viewvecseq&soid=5475&view=Draw&f=v)
2.4 Bacteria Used for Cloning and Plant Transformations

2.4.1 *Escherichia coli*

The *E. coli* strains DH5α™ (Invitrogen), DH10B™ (Invitrogen) and BL21-CodonPlus® (DE3)-RIL (Stratagene) were used as bacterial transformation hosts in the research thesis. The genotypes of these bacterial strains were as follows:

DH5α™  
F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA 
supE44 λ- thi-1 gyrA96 relA1

DH10B™  
F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 
Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG

BL21-CodonPlus® (DE3)-RIL  
F- ompT hsdS(rB- mB-) dcm+ Tet⁰ gal λ(DE3) endA Hte [argU ileY leuW Cam⁰]

2.4.2 *Agrobacterium tumefaciens*

The avirulent *A. tumefaciens* strain GV3101 was used for the transformation of *Arabidopsis* plant cells. GV3101 contains a rifampicin resistance gene as a chromosomal marker and a disarmed Ti-plasmid pMP90 which does not have its own T-DNA but has retained the functional *vir* region that is capable of transferring T-DNA into plants (Hellens, Edwards, Leyland, Bean, & Mullineaux, 2000).
2.4.3 Bacterial Growth Media

All media were autoclaved for 15 min at 121°C before use.

Luria-Bertani (LB) broth 1% (w/v) bacto-tryptone (Difco)
0.5% (w/v) yeast extract (Difco)
1% (w/v) NaCl, pH 7.0

LB plates 1.5% (w/v) bacto-agar (Difco)
*Made up in LB broth*

SOC medium 2% (w/v) bacto-tryptone (Difco)
0.5% (w/v) yeast extract (Difco)
8 mM NaCl, pH 7.0
2.5 mM KCl
20 mM glucose*

2YT broth 1.6% (w/v) bacto-tryptone (Difco)
1% (w/v) yeast extract (Difco)
0.5% (w/v) NaCl, pH 7.0

2.4.4 Maintenance and Selection of Bacterial Strains and Plasmids

The plasmids pCR®2.1-TOPO®, pART7, and pTYB2 were maintained in *E. coli* using ampicillin (100 mg/L). The plasmid pART27 was maintained in *E. coli* and *A. tumefaciens* using spectinomycin (100 mg/L). *A. tumefaciens* was maintained using rifampicin (100 mg/L). *E. coli* BL21 cells were maintained using chloramphenicol (34 mg/L).
2.5 Manipulation of Bacteria

2.5.1 *Escherichia coli* transformation

2.5.1.1 Heat-shock Transformation

*E. coli* MAX Efficiency® DH5α™ Chemically Competent cells (Invitrogen) and BL21-CodonPlus® (DE3)-RIL competent cells (Stratagene) were chemically transformed with plasmid DNA using the heat-shock method. DNA (1 to 2 μL, 10 to 20 ng) was diluted 50-fold in competent cells. The cells were incubated on ice for 30 min, heat-shocked in a 42°C water bath (Julabo® TW20 Water Bath) for 45 sec, and immediately placed on ice for at least 1 min. The heat-shocked cells were then incubated in 0.5 mL SOC medium for 1 hr at 37°C, shaking at 225 rpm (Gallenkamp Orbital Incubator). Incubated cells (200 μL) were plated on LB plates supplemented with the appropriate antibiotic(s). Small colonies appeared on the surface of the agar after an overnight incubation at 37°C.

2.5.1.2 Electroporation Transformation

*E. coli* One Shot® TOP10 Electrocomp™ DH10B™ Electrocompetent cells (Invitrogen) were electroporated with plasmid DNA using the GibBiorBRL Cell Porator® (Life Technologies). The settings were 330 μF capacitance, low Ω (impedance), fast charge rate and 4 kΩ on the voltage booster. DNA (1 μL, 10 to 20 ng) was added to 20 μL of cells. The cells were electroporated once (one pulse) and then immediately incubated in 0.5 mL SOC medium for 1 hr at 37°C, shaking at 225 rpm (Gallenkamp Orbital Incubator). Incubated cells (100 to 150 μL) were plated on LB plates supplemented with the appropriate antibiotic(s). Small colonies appeared on the surface of the agar after an overnight incubation at 37°C.

2.5.1.3 Blue/White Screening

For plasmids pCR®2.1-TOPO® and pART27 where the α-component of the lacZ gene was located around the multiple cloning site of the plasmid, blue/white screening of transformant bacterial cells was applied. *E. coli* cells transformed with the lacZ gene-containing plasmids were plated on LB/antibiotic(s) media supplemented with 1 mg X-gal per plate (Duchefa Biochemie).
and incubated overnight at 37°C. The lacZ gene encodes for a β-galactosidase which hydrolyses the histochemical substrate X-gal into a blue precipitate. Non-transformant colonies have their lacZ gene intact and appear blue on culture medium supplemented with X-gal, whereas transformant colonies, carrying an additional DNA fragment that disrupts the lacZ gene, appear white.

2.5.2 Agrobacterium tumefaciens Transformation

*Agrobacterium tumefaciens* GV3101 electrocompetent cells were electrotransformed with plasmid DNA using the GIBROBRL Cell Porator® (Life Technologies). The settings were 330 μF capacitance, low Ω (impedance), fast charge rate and 4 kΩ on the voltage booster. DNA (1 μL, 10 to 20 ng) was added to 20 μL of cells. The cells were electroporated once (one pulse) and then immediately incubated in 0.5 mL SOC medium for 4 hr at 28°C, shaking at 225 rpm (Gallenkamp Orbital Incubator). Incubated cells (100 μL) were plated on LB plates supplemented with the appropriate antibiotic(s). Small colonies appeared on the surface of the agar after 48 hr of incubation at 28°C.

2.6 Nucleic acid manipulations

2.6.1 Polymerase Chain Reaction

The DNA of genes of interest was obtained for cloning through DNA amplification by PCR using specific primers. Table 2.2 lists the oligonucleotide primers which were used for DNA amplification in the research thesis. A typical PCR reaction mix contained 2 μL DNA template (approximately 500 ng), 5 μL 10x PCR buffer, 1.5 mM MgCl₂, 200 μM 2’-deoxynucleotide 5’-triphosphates (dNTPs), 500 μM of each primer, and 1 unit Platinum® Taq DNA Polymerase (Invitrogen) in a 50 μL total reaction volume. Tubes containing water as template acted as negative controls.

The PCR reaction was performed in a Mastercycler (Eppendorf) with the following programme: one initiation cycle at 94°C for 10 min, 30 cycles at 94°C, 58°C, and 72°C for 30 sec each or longer at 72°C as required to provide 1 min per kilobase to be amplified, then one termination cycle at 72°C for 10 min.
Table 2.2 Oligonucleotide primers used for DNA amplification in the research thesis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Targeted gene nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>42563378 F 1296</td>
<td>ATT TGG AGT CAT CCT GCT CGA ACT TCT C</td>
<td><em>Arabidopsis</em> kinase with unknown function (At1g80640) nt 1296 - 1323</td>
</tr>
<tr>
<td>28950719 R 171</td>
<td>CTT GAA GTC AGT CTT CTC TTG GAA TCT C</td>
<td><em>Arabidopsis</em> dsRBD-containing protein (At1g80650) nt 171 - 144</td>
</tr>
<tr>
<td>At eIF2A F EcoRI NcoI</td>
<td>CCC TTG AAT TCC *ATG GCG AAT CCT GCT</td>
<td><em>Arabidopsis</em> eIF2α (At5g05470) nt 1 – 15</td>
</tr>
<tr>
<td>At eIF2A R BamHI</td>
<td>CCC TTG GAT CC^T CAT TCA ATT ATC CGG CTA</td>
<td><em>Arabidopsis</em> eIF2α (At5g05470) nt 1335 - 1317</td>
</tr>
<tr>
<td>At eIF2A R 181-148 noSacI WT</td>
<td>AAT CCG ACG GCG AGA GAG tTC GGA GAA CAG GAT</td>
<td><em>Arabidopsis</em> eIF2α (At5g05470) nt 181 – 148; mutation at nt 162</td>
</tr>
<tr>
<td>At eIF2A R 181-148 noSacI S56A</td>
<td>AAT CCG ACG GCG Agc GAG tTC GGA GAA CAG GAT</td>
<td><em>Arabidopsis</em> eIF2α (At5g05470) nt 181 – 148; mutation at nt 162 &amp; 166</td>
</tr>
<tr>
<td>At eIF2A R 181-148 noSacI S56D/E</td>
<td>AAT CCG ACG GCG mtc GAG tTC GGA GAA CAG GAT</td>
<td><em>Arabidopsis</em> eIF2α (At5g05470) nt 181 – 148; mutation at nt 162 &amp; 166 – 168</td>
</tr>
<tr>
<td>At eIF2A F 170-197</td>
<td>GCC GTC GGA TTC GTA GTA TCA GTA GCT</td>
<td><em>Arabidopsis</em> eIF2α (At5g05470) nt 170 – 197</td>
</tr>
<tr>
<td>At GCN2 5’ EcoRI Ndel</td>
<td>ATT TGA ATT CAT *ATG GGT CGC AGC AGT TC</td>
<td><em>Arabidopsis</em> GCN2 (At3g59410) nt 1 – 17</td>
</tr>
<tr>
<td>At GCN2 3’ BglII</td>
<td>AAC CAA AGA TC^T TAG CTC CAA ACA GAG G</td>
<td><em>Arabidopsis</em> GCN2 (At3g59410) nt 3726 – 3710</td>
</tr>
<tr>
<td>At GCN2 hp1 5’ Xhol</td>
<td>TCT TCT CGA GGA CCA GCC AAA TTC CAA</td>
<td><em>Arabidopsis</em> GCN2 (At3g59410) nt 351 – 377</td>
</tr>
<tr>
<td>At GCN2 hp1 3’ BglII</td>
<td>CAA CAG ATC TTT CTT GCT AAT CTG AGG</td>
<td><em>Arabidopsis</em> GCN2 (At3g59410) nt 1005 – 979</td>
</tr>
<tr>
<td>At GCN2 hp2 5’ BamHI</td>
<td>AAT TGG ATC CTT GAA GAT TGG ATT CCT</td>
<td><em>Arabidopsis</em> GCN2 (At3g59410) nt 892 – 875</td>
</tr>
<tr>
<td>At GCN2 hp2 3’ XbaI</td>
<td>AAC CTC TAG ACC AGG CAA ATT CCA ATG</td>
<td><em>Arabidopsis</em> GCN2 (At3g59410) nt 361 - 379</td>
</tr>
<tr>
<td>At IPK hp1 5’ XhoI</td>
<td>AAT TCT CGA GCA GAG AGG AGA AGC TAA</td>
<td><em>Arabidopsis</em> p58&lt;sup&gt;IPK&lt;/sup&gt; (At5g03160) nt 964 – 980</td>
</tr>
<tr>
<td>At IPK hp1 3’ BglII</td>
<td>AAC CAG ATC TCC AGC AAA ACC ACC CTC</td>
<td><em>Arabidopsis</em> p58&lt;sup&gt;IPK&lt;/sup&gt; (At5g03160) nt 1418 – 1399</td>
</tr>
<tr>
<td>At IPK hp2 5’ BamHI</td>
<td>AAT TGG ATC CAC CCA TAT CTT CAA GAT</td>
<td><em>Arabidopsis</em> p58&lt;sup&gt;IPK&lt;/sup&gt; (At5g03160) nt 1329 – 1310</td>
</tr>
<tr>
<td>At IPK hp2 3’ XbaI</td>
<td>CCG CTC TAG AAG CTA AAC TGT TGC TTG</td>
<td><em>Arabidopsis</em> p58&lt;sup&gt;IPK&lt;/sup&gt; (At5g03160) nt 972 – 991</td>
</tr>
</tbody>
</table>

Underlined nucleotides, location of restriction enzyme sites; bold text, sense coding sequence; italic text, antisense coding sequence; *, start codon of target gene; ^, stop codon of target gene; lower case letters, mutated base; nt, nucleotide
2.6.2 Gel Electrophoresis of Nucleic Acids

Agarose gel electrophoresis was performed using submerged horizontal gels. DNA preparations containing 10% (v/v) agarose gel loading buffer were loaded in 1% (w/v) UltraPure™ agarose (Invitrogen) TAE-buffered gels. DNA fragments were separated at 110 V for 30 to 45 min. The 1 kb Plus DNA Ladder (Invitrogen) was used for size determination of the DNA fragments. Agarose gels were stained for 30 min in 0.5 mg/L ethidium bromide solution in TAE. Nucleic acid bands were visualised under short wave UV light (250 – 310 nm) and digital images were taken using the Gel Doc 1000 imaging system (Bio-Rad).

2.6.3 DNA Ligation

2.6.3.1 DNA Ligation Using the pCR®2.1-TOPO® System

The pCR®2.1-TOPO® system was used mainly for the subcloning of PCR fragments. PCR products were first A-tailed by extension at 72°C for 10 min in the presence of the PCR reaction mix (Section 2.6.1) and 1 unit Taq DNA Polymerase (Invitrogen). A-tailing results in the addition of a single deoxyadenosine to the 3’ ends of PCR products and allows the A-tailed fragments to ligate efficiently to the single 3’deoxythymidine overhangs on the pCR®2.1-TOPO® vector. Following A-tailing, PCR products were ligated to pCR®2.1-TOPO® in a ligation reaction containing 1 μL Salt Solution, 10 ng pCR®2.1-TOPO® vector, and 50 to 100 ng PCR product in a 6 μL total reaction volume. The reaction mix was incubated for 5 to 30 min at room temperature.

2.6.3.2 DNA Ligation into Other Plasmid Vectors

For the ligation of DNA into plasmid vectors such as pART7, pART27, and pTYB2, a typical ligation reaction containing 1 μL 5x T4 DNA ligase reaction buffer, 1 unit T4 DNA ligase (Invitrogen), 100 ng plasmid vector, and 50 to 200 ng DNA insert in a 10 μL total reaction volume was incubated overnight in wet ice that increased from 4 to 16°C. Control tubes containing water in place of either insert or ligase enzyme acted as an indication of vector self-igation rates or amount of uncut vector, respectively.
2.6.4 Extraction of Plasmid DNA from *Escherichia coli* Cultures

Plasmid DNA was extracted from bacterial cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Aliquots (2 mL) of LB broth supplemented with the appropriate antibiotic(s) were inoculated with single colonies grown on LB/antibiotic(s) plates and incubated overnight at 37°C, shaking at 225 rpm (Gallenkamp Orbital Incubator). The overnight cultures were then collected by centrifugation and the bacterial sediments were resuspended, alkaline lysed, and neutralised to precipitate the cellular debris. Following centrifugation, the aqueous phase was transferred to a supplied spin column and washed with the Column Wash Solution. The resultant plasmid DNA was eluted with 100 μL distilled water and stored at -20°C.

2.6.5 Determination of DNA Concentration

The concentration of the prepared DNA was determined by measuring the absorbance at 260 nm in a ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies). An absorbance of 1.0 at 260 nm (A_{260}) corresponded to 50 μg/μL of DNA.

2.6.6 Restriction Enzyme Digestion

The digestion of plasmid DNA by restriction enzymes was used to excise DNA for subsequent subcloning procedures and to assess the correct subcloning of DNA inserts into a specific vector.

2.6.6.1 Restriction Enzyme Digestion for the Excision of DNA

Plasmid DNA was digested for at least 1 hr at the temperature recommended by the supplier, in a typical 20 μL reaction containing 10 μL DNA sample (approximately 2 μg), 2 μL 10x buffer as supplied with the restriction enzyme, 100 μg/mL bovine serum albumin (BSA, when required), and 2 units restriction enzyme.

2.6.6.2 Restriction Enzyme Digestion for Assessment of Correct DNA Insertion

Plasmid DNA was digested for at least 1 hr at the temperature recommended by the supplier, in a typical 10 μL reaction containing 1 μL DNA sample (approximately 200 ng), 1 μL 10x buffer as
supplied with the restriction enzyme, 100 μg/mL BSA (when required), and 0.5 to 1 unit restriction enzyme.

2.6.7 Ethanol Precipitation of DNA

DNA preparations were purified and adjusted to the desired concentration using ethanol precipitation. Sodium acetate was added to the sample to a final concentration of 0.3 M, together with 2.5 volumes of 100% (v/v) ethanol. The nucleic acid/ethanol mix was incubated at -80°C for at least 30 min and the DNA was then pelleted at 15 700 x g for 15 min at 4°C (Centrifuge 5415 R, Eppendorf). The pellet was washed with 70% ethanol, air dried and resuspended in the desired volume of sterile distilled water.

2.6.8 Gel Extraction of DNA

The extraction of DNA from agarose gels was performed using the Perfectprep® Gel Cleanup kit (Eppendorf). The desired DNA band was first excised from the agarose gel and weighed. Three gel volumes of Binding Buffer was added to the gel and the gel buffer mix was incubated at 50°C for 10 min. One gel volume of isopropanol was subsequently added to the gel buffer mix. The mixture was transferred to a supplied spin column and washed with the Wash Buffer. The resultant plasmid DNA was eluted with 20 μL distilled water and stored at -20°C.

2.6.9 DNA Sequencing Analyses

The sequences of DNA plasmid clones were determined by conducting sequence analyses at the DNA Sequencing Facilities at The New Zealand Institute for Plant and Food Research Ltd (Plant and Food Research, Mt Albert) or the Allan Wilson Centre of Massey University (Albany or Palmerston North). Template and primers were prepared to the required purity and free of contaminating salts, solvents, RNA, proteins and chelating agents. Table 2.3 lists the sequencing primers used in the research thesis. Sequence data was examined using ContigExpress (VectorNTI Advance 10, Invitrogen).
Table 2.3 Oligonucleotide primers used for the sequencing of DNA plasmid clones in the research thesis

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence (5' → 3')</th>
<th>Sequencing of</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 Forward</td>
<td>GTA AAA CGA CGG CCA G</td>
<td>pCR®2.1-TOPO®-cloned genes</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>CAG GAA ACA GCT ATG AC</td>
<td></td>
</tr>
<tr>
<td>T7 Promoter</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
<td></td>
</tr>
<tr>
<td>Sp6</td>
<td>ATT TAG GTG ACA CTA TAG</td>
<td></td>
</tr>
<tr>
<td>3SS</td>
<td>ACA GTG GTC CCA AAG ATG GAC</td>
<td>pART7-cloned genes</td>
</tr>
<tr>
<td>OCS</td>
<td>AGA ATG AAC CGA AAC CGG CG</td>
<td></td>
</tr>
<tr>
<td>At GCN2 5’ 201bp</td>
<td>GCC GTA CTC AAA GGACAT GGG</td>
<td>Arabidopsis GCN2</td>
</tr>
<tr>
<td>At GCN2 5’ 601bp</td>
<td>GCA AGA AAT TGG AGT CTG ACT</td>
<td></td>
</tr>
<tr>
<td>At GCN2 5’1001bp</td>
<td>TGT TGA TGG TCC ATT TAC TTC</td>
<td></td>
</tr>
<tr>
<td>At GCN2 5’ 1401bp</td>
<td>TCG GAT AGT TCG AGA AGT AGC</td>
<td></td>
</tr>
<tr>
<td>At GCN2 5’ 2701bp</td>
<td>TCC CTG ACA GAG GCA GAA GTT</td>
<td></td>
</tr>
</tbody>
</table>

2.7 Protein manipulations

2.7.1 Extraction of Proteins from Plant Tissue

Plant tissue was harvested and homogenised in Buffer A (refer to Section 2.1) unless otherwise stated. For small tissue samples (1 to 2 mL), the lysed plant extract was centrifuged at 15 700 x g for 5 min at 4°C (Centrifuge 5415 R, Eppendorf) to remove any plant debris. For larger tissue samples, the lysed plant extract was pressed through miracloth and ultracentrifuged at 35 000 x g for 20 min at 4°C (CP100MX, Hitachi Koki). The resultant supernatant was termed the clarified plant extract. Protein samples to be stored for future use were mixed with glycerol to a final concentration of 10% (v/v), frozen in liquid nitrogen, and stored at -80°C.

2.7.2 Subcellular Fractionation of Proteins Extracted from Plant Tissue

The clarified plant extract was separated into cytoplasmic and ribosomal fractions by ultracentrifugation at 490 000 x g for 33 min at 4°C (CP100MX, Hitachi Koki). The resultant supernatant was the cytoplasmic fraction and the pellet comprised the ribosomes. The ribosomal pellet was mixed gently in Ribosomal Salt Wash buffer using a glass rod and fully resuspended by
stirring with a magnetic stir bar for 2 hr at 4°C. The ribosome suspension was then ultracentrifuged at 220 000 x g for 40 min at 4°C (CP100MX, Hitachi Koki). The resultant supernatant was the ribosomal fraction.

2.7.3 Expression and Purification of Recombinant Proteins in *Escherichia coli*

2.7.3.1 Growth and Induction of Bacteria

Protein expression was performed in *E. coli* BL21 cells containing the gene construct of interest. A single colony of bacteria was used to inoculate 5 mL of 2YT media, supplemented with 100 mg/L ampicillin and 34 mg/L chloramphenicol, and incubated overnight at 37°C, shaking at 225 rpm (Gallenkamp Orbital Incubator). Subsequently, 1 mL of the overnight culture was used to inoculate 100 mL 2YT media supplemented with the same antibiotics, and the culture was grown at 37°C until it reached an A₆₀₀ of 1.0. The culture was then transferred to a 20°C shaking incubator, shaking at 225 rpm (Gallenkamp Orbital Incubator) and allowed to equilibrate to that temperature for 20 min. Expression was induced by the addition of IPTG to a final concentration of 0.3 mM and the culture was grown for a further 18 hr at 20°C.

2.7.3.2 Extraction of Soluble Protein from *Escherichia coli* Cells

Following the induction of protein expression, cells were harvested by centrifugation at 5000 x g for 10 min at 4°C (Sorvall® RC-5B Refrigerated Superspeed Centrifuge, Du Pont). The pelleted cells were resuspended in 20 mL IMPACT column buffer and were lysed by passing the whole 20 mL cell suspension twice through a gas powered emulsifier Emulsiflex C5 (Avestin), at a pressure of 15 000 p.s.i. The bacterial suspension was passed twice through the emulsifier to ensure efficient lysis of the cells. The lysates were subsequently centrifuged at 20 000 x g for 30 min at 4°C (Sorvall® RC-5B Refrigerated Superspeed Centrifuge, Du Pont). The resultant supernatant represented soluble proteins, while the pelleted fraction represented the insoluble proteins.

2.7.3.3 Purification of Recombinant Intein-tagged Protein

The soluble protein extract was purified using the Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT)™-CN System (New England Biolabs), which has the ability to
purify, in a single chromatographic step, a native recombinant protein without the use of a protease. Chitin resin (2 mL) was loaded into a 50 mL syringe barrel and covered with Whatman Grade No. 1 filter paper (Whatman). Column flow was gravity driven and the chitin column was pre-equilibrated with 10 volumes of IMPACT column buffer. The protein sample was slowly loaded onto the column and allowed to flow-through. The column was washed with 15 volumes of IMPACT column buffer and self-cleavage activity of the intein was induced with three volumes of IMPACT cleavage buffer. A few drops were allowed to pass through the column before the flow was stopped and the column was left to incubate in the cleavage buffer overnight at 4°C. The cleaved proteins were then eluted in 1 mL fractions. Cleavage efficiency was assessed by taking 200 μL of chitin resin and running the bound proteins on a protein gel.

2.7.4 Desalting of Protein

Salts or ATP were removed from protein samples using Bio-Spin® P-6 Columns (Bio-Rad). These chromatography columns allowed rapid and efficient cleanup and purification of proteins and retained proteins or other soluble compounds under the molecular weight of 6000 Da. The column was first drained by gravity of its excess packing buffer and equilibrated with four washes of 0.5 mL Buffer A unless otherwise stated. The buffer was drained by gravity except for the last wash where the column was centrifuged using a swinging bucket centrifuge at 1000 x g for 1 min (MSE Centaur 2). Protein sample (50 to 100 μL) was then loaded onto the column and the column was centrifuged at 1000 x g for 4 min. The eluted protein was collected in a 1.5 mL microcentrifuge tube.

2.7.5 Determination of Protein Concentration

The concentration of proteins was evaluated using the Bio-Rad Protein Assay (Bio-Rad) which measures the differential absorbance at 595 nm due to the color change of a dye in response to various protein concentrations. The diluted Dye Reagent was prepared by mixing one part Dye Reagent Concentrate with four parts distilled water and filtering the diluted Dye Reagent through Whatman Grade No. 1 filter paper (Whatman). Protein standards were prepared by diluting concentrated BSA to 0.2, 0.4, 0.6, and 0.8 mg/mL BSA. Each standard and sample solution were then added to the diluted Dye Reagent in a ratio of one part protein sample to 50 parts diluted Dye Reagent and incubated at room temperature for at least 5 min. The absorbance of the samples was then measured at 595 nm using a PYE Unicam PU 8600 UV/VIS Spectrophotometer (Philips).
2.7.6 Protein Kinase Assay

Protein kinase phosphorylation was performed by adding 2.5 μM ATP (Sigma) and 200 μCi/mL γ^{32}P-ATP (Amersham Biosciences or Perkin Elmer) to the protein extract and incubating the reaction mix for 5 min at room temperature.

2.7.7 Denaturing Gel Electrophoresis

2.7.7.1 Denatured Protein Sample Preparation

Protein samples were mixed with 2x SDS gel loading buffer in a ratio of one part protein sample to one part loading buffer. After mixing, the samples were placed in a boiling water bath for 5 min to denature the protein structure and then centrifuged at maximum speed for 1 min.

2.7.7.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Denatured protein samples were analysed on a 10% SDS polyacrylamide gel which consisted of a 10% separating gel and a 5% stacking gel. The SDS polyacrylamide gels were hand-cast and assembled with the Bio-Rad gel apparatus. The recipes for the polyacrylamide gels were as follows:

10% separating gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% (v/v) Acrylamide/Bis Solution</td>
<td>29:1 (Bio-Rad)</td>
</tr>
<tr>
<td>380 mM Tris (pH 8.8)</td>
<td></td>
</tr>
<tr>
<td>1% (w/v) SDS</td>
<td></td>
</tr>
<tr>
<td>1% (w/v) ammonium persulfate (Bio-Rad)</td>
<td></td>
</tr>
<tr>
<td>0.08% (v/v) TEMED (Bio-Rad)</td>
<td></td>
</tr>
</tbody>
</table>

5% stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% (v/v) Acrylamide/Bis Solution</td>
<td>29:1 (Bio-Rad)</td>
</tr>
<tr>
<td>125 mM Tris (pH 6.8)</td>
<td></td>
</tr>
<tr>
<td>1% (w/v) SDS</td>
<td></td>
</tr>
<tr>
<td>1% (w/v) ammonium persulfate (Bio-Rad)</td>
<td></td>
</tr>
<tr>
<td>0.2% (v/v) TEMED (Bio-Rad)</td>
<td></td>
</tr>
</tbody>
</table>
Denatured proteins (20 to 30 μL) was loaded onto the SDS polyacrylamide gel and proteins were separated in 1x SDS PAGE running buffer at 130 V for 1 hr 20 min or until the dye front had left the bottom of the gel. The Precision Plus Protein™ Dual Color Standards (BioRad) was used for size determination of the protein samples.

2.7.8 Native Gel Electrophoresis

Two different electrophoresis systems were used for the analysis of native protein samples. Originally the NativePAGE™ (Invitrogen) system was used (Section 2.7.8.2). This method was later improved by using a clear native electrophoresis technique (Wittig, Karas, & Schagger, 2007) (Section 2.7.8.3).

2.7.8.1 Native Protein Sample Preparation

Protein samples to be analysed with the NativePAGE™ system were mixed with 1x NativePAGE™ Sample Buffer and 0.25% (v/v) NativePAGE™ 5% G-250 Sample Additive.

Protein samples to be analysed with the clear native electrophoresis system were mixed with hrCNE sample buffer in a ratio of one part sample buffer to eight parts protein sample.

2.7.8.2 NativePAGE™ Gel Electrophoresis

The NativePAGE™ Gel system was based on the Blue Native Polyacrylamide Gel Electrophoresis (BN PAGE) technique developed by Schägger and von Jagow (1991) that uses Coomassie® G-250 as a charge-shift molecule. Native protein samples were analysed on pre-cast NativePAGE™ Novex® 4-16% Bis-Tris Gels (Invitrogen). The NativePAGE™ gels were assembled in the XCell SureLock™ Mini-Cell (Invitrogen) apparatus. The upper buffer chamber was filled with 1x NativePAGE™ Cathode Buffer (1x NativePAGE™ Running Buffer with 1x NativePAGE™ Cathode Additive) and the lower buffer chamber was filled with 1x NativePAGE™ Anode Buffer (1x NativePAGE™ Running Buffer). Protein sample (10 to 20 μL) was loaded onto the NativePAGE™ gel and proteins were separated at 150 V for 2 hr at 4°C or until the dye front had left the bottom of the gel. The NativeMark™ Unstained Protein Standard (Invitrogen) was used for size determination of the protein samples.
2.7.8.3 High Resolution Clear Native Electrophoresis (hrCNE)

The hrCNE system is based on the BN PAGE technique but uses non-coloured mixtures of anionic and neutral detergents as the charge-shift molecule, in place of Coomassie® G-250, and this allows high resolution of protein separation by clear native electrophoresis. Similar to the NativePAGE™ Gel system, native protein samples were analysed on pre-cast NativePAGE™ Novex® 4-16% Bis-Tris Gels (Invitrogen) and the NativePAGE™ gels were assembled in the XCell SureLock™ Mini-Cell (Invitrogen) apparatus. However, the upper buffer chamber was filled with hrCNE cathode buffer and the lower chamber was filled with hrCNE anode buffer. Protein sample (10 to 20 μL) was loaded onto the NativePAGE™ gel and proteins were initially separated at 100 V. When the sample had entered the gel, the proteins were separated at 500 V with the current limited to 15 mA for 3 hr at 4°C or until the dye front had left the bottom of the gel. The NativeMark™ Unstained Protein Standard (Invitrogen) was used for size determination of the protein samples.

2.7.9 Staining of Protein Gels

2.7.9.1 Colloidal Coomassie® Staining

Protein gels were stained for 1 hr to overnight at room temperature in 20 mL Colloidal Coomassie G-250 stain with gentle shaking (ProBlot Rocker 25). Subsequently, gels were washed with fresh changes of water for 1 to 2 days to remove excess stain.

2.7.9.2 Deep Purple™ Staining

Protein gels were fixed for 1 hr to overnight at room temperature in 50 mL gel fixation solution with gentle shaking (ProBlot Rocker 25). Subsequently, gels were stained for 1 hr at room temperature in 20 mL staining solution comprising one part Deep Purple™ stain (Amersham Biosciences) to 200 parts 100 mM sodium borate, pH 10.5, with gentle shaking (ProBlot Rocker 25) and covered with foil. Protein gels were then washed with 15% (v/v) ethanol for 30 min and acidified by placing the gels in 15% (v/v) ethanol/1% (v/v) citric acid, pH 2.3, for 30 min. The gels were visualised using a flat-bed laser based fluorescence Typhoon 9400 Variable Mode Imager (Amersham Biosciences).
The settings were as follows:

Excitation: Green laser (532 nm)
Emission: 560 LP general filter
PMT voltage rating: 550 V

Images were pre-scanned at 1000 μm resolution and then scanned using 100 μm resolution. Images were viewed using ImageQuant TL (Amersham Biosciences).

2.7.10 Immunoblotting

Following separation by gel electrophoresis, proteins were transferred from the polyacrylamide gel to a membrane to assess their immunoreactivity to antibodies specific to a target protein. An 8 x 10 cm Immun-Blot™ polyvinylidene fluoride (PVDF) Membrane (0.2 μm, Bio-Rad) was pre-soaked in methanol for 5 min and subsequently equilibrated in western transfer buffer. A gel blot sandwich was then assembled, immersed in western transfer buffer, using the Bio-Rad immunoblot apparatus. The bottom layer of the sandwich consisted of a fibre pad with three pieces of 8 x 10 cm Whatman Grade No. 3 filter paper (Whatman) laid on top; the middle layer comprised the polyacrylamide gel on which the pre-wetted PVDF membrane was placed; and the top layer consisted of another three pieces of 8 x 10 cm Whatman Grade No. 3 filter paper (Whatman) with a fibre pad laid on top. The separated proteins were transferred to the PVDF membrane at 300 mA for 1 hr 10 min at 4°C.

The blotted membrane was then blocked with blocking buffer for 1 to 2 hr at room temperature. For detection of proteins immunologically similar to human PKR, the blocked blots were incubated overnight at 4°C with either the anti-mPKR polyclonal antibody (Cell Signaling Technology, diluted 1:2000 in blocking buffer) or the anti-mPKR monoclonal antibody (BD Transduction Laboratories, diluted 1:5000 in blocking buffer). The membrane was then washed three times for 30 min each with TBS/0.05% (v/v) Tween 20. This was followed by 1 hr incubation at room temperature with ECL™ anti-rabbit IgG, horseradish peroxidase linked whole antibody from donkey (Amersham Bioscience, diluted 1:2000 in blocking buffer) or ECL™ anti-mouse IgG, horseradish peroxidase linked whole antibody from sheep (Amersham Bioscience, diluted 1:5000 in blocking buffer), respectively. The membrane was again washed three times for 30 min each with TBS/0.05% (v/v) Tween 20.
Protein immunoreactivity on the blotted membrane was detected using the ECL Plus™ Western Blotting Detection system (Amersham Biosciences). ECL Plus™ Detection Reagents were mixed at a ratio of 40 parts Solution A to one part Solution B and were incubated with the blotted membrane for 5 min. Excess liquid was drained and the membrane was sealed into a plastic bag and exposed to high performance chemiluminescence Hyperfilm ECL™ (Amersham Biosciences) for 1 to 20 min. The exposed film was developed using a CURIX 60 Table-Top Processor (Agfa).

2.7.11 Phosphorimaging

Proteins that were assayed for protein kinase activity (Section 2.7.6) were analysed by phosphorimaging. Following protein separation by gel electrophoresis, the polyacrylamide gel was incubated in methanol destain for 30 min and vacuum dried in a Dual Temperature Slab Gel Dryer (Bio-Rad) for 3 hr to overnight. The dried gel was then exposed to a Storage Phosphor Screen (Molecular Dynamics) for at least 3 days and the developed phosphorimage was visualised with the Typhoon 9400 Variable Mode Imager (Amersham Biosciences). Phosphorimages were scanned using 50 μm resolution and images were viewed using ImageQuant TL (Amersham Biosciences).

2.7.12 Immunoprecipitation

Protein from plant tissue was extracted as described in Section 2.7.1 and was incubated with gentle shaking overnight at 4°C with the anti-mPKR polyclonal antibody (Cell Signaling Technology, diluted 1:100 in Buffer A).

For each immunoprecipitation assay, 20 – 40 μL of Protein A-Sepharose™ 4B Conjugate (Zymed Laboratories) was used and was prepared by pre-washing twice in Buffer A. The antibody/protein extract was added to the Protein A agarose and incubated with gentle shaking for 3 hr at 4°C. Subsequently, the agarose was pelleted by centrifugation at 300 rpm for 3 min at 4°C (MSE Centaur 2) and washed three times in Buffer A to remove unbound antibody and/or unbound antibody/protein complexes. The protein was then released from the antibody-bound agarose by denaturation and analysed by SDS PAGE as described in Sections 2.7.7.1 and 2.7.7.2, respectively. The presence of proteins was detected by Deep Purple™ staining and the immunoreactivity of the purified proteins with the anti-mPKR antibody was assessed as described in Sections 2.7.9.2 and 2.7.10, respectively.
2.7.13 Mass Spectrometry

MS was used to identify bands of interest from protein gels. The proteins were digested with trypsin and the resulting peptides were run on a LCQ Deca ion trap mass spectrometer (ThermoFinnigan) to generate MS data which was subsequently searched against the *Arabidopsis* genome sequence.

2.7.13.1 In-gel Digestion of Proteins for Mass Spectrometry

Proteins were processed and digested for electrospray ionisation-MS using a protocol adapted from the *Current Protocols in Protein Science* (1998). Protein bands of interest from Colloidal Coomassie® and Deep Purple™ stained polyacrylamide gels were excised using a scalpel blade. The excised bands were then cut into smaller pieces of about 1 mm³ and placed in a 1.5 mL microcentrifuge tube. Approximately 100 μL of 25 mM ammonium bicarbonate/50% (v/v) acetonitrile solution was added to cover the gel particles and the tube was incubated for 10 min at room temperature. Fine tipped pipette tips were used to remove the solution and this wash/dehydration step was repeated three times or until the gel pieces became opaque. The gel particles were then dried for 30 min to 1 hr in a SpeedVac Concentrator (Savant) before in-gel digestion was performed with sequencing grade trypsin (Roche Diagnostics). Trypsin, stored at -20°C in aliquots of 0.5 μg/μL in 1 mM HCl, was diluted to a concentration of 0.05 μg/μL with 25 mM ammonium bicarbonate solution immediately before use. 5% (v/v) 25 mM ammonium bicarbonate/50% (v/v) acetonitrile was added to the diluted trypsin to enhance the function of the enzyme. The gel particles were rehydrated in 20 μL trypsin mix and incubated overnight at 37°C. To recover the digested peptides from the gel particles, 50 μL of water was added to the tube and the sample sonicated for 5 min in an Ultrasonic Cleaner (Mettler Electronics Corp.). The peptide solution was collected using a fine tipped pipette tip and transferred to a fresh 1.5 mL microcentrifuge tube. Two additional extractions were then performed using 50 μL and 25 μL 5% (v/v) formic acid/50% (v/v) acetonitrile, respectively. The recovered peptides were concentrated by reducing the final volume of the extracts to 30 μL in a SpeedVac Concentrator (Savant).
2.7.13.2 Mass Spectrometry Run and Analysis

The digested protein samples were sent for MS analysis to either the Protein Analysis Facility (Plant and Food Research, Ruakura) or the Centre for Genomics & Proteomics (University of Auckland).

MS data were analysed using the Bioworks 3.1 and Xcalibur packages (ThermoFinnigan) and the data collected for each protein sample, including information about the full scan MS spectra for total ion current scans and individual tandem MS spectra for selected precursor ions, was presented as an Xcalibur binary RAW file. The data was then searched against the *Arabidopsis* genome sequence and matches were presented in a hierarchical list on the summary pages. A peptide match was deemed significant using a number of factors. Firstly, the raw cross correlation (Xcorr) values had to be above certain thresholds depending on the charge state of the ion. These Xcorr threshold values were 1.5 (for M⁺), 2.0 (for M²⁺), and 2.5 (for M³⁺). As the experimental MS/MS data was cross correlated to the candidate peptides from the database, the higher the Xcorr score, the more likely the experimental data fitted the theoretical data. Secondly, the preliminary raw score (Sp) value needed to be above 300 to be considered acceptable and the delta correlation (ΔCn) score had to be above 0.1. The Sp value was calculated based on the number of ions from the experimental MS/MS data that fitted the expected ions from a candidate peptide in the database. The ΔCn score indicated the difference between the Xcorr of the two top ranked candidate peptides and therefore the higher the value, the higher probability that the top ranked sequence was correct.

2.8 Plant Protein Kinase R Purification Methods

2.8.1 Partial Purification of Double-stranded RNA Binding Proteins

2.8.1.1 Preparation of Double-stranded RNA Agarose

PolyI-PolyC (2mg, Sigma) was resuspended in 803 µL 0.1 M NaOAC, pH 5.0, boiled for 10 min and allowed to cool for 2 to 3 hr. The PolyI-PolyC was either stored at 4°C or immediately prepared to make the dsRNA agarose. 0.1 M Sodium m-Periodate (38.4 µL, Sigma) was added to the PolyI-PolyC mix and incubated in the dark for 1 hr at room temperature. The PolyI-PolyC mix was then ethanol precipitated twice by adding 2.5 volumes of ethanol to one volume of PolyI-
PolyC, incubating the mix at -80°C for at least 30 min, precipitating the PolyI-PolyC by centrifugation at 9300 x g for 20 min at 4°C (Centrifuge 5415 R, Eppendorf), and resuspending the PolyI-PolyC pellet in 803 µL and 2mL 0.1 M NaOAc, respectively. Subsequently, the prepared PolyI-PolyC was gently mixed for 3 hr at 4°C with 1.25 mL Adipic acid dihydrazide Agarose (Sigma) that was pre-washed twice in 0.1 M NaOAc. The PolyI-PolyC/agarose mixture was adjusted to 2 M NaCl and gently mixed for a further 30 min at 4°C. The mixture was then pelleted by centrifugation at 300 rpm for 3 min (MSE Centaur 2) and washed twice in Buffer A. The PolyI-PolyC/agarose mixture was resuspended at a final volume of 1.25 mL Buffer A and stored at 4°C.

2.8.1.2 Double-stranded RNA Agarose Binding Assay

Protein from plant tissue was extracted and fractionated as described in Sections 2.7.1 and 2.7.2, respectively. The cytoplasmic fraction was used immediately while the ribosomal fraction was diluted with 14 volumes of Buffer A (-salt) before use to adjust the salt concentration from 1.5 M to 100 mM.

For each binding assay, 100 µL of dsRNA agarose was used and prepared by pre-washing twice in Buffer A. The protein extract was pre-incubated with 1 µg poly(rA) (Sigma) for 5 min at 4°C and then added to the dsRNA agarose and incubated with gentle shaking for 30 min at 4°C (ProBlot Rocker 25). The agarose was pelleted by centrifugation at 300 rpm for 3 min at 4°C (MSE Centaur 2) and washed three times in Buffer A to remove unbound proteins. Following the third wash, the agarose was resuspended in 0.5 mL Buffer A and transferred to a 1.5 mL microcentrifuge tube. The agarose was then pelleted at 4°C by short centrifugal spins and 90° rotation of the tubes following each spin. The protein-bound agarose was resuspended in Buffer A and used for subsequent protein assays and analyses.

For competition assays, the protein extract was incubated with the competing ligand for 10 min at 4°C before the addition of the extract to dsRNA agarose.
2.8.2 Partial Purification of Protein Kinases

The KinaseBind™ γ-phosphate-linked ATP resin (Innova Biosciences) was used to affinity purify protein kinases from plant extracts. A low ligand density (1 – 2 μmol/mL ATP) and high ligand density (8 – 12 μmol/mL ATP) resin was used together with a control resin with no ligand present.

For each binding assay, 50 μL of ATP resin was used and was prepared by pre-equilibrating in Buffer A/DTT. The clarified protein extract (homogenised in Buffer A/DTT and prepared as described in Section 2.7.1) was then added to the ATP resin and incubated with gentle shaking for 1 hr at 4°C (ProBlot Rocker 25). The resin was pelleted by centrifugation at 300 rpm for 3 min at 4°C and washed five times in Buffer A/DTT to remove unbound proteins. The ATP resin-bound proteins were then competed from the resin with 5 to 10 mM ATP (Sigma) with gentle shaking for 30 min at 4°C. The resin was pelleted by centrifugation at 300 rpm for 3 min at 4°C and the resultant supernatant comprised the dissociated proteins. The eluted protein fraction was used for subsequent protein assays and analyses.

2.8.3 Size Exclusion Chromatography

Plant-extracted proteins were separated via their molecular size by performing size exclusion chromatography on a Ti-Series 1050 High Pressure Liquid Chromatograph system (Hewlett Packard) using a BioSep™-SEC-S3000 silica-based gel filtration column (Phenomenex). The size exclusion column was pre-equilibrated with Buffer A/DTT. Clarified protein extract (100 μL, homogenised in Buffer A/DTT and prepared as described in Section 2.7.1) was then loaded into the column using a fine needle syringe. The flow rate was set at 0.6 mL per min with a pressure limit of 200 bars and the presence of protein in elution fractions was detected at absorbances of A_{218} and A_{280}. Eluted proteins were collected in 1.5 mL microcentrifuge tubes at a time period of 15 or 30 sec and the fractions were used for subsequent protein assays and analyses.

2.8.4 Ion Exchange Chromatography

Plant-extracted proteins were separated via their ionic strength by performing ion exchange chromatography on an ÄKTA™prime liquid chromatograph system (Amersham Biosciences) using various HiTrap™ ion exchange columns (Amersham Biosciences). All ion exchange columns used were 1 mL in column volume and included a HiTrap™ Q Sepharose HP and a
HiTrap™ SP Sepharose XL column. The column was pre-equilibrated with Buffer A/DTT/20 mM KCl unless otherwise stated. Clarified protein extract (1 to 2 mL, homogenised in Buffer A/DTT and prepared as described in Section 2.7.1) was loaded into the column using a 2 or 5 mL loop and washed with five column volumes of Buffer A/DTT/20 mM KCl. Proteins were then eluted using a KCl gradient of 20 mM to 500 mM over 20 column volumes. The presence of protein in elution fractions was detected at an absorbance of A$_{280}$ and eluted proteins were collected in 1.5 mL microcentrifuge tubes as 0.5 mL fractions. Protein fractions were then desalted as described in Section 2.7.4 and used for subsequent protein assays and analyses.

### 2.9 Immobilised eIF2α Peptide Assay

#### 2.9.1 Synthesis of eIF2α Peptides

Two peptides (Peptide S and Peptide A) were synthesised and obtained from Auspep Pty Ltd. The synthetic peptides contained sequences derived from residues 50 – 60 of the *Arabidopsis* eIF2α protein (At5g05470) and were as follows:

- Peptide S: Ac-ILFSELSSRRIRC
- Peptide A: Ac-ILFSELARRRIRC

Peptide S contained the wildtype sequences of eIF2α aa 50 – 60, while Peptide A carried an Ala substitution in place of the putative phosphoacceptor Ser56 to distinguish non-Ser56 phosphorylation. The peptides were modified by acetylation at the amino termini to ensure specific immobilisation of the peptides via its carboxyl termini and an Arg and Cys residue were included at the carboxyl termini to provide side chains that would mediate linking of the peptides to the activated magnetic beads.

0.01 M peptide stocks were prepared by resuspending the synthesised peptide in distilled water and were stored at -80°C.
2.9.2 Preparation of eIF2α Peptide Beads

eIF2α peptide beads were prepared by resuspending the peptide in M-280 Tosylactivated Dynabeads® (Invitrogen) that were pre-equilibrated with 0.05 M borate buffer, pH 9.5. When required, the beads were congregated with a Magnetic Particle Concentrator (MPC, Dynal Biotech ASA). A typical reaction mix contained 676 μg peptide and 100 μL beads in a total reaction volume of 1.2 mL 0.05 M borate buffer, pH 9.5. The peptide beads were incubated for 24 hr at 37°C, shaking at 225 rpm (Gallenkamp Orbital Incubator) and then washed three times with PBS/1% (w/v) BSA with 10 min incubation between each wash. Subsequently, the peptide beads were incubated overnight at room temperature in 0.2 M Tris buffer and resuspended in 1.2 mL PBS. The prepared peptide beads were stored at 4°C. A Beads only negative control containing 0.05 M borate buffer, pH 9.5 in place of the peptide was also prepared.

2.9.3 Determination of Peptide Binding Efficiency

The binding efficiency of the eIF2α peptide to magnetic beads was evaluated by measuring the absorbance of the peptide bead mix at 230 nm using an 8453 UV-Visible Spectrophotometer (Agilent). Absorbance was measured directly before and after the 37°C incubation (24 hr), where the first measurement indicated the amount of peptide available for binding and the latter measurement represented the amount of peptide which did not bind to the beads.

2.9.4 Immobilised eIF2α Peptide Assay

Protein samples were mixed with the eIF2α peptide beads in the presence of radiolabelled-ATP to assess for its ability to phosphorylate eIF2α. A duplicate of each eIF2α peptide bead (Peptide S, Peptide A, and Beads only) was used to assess each protein sample. Plant proteins were extracted, fractionated, and partially purified as described in Sections 2.7.1, 2.7.2, and 2.8, respectively, while mammalian proteins were expressed and purified as described in Section 2.7.3. A typical reaction mix contained 2.5 μM ATP (Sigma) and 200 μCi/mL γ32P-ATP (Amersham Biosciences and Perkin Elmer) in a total reaction volume of 5 μL protein sample and mixed with 15 μL of peptide beads. The protein/peptide beads mix was incubated for 5 min (unless otherwise stated) at room temperature and washed twice for 10 min with 200 μL 0.1% (w/v) pentasodium triphosphate (PSTP). Subsequently, the beads were incubated with 10 μg/mL endoproteinase Glu-C
Staphylococcus aureus V8 protease (Roche Diagnostics) diluted in 0.1 M ammonium bicarbonate, pH 7.8, for 2 hr at 37°C and washed twice for 10 min with 200 μL 0.1% (w/v) PSTP. The protein/peptide beads mix was then transferred to a fresh 1.5 mL microcentrifuge tube and the amount of radioactivity present on the peptide due to the transfer of radiolabelled phosphate via kinase phosphorylation was measured with a 1214 RackBeta liquid scintillation counter (Wallac) as counts per minute (CPM) by Cerenkov counting using a one min open window per sample. The CPM of each protein sample was determined to be directly proportional to the amount of phosphorylation activity present.

For enzyme kinetics and ion preference studies, the protein extract was incubated with the solution of interest for one to 30 min at 4°C before the addition of ATP and incubation with peptide beads.

2.10 Plant Culture

2.10.1 Plant Material

Wildtype Arabidopsis and N. benthamiana plants were grown in a Physical Containment Level 2 (PC2) containment glasshouse at Plant and Food Research (Mt Albert) and maintained at 20°C with 16 hr light and eight hr dark.

Transgenic Arabidopsis T-DNA insertion lines were obtained as seed stocks from the Arabidopsis Biological Resource Centre (ABRC) and were grown and maintained in a PC2 containment glasshouse at Plant and Food Research (Mt Albert) at 20°C with 16 hr light and eight hr dark.

2.10.2 Plant Basal Media and Antibiotics

Seeds were planted on Plant Basal Medium containing 0.44% (w/v) Murashige & Skoog medium including vitamins (Duchefa Biochemie), 1% (w/v) sucrose, pH 5.6 – 6.0, and 1.5% (w/v) standard agar (Danisco). The plant medium was autoclaved for 15 min at 121°C, supplemented with the appropriate antibiotic(s), and poured into 85 mm growth tubs (Schering-Plough) as prepared Plant Basal Medium plates. Transgenic Arabidopsis lines were maintained using kanamycin (50 mg/mL for fourth generation lines (T4) and 100 mg/mL for all other generations).
2.10.3 *Agrobacterium tumefaciens*-mediated Transformation of *Arabidopsis thaliana* plants

The transformation of *Arabidopsis* was performed using the floral dip method (Clough & Bent, 1998).

2.10.3.1 Preparation of *Agrobacterium tumefaciens* cells

*A. tumefaciens* GV3101 transformed with the gene construct of interest, derived from single cell isolates, were grown overnight at 28°C on LB plates supplemented with the appropriate antibiotic(s). Aliquots (1 L) of LB broth (with the same antibiotics) were then inoculated with two full loops of the grown *A. tumefaciens* and incubated overnight at 28°C, shaking at 225 rpm (Gallenkamp Orbital Incubator). Cells were harvested by centrifugation at 4400 x g for 10 min at 4°C (Sorvall® RC-5B Refrigerated Superspeed Centrifuge, Du Pont) and the pelleted cells were resuspended in 200 mL 5% sucrose per 1 L bacteria. 100 μL Silwet L-77 (Lehle Seeds) was added per 1 L bacterial suspension to enhance the efficiency of the transformation.

2.10.3.2 Floral dipping of *Arabidopsis thaliana* plants

Three pots of *Arabidopsis* with young inflorescences, approximately 50 plants per pot, were chosen for each transformation line. Developed siliques were removed from the plants to minimise the number of untransformed wildtype seeds. The inflorescences were submerged and agitated in the *A. tumefaciens* suspension for 30 sec. Dipped plants were subsequently placed separately under a plastic cover for three to four days to avoid dehydration and then transferred to the containment glasshouse bench to allow for growth.

2.10.4 Collection of Seeds from *Arabidopsis thaliana*

When the siliques of the floral dipped *Arabidopsis* plants were fully developed, the plants were left unwatered and allowed to dry for two to three weeks. The seeds were then released from the siliques by hand and sieved to isolate the seeds from other plant material. Collected seeds were transferred to 1.5 mL microcentrifuge tubes and stored at 4°C.
2.10.5 Sterilisation and Culture of Transgenic Seeds

*Arabidopsis* seeds were surface sterilised by immersion in isopropanol for 10 min, followed by immersion in 2.5% (v/v) bleach/0.05% (v/v) Tween 20 for 10 min. The seeds were then washed three times with sterile distilled water. To assist with even distribution of seeds on the plates, 500 μL of 0.1% (w/v) agarose was added to the seeds which were then planted onto Plant Basal Medium plates supplemented with the appropriate concentration of kanamycin as described in Section 2.10.2. The planted seeds were then placed into a temperature- and light-controlled growth room set at 20°C with 16 hr of light per day and allowed to grow. If the seed did not contain the kanamycin resistance gene, the leaves of the seedling became white and died.

2.10.6 Transfer of Transgenic Seedlings to Soil

When root development was observed in the tissue cultured seedlings (usually within two to three weeks), the plants were transferred to soil. The soil for planting seedlings was first sieved and the coarse soil was placed at the bottom of the pots while the finer soil was placed at the top. The soil was then fully moistened with water supplemented with the insecticide Dimilin and the seedling was loosened from the Plant Basal Medium and transferred onto the soil. Planted seedlings were then grown in a mister chamber for four to five days where a moist surrounding was provided by a constant water mist. When the seedlings had established growth in the soil, the plants were transferred to the containment glasshouse bench to allow for growth under the 16 hr light and eight hr dark cycle.
Development of the Immobilised eIF2\(\alpha\) Peptide Assay

The major assay used in this research thesis to determine the presence and enrichment of putative PKR in plants was the quantification of its eIF2\(\alpha\) phosphorylation activity. The immobilised eIF2\(\alpha\) peptide assay was initially developed by myself and Prakash (2006) and allowed the rapid detection and quantification of eIF2\(\alpha\) kinase activity in plants. The mechanisms of the assay as well as its ability to identify and quantify the level of eIF2\(\alpha\) kinase activity present are briefly described in Sections 3.1 and 3.2, respectively.

As part of this research thesis, the immobilised eIF2\(\alpha\) peptide assay was optimised and validated to reliably detect the activity of eIF2\(\alpha\) kinase activity in vitro. Four different aspects were examined and results from these studies are described in Section 3.3:

a. Optimal time for incubation of the plant sample with the peptide beads (Section 3.3.1)
b. Type of wash buffer used to wash the peptide beads (Section 3.3.2)
c. Distinguishing nonspecific binding of ATP to the peptide beads from kinase-dependent phosphorylation activity (Section 3.3.3)
d. Specificity and quantification of eIF2α peptide phosphorylation by human PKR (Section 3.3.4)

3.1 Mechanism of the Immobilised eIF2α Peptide Assay

The immobilised eIF2α peptide assay provides an indirect method of quantifying eIF2α kinase activity through the ability of the kinase to phosphorylate its substrate eIF2α and essentially involves the use of magnetic beads coated with a short peptide of the substrate (generated as described in Section 2.9.2). The peptide, known as Peptide S, contains the residues at aa 50 – 60 of the *Arabidopsis* eIF2α protein (At5g05470) that is recognised by eIF2α kinases and phosphorylated at Ser56 (Fig. 3.1). Additional Arg and Cys residues are present at the carboxyl terminus of the synthesised peptide to provide the respective amino and sulphydryl groups for the linking of the peptide to the tosyl-activated magnetic Dynabeads®. Moreover, the amino terminus of the peptide is acetylated while the carboxyl terminus has an attached carboxyamide group which serves to ensure that the peptide is covalently immobilised to the magnetic beads in the correct orientation, specifically via the sulphhydryl/amide/tosyl linkage.

The eIF2α peptide-coated magnetic beads serve effectively as a substrate delivery and retrieval tool. Firstly, the beads are incubated, in the presence of γ^32P-ATP, with either clarified plant extract (extracted as described in Section 2.7.1) or partially purified plant extract (partially purified as described in Sections 2.8.1 to 2.8.4). This step delivers the substrate eIF2α to the plant extract and, where eIF2α kinases are present, phosphorylation occurs on Ser56 of the eIF2α peptide. In a typical reaction, approximately 300 μg of peptide (equivalent to 15 μL of peptide beads) was delivered to 20 – 100 μg of protein sample and incubated in the presence of 2.5 μM ATP and 200 μCi/mL γ^32P-ATP to allow for kinase phosphorylation.

The phosphorylated eIF2α peptide is then washed and retrieved from the extract by congregating the magnetic beads on a Magnetic Particle Concentrator (MPC) and the amount of phosphorylation is quantified by counting the amount of radiolabel on the peptide. However, the eIF2α peptide contains two potential phosphorylation sites; Ser56, which is phosphorylated by eIF2α kinases such as PKR, and Ser53, which is nonspecifically phosphorylated by other protein kinases present in the protein extract (Chang et al., 1999). Hence, subsequent to the kinase reaction, Ser53, which has the potential for non-eIF2α kinase phosphorylation, is removed from the magnetic beads through digestion with endoproteinase Glu-C *Staphylococcus aureus* V8 protease. The V8
protease cleaves between the Glu and Leu residues of the eIF2α peptide and leaves, attached to the magnetic beads, a shortened peptide containing only the single, potentially eIF2α kinase-phosphorylated Ser residue, Ser56 (Fig. 3.1). Essentially, this V8 digestion increases the specificity for the quantification of eIF2α kinase activity.

![Figure 3.1 Schematic diagram of the immobilised eIF2α peptide assay.](image)

In addition to Peptide S, two negative controls are included in this assay (Fig. 3.2). The first negative control (Peptide A) consists of magnetic beads coated with a synthesised peptide which contains a sequence identical to Peptide S except for a substitution of Ser56 to Ala. As demonstrated by previous *in vitro* experiments using mammalian eIF2α peptides (Nanduri et al., 2000), this substitution renders the peptide unphosphorylatable by eIF2α kinases. The second negative control (Beads only) consists of magnetic beads with no peptide attached and thus also cannot be phosphorylated by eIF2α kinases.
Figure 3.2 Magnetic beads and types of linked eIF2α peptides. Three different forms of magnetic beads (eIF2α peptide-coated, or uncoated) were used in the immobilised eIF2α peptide assay. Peptide S represents the wildtype sequence of Arabidopsis eIF2α that is recognised and phosphorylated by eIF2α kinases. Peptide A contains an Ala in place of the eIF2α kinase-phosphorylated Ser56 such that no phosphorylation by eIF2α kinases would occur on this peptide. Beads only consist of uncoated magnetic beads and no phosphorylation occurs on this negative control.

3.2 Detection of Plant eIF2α Kinase Activity

The immobilised eIF2α peptide assay allows the detection and quantification of eIF2α kinase activity by counting the amount of radiolabelling (CPM) on the V8-cleaved peptide. For each sample in an experiment, two biological replicates were performed and the level of radioactivity detected in each of these was then counted four times to produce four technical replicates per biological replicate. The level of error for each sample was thus calculated from the eight combined CPM readings. The standard error, presented in the bar graphs, was calculated using the following formula:

\[
\text{standard error} = \frac{\sigma}{\sqrt{n}} \quad \text{where} \quad \sigma = \text{standard deviation} \quad \text{n = number of samples}
\]

Due to the short half life of the radioactive phosphate used in these experiments and the relative signal of the background, results for each sample could only be compared quantitatively within a given experiment rather than between experiments. Therefore, all results shown in this research thesis were of a single experiment, representative of the pattern of results obtained for a set of
experiments. Each set of experiments included at least three repeats of the same experiment with a similar pattern of results observed between these experiments.

Figure 3.3 is a typical representation of the level of phosphorylation that is detected in Peptide S, as compared to the two negative controls (Peptide A and Beads only), when incubated with partially purified Arabidopsis flower extract. The amount of phosphorylation activity is measured as the number of CPM measured in each sample. A low level of phosphorylation is detected in the Peptide A and Beads only controls which is indicative of the basal amount of $\gamma^{32}$P-ATP that is nonspecifically bound to the peptide and/or magnetic beads.

![Figure 3.3](image)

**Figure 3.3 Quantification of eIF2\(\alpha\) kinase activity using the immobilised eIF2\(\alpha\) peptide assay.** Bar graph showing a typical example of the amount of eIF2\(\alpha\) phosphorylation activity that is detected on the eIF2\(\alpha\) peptide-coated, or uncoated, magnetic beads when incubated with partially purified Arabidopsis flower extract. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S. Numerical values (coloured) above each bar represent actual counts per minute detected in each peptide bead sample.
3.3 Optimisation and Validation of the Immobilised eIF2α Peptide Assay

Although phosphorylation activity was initially detected on the eIF2α peptide beads, it was essential to optimise the assay for maximal and reproducible detection of eIF2α phosphorylation and to validate the assay for the specific detection of eIF2α kinase activity. Two approaches were implemented to optimise the assay (Sections 3.3.1 and 3.3.2) and two approaches were used to determine that the assay measured eIF2α kinase activity specifically (Sections 3.3.3 and 3.3.4).

3.3.1 Optimal Time for Incubation of Protein Sample with the Peptide-Coated Beads

To maximise the detection of eIF2α kinase activity above background, the optimal length of incubation time was determined. Three samples were tested for the presence of eIF2α phosphorylation activity using Peptide S beads. These included two protein samples purified by size exclusion chromatography (as described briefly in Section 2.8.3 and more fully in Section 4.6) and a sample containing Buffer A only. The two protein samples were representative of the fractions which had the highest and lowest kinase activity from the size exclusion chromatography purification (Section 4.6.2), and were termed eIF2α kinase positive and eIF2α kinase negative, respectively.

Figure 3.4 illustrates the ratios of eIF2α phosphorylation activity that were observed in the two protein samples relative to background phosphorylation activity in the presence of Buffer A only. The optimal incubation time for measuring maximal eIF2α kinase activity above background was determined to be 3 – 10 min and incubation at some time point beyond 5 min displayed a saturation of enzyme activity above background. For convenience and to minimise the saturation of eIF2α kinase activity while maximising the sensitivity of eIF2α kinase detection, subsequent experiments were performed with an incubation time between 5 – 10 min.
3.3.2 Type of Wash Buffer for the Washing of the Peptide-Coated Beads

A crucial step in the immobilised eIF2α peptide assay is the washing of any nonspecifically bound radiolabelled ATP from the peptide beads following incubation with potential kinases in the protein extracts. Optimisation of this step was important as it would minimise the level of background CPM and maximise the reproducibility of the assay. An effective washing solution was determined.

PBS was previously shown to be most effective in removing nonspecifically bound $\gamma^{32}$P-ATP (Prakash, 2006). However, further testing with three different buffers showed that PSTP (average of 399.14 CPM) was able to reduce the level of CPM significantly more than PBS (average of 772.90 CPM), while PBS/0.1% (v/v) Tween 20 (PBST, average of 679.85 CPM) showed minor differences in comparison to PBS (Fig. 3.5). Presumably, the same amount of covalently and nonspecifically bound $\gamma^{32}$P-ATP was initially present for each wash treatment and PSTP was most
efficient at removing nonspecifically bound $\gamma^{32}$P-ATP from the magnetic beads. This indicated that PSTP was a better wash buffer than PBS as it provided the data for the covalent binding of $^{32}$P to the substrate and thus most accurately reflected the activity of the kinase. The use of PSTP also resulted in a lower CPM for Peptide S. Although an apparently higher ratio of background were observed in the Peptide A (0.22) and Beads only (0.10) controls, this higher ratio was due to the amount of remaining associated radioactivity that could not be further reduced by any of the wash buffers.

It is important to note that due to the initial use of PBS as the washing buffer, some results shown in this research thesis had CPM levels above 1000 for Peptide S (washed using PBS as annotated in figure legends), while the majority of results ranged between 300 – 400 CPMs when washed using PSTP. However, even with the use of different buffers, a similar phosphorylation pattern was observed between these experiments.

Figure 3.5 Quantification of the effect of different wash buffers on eIF2$\alpha$ kinase activity. Bar graph showing the ability of different buffers to remove nonspecifically bound radiolabelled ATP. Clarified Arabidopsis flower extract was incubated with eIF2$\alpha$ peptide beads and washed with either phosphate buffered saline (PBS), PBS/0.1% (v/v) Tween 20 (PBST), or pentasodium triphosphate (PSTP). The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (coloured) above each bar represent actual counts per minute detected in each peptide bead sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
Interestingly, it was observed that when the peptide beads were incubated with \( \gamma^{32}\text{P}-\text{ATP} \) and water instead of the standard plant extraction buffer, Buffer A, the level of measured phosphorylation increased significantly despite the absence of plant tissue and thereby kinase activity (Fig. 3.6). This effect may be the generation of a chemical reaction, possibly electrostatic and ionic strength-related, between the water, \( \gamma^{32}\text{P}-\text{ATP} \), and peptide beads, which resulted in a very high amount of radiolabelled ATP nonspecifically binding to the peptide beads. However, Figure 3.6 shows that when PSTP was used as the wash buffer, the radiolabelled background generated by incubating the peptide beads in water was significantly reduced. These results again demonstrated that PSTP was the most effective wash buffer for the immobilised eIF2\( \alpha \) peptide assay.

**Figure 3.6 Quantification of the effect of water on eIF2\( \alpha \) kinase activity.** Bar graph showing the increase in radiolabelled background and the significant reduction of this background with the use of PSTP when eIF2\( \alpha \) peptide beads were incubated with *Arabidopsis* flower extract (Plant) compared with water only (Water). The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (coloured) above each bar represent actual counts per minute detected in each peptide bead sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only. PBS, phosphate buffered saline; PSTP, pentasodium triphosphate.
3.3.3 Distinguishing Between Nonspecific Binding of ATP and Kinase-dependent Transfer of γ Phosphate

As well as selecting an effective wash buffer for the immobilised eIF2α peptide assay, it was also important to distinguish whether the detected radioactivity on the peptide beads was present due to a nonspecific attachment or a kinase-dependent transfer of radiolabelled phosphate. In order to make this distinction, two forms of radiolabelled ATP were used; ATP that was radiolabelled either on the α phosphate (αP) or the γP (Fig. 3.7). Kinase-dependent phosphorylation involves the transfer of the terminal phosphate of ATP, i.e., γP, to the target site, leaving behind the α and β phosphate within the ADP by-product of phosphorylation. Thus, kinase-dependent transfer of radiolabelled ATP would only be observed with γ32P-ATP and not with α32P-ATP.

Figure 3.7 Structure of adenosine 5'-triphosphate (ATP). ATP consists of an adenine ring and a ribose sugar (adenosine) and three phosphate groups (triphosphate). The phosphoryl groups are referred to as the alpha (α), beta (β), and gamma (γ) phosphates. (modified from http://en.wikipedia.org/wiki/Adenosine_triphosphate)

Figure 3.8 shows the observed level of radioactivity detected in peptide beads incubated with clarified Arabidopsis flower extract or Buffer A only, in the presence of either α-labelled ATP or γ-labelled ATP. When α-labelled ATP was used, minimal levels of radioactivity were detected in the plant extract and Buffer A only samples (average of 132.33 and 109.88 CPM, respectively). On the other hand, in the presence of γ-labelled ATP, significantly higher levels of radioactivity were observed in the peptide beads incubated with the plant extract (average of 318.74 CPM) as compared to the Buffer A only sample (average of 141.43 CPM). In fact, the level of radiation
detected in the $\gamma^{32}$P-ATP-incubated Buffer A only sample was comparable to the low level of radioactivity detected in the $\alpha^{32}$P-ATP-incubated plant extract and Buffer A only samples. These results validate that the radioactivity detected on plant extract-incubated peptide beads as kinase-dependent phosphorylation and this kinase activity occurs via the transfer of the $\gamma$P of ATP to the peptide. However, it is interesting to note that the amount of nonspecifically bound radiolabelled ATP was consistently higher in the Peptide S samples relative to the negative controls, regardless of whether $\alpha^{32}$P- or $\gamma^{32}$P-ATP was used. The only difference that is present between the Peptide S and Peptide A beads are the respective substitution of the Ser56 to Ala residue in the eIF2$\alpha$ peptide. A possible rationale for this phenomenon is that the Ser residue may have a higher attraction for the nonspecific binding of ATP. Although this issue remains unresolved, the above study does provide evidence that a higher level of radioactivity is observed when kinase-dependent phosphorylation is present and that this is statistically significant from the detected levels of radiation that is bound nonspecifically to the peptide beads.

![Figure 3.8](image-url)

**Figure 3.8 Quantification of specific and nonspecific binding of radiolabelled ATP to eIF2$\alpha$ peptide beads.** Bar graph showing the effect on the radiolabelled background with the incubation of eIF2$\alpha$ peptide beads and *Arabidopsis* flower extract (Plant) or Buffer A, with either $\alpha$-labelled ATP ($\alpha$P-ATP) or $\gamma$-labelled ATP ($\gamma$P-ATP). The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (coloured) above each bar represent actual counts per minute detected in each peptide bead sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
3.3.4 Validation of Phosphorylation of eIF2α Peptide Beads by Human Protein Kinase R

The assessment of phosphorylation activity observed from the incubation of the eIF2α peptide beads with expressed human PKR protein provides an ideal positive control for the immobilised eIF2α peptide assay and validates the detected kinase activity as PKR-related.

3.3.4.1 Expression of Human Protein Kinase R

A plasmid construct containing the cDNA of human PKR was obtained from Dr. Graeme Conn (University of Manchester, personal communication) (Fig. 3.9). The expressed human PKR protein from the pTYB2 vector carries a C-terminal intein tag fusion and facilitates the E. coli-expressed protein to be purified by the intein using a single chromatographic step (Fig. 3.10). The plasmid was transformed into E. coli BL21 cells by electroporation and a single colony of bacteria was selected for the inoculation and subsequent growth of bacteria for induction of the expression of the human PKR protein (Section 2.7.3.1 and 2.7.3.2). The expressed protein was harvested and subjected to purification using the IMPACT™-CN System as described in Section 2.7.3.3 and shown in Figure 3.10. Briefly, the expressed soluble protein fraction was loaded onto a chitin column. Recombinant proteins containing the intein tag and chitin binding domain, i.e., expressed human PKR, bound to the chitin column, while unbound proteins were allowed to flow-through. Self-cleavage activity of the intein was then induced in the presence of dithiothreitol (DTT) and the human PKR protein was released from the chitin-bound intein tag. The resulting eluant contained the released protein and allowed a single-column purification of the expressed human PKR protein.

Figure 3.9 Plasmid construct for the expression of human PKR. The construct was built based on the IMPACT™-CN System vector pTYB2. The human PKR gene (PKR(PP)) is inserted before an intein tag which facilitates the purification of the expressed protein. Since PKR can autophosphorylate, a λ-protein phosphatase (λ-PPase) is included in the construct to obtain expressed PKR in a nonphosphorylated form. (Conn, 2003)
Figure 3.10 Schematic diagram of the IMPACT™-CN System. The human PKR construct consists of a C-terminal tag fusion and was expressed and purified using the IMPACT™-CN System. See text for details. (www.neb.com/nebcomm/products/productE6900.asp)

Approximately 0.3 mg of soluble human PKR protein was expressed from 100 mL of bacteria. The purification of human PKR is shown in Figure 3.11a (lanes 7-12, red arrow). Another protein, MYB5, was also expressed as a positive control for the expression and purification system (Fig. 3.11b lanes, 7-12, red arrow). The removal of proteins that remained bound to the chitin column (Fig. 3.11a and b, lane 13) indicated that the self-induced cleavage of the intein tag was relatively efficient. Only a small subset of the bound proteins were uncleaved expressed proteins (human PKR, 130 kDa, and MYB5, 100 kDa, in Fig. 3.11a and b, respectively), while the other bound protein observed in lane 13 of Figure 3.11a and b (purple asterisks) was the 55 kDa cleaved intein-chitin binding domain fusion.
Figure 3.11 Expression and purification of human PKR and MYB5. Colloidal Coomassie® stained protein gel showing the expression of (a) human PKR and (b) MYB5 protein in Escherichia coli and their purification via the intein tag using the IMPACT™-CN System. Purple asterisk (*) indicates the cleaved intein-chitin binding domain fusion. Red arrows indicate the expressed protein of interest. Lanes: 1, before IPTG induction; 2, 16 hours after IPTG induction; 3, soluble fraction; 4, flow-through; 5, wash; 6, addition of dithiothreitol; 7-12, elution fractions; 13, remaining chitin resin. Protein markers indicate molecular sizes in kDa.
3.3.4.2 Incubation of elf2α Peptide Beads with Human Protein Kinase R

The immobilised elf2α peptide assay was performed with 2 µg of purified human PKR protein in the presence of increasing concentrations of dsRNA. The human PKR protein was first mixed with dsRNA for 30 min at 4°C, and then incubated with the elf2α peptide beads in the presence of γ^{32}P-ATP. As discussed in Section 1.2.1.2, the activity of PKR is activated predominantly by dsRNA and results in its autophosphorylation and subsequent trans-phosphorylation of elf2α. This activation also fits a bell-shaped curve whereby both concentrations of dsRNA too low and too high lead to the inhibition of PKR activity.

The bell-shaped activation of human PKR was observed using the immobilised elf2α peptide assay (Fig. 3.12). At concentrations of dsRNA below 0.001 µg/mL and above 1 mg/mL, no elf2α phosphorylation was detected. However, significant elf2α phosphorylation was seen when 0.01 – 100 µg/mL of dsRNA was added to human PKR, with the highest activity recorded in the presence of 1 µg/mL of dsRNA. These findings indicate that human PKR was activated by the presence of adequate amounts of dsRNA and trans-phosphorylation of its substrate elf2α had occurred (as similarly observed in Conn, 2003). In comparison, very low phosphorylation activity, possibly radiolabelled background, was observed in the control protein MYB5 with or without the presence of dsRNA.

3.3.4.3 Summary of the Phosphorylation of elf2α Peptide Beads by Human Protein Kinase R

The results achieved from the phosphorylation of the elf2α peptide-coated beads by human PKR were similar to the human PKR activation and elf2α phosphorylation profiles observed by Conn (2003) where activation of human PKR via autophosphorylation and peak activity of elf2α phosphorylation were detected in the presence of 0.1 – 100 µg/mL dsRNA (Fig. 3.13). The peak activity of elf2α phosphorylation was determined by Conn (2003) to be at 1 µg/mL dsRNA, which resembles the phosphorylation profile achieved by the immobilised elf2α peptide assay described in this chapter. In fact, the immobilised elf2α peptide assay was more sensitive than the phosphorylation observed by Conn (2003) for the detection of human PKR trans-phosphorylation activity. Using the immobilised elf2α peptide assay, an increase in elf2α phosphorylation was already detected in the presence of as little as 0.01 µg/mL dsRNA (Fig. 3.12).
Figure 3.12 Quantification of human PKR (hPKR) activity in the presence of dsRNA. Bar graph showing the bell-shaped effects of increasing concentrations of dsRNA on the eIF2α phosphorylation activity of expressed hPKR. Another expressed protein, MYB5, and a Buffer A only sample were assayed as negative controls. Orange bars represent the amount of dsRNA added to each sample. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Peptide S, blue bar; Peptide A, red bar; Beads only, green bar.
Figure 3.13 Autophosphorylation and trans-phosphorylation activity of expressed human PKR. Phosphorimage showing the autophosphorylation activity of human PKR (PKP (PP)) and the trans-phosphorylation activity of its substrate eIF2α in the presence of increasing poly(I).poly(c) RNA. (Conn, 2003)

3.4 Conclusions

The immobilised eIF2α peptide assay, developed for the rapid detection of eIF2α kinase activity in vitro, has been refined to achieve a significantly reduced radiolabelled background and has been validated for its ability to detect and quantify kinase-dependent, dsRNA-dependent, and PKR-phosphorylation of eIF2α. A positive control for the assay has also been developed through the use of expressed human PKR. The assay is therefore suitable for detecting the presence of eIF2α kinases in experiments involving the detection and purification of the plant-functional equivalent of PKR.
Previously, attempts by Langland et al. (1995) had failed to identify the plant-functional homolog of PKR by sequence homology using the mouse and human PKR sequences to search the Arabidopsis, rice, and tomato databanks. This genomics approach was further investigated by myself and Kemeter (2005), whereby the Arabidopsis genome was examined for potential pPKR candidates via the known and/or assumed characteristics of the protein kinase. These included features such as the presence of a kinase domain, nucleic acid binding domain, a molecular size of 68 – 70 kDa, and a pI of 7 or higher. Specific sequences postulated or shown to be related to pPKR were also used to search through the Arabidopsis database for potential pPKR candidates. These included three peptide sequences previously identified by Professor Donald Roth (University of Wyoming, personal communication) that were believed to reside in pPKR; and an epitope sequence encompassing the second dsRBM and third basic aa region of human PKR that is recognised by a monoclonal anti-mPKR antibody and which Langland et al. (1995) had previously observed immunoreactivity with a 68 – 70 kDa plant protein that was present in an extract
comprising of pPKR-like activity. Furthermore, as there are no annotated proteins encoded by the *Arabidopsis* genome that contain both a dsRBM and a kinase domain, it was of interest to investigate the possibility of misannotations in the *Arabidopsis* genome. Proteins annotated as protein kinases in the *Arabidopsis* genome were analysed for the presence of upstream or downstream neighbouring ORFs containing a dsRBM which may indicate misannotation of the two domains as separate genes. Unfortunately, none of the genomics studies identified a gene with high potential as a pPKR candidate.

Although attempts were unsuccessful in identifying pPKR via a genomics approach searching through the *Arabidopsis* sequence genome, there is clear biochemical evidence for the presence of pPKR activity in plants. A proteomics approach was proposed to identify pPKR whereby the protein of interest is first purified through the correlation of its presence with the detection of its activity, and then sequenced. The identified sequence of the protein would then be utilised to locate the gene of pPKR in the *Arabidopsis* genome.

As described in Section 1.3, pPKR activity detected in barley and wheat has previously been shown to reside in a protein with a denatured size of 68 – 70 kDa and has the ability to bind and be activated by dsRNA, autophosphorylate, and *trans*-phosphorylate the substrates eIF2α and histones (Langland et al., 1995; Langland et al., 1996). These known characteristics of pPKR allow a variety of techniques to be exploited for the purification of pPKR. In addition, these purification tools could be carried out with the plant model *Arabidopsis* as its genome is fully sequenced and allows a protein sequence to be matched to a specific gene sequence. Hence, *Arabidopsis* provides a useful system for the identification of candidates for the protein and gene sequence of PKR in plants.

Seven different approaches, including six purification protocols, were utilised to aid the partial purification of pPKR from *Arabidopsis* and results from these studies are described and discussed in Sections 4.2 to 4.8:

- a. Identification of plant tissues with the highest putative pPKR specific activity (Section 4.2)
- b. Partial purification of dsRNA binding proteins (Section 4.3)
- c. Immunoprecipitation of proteins immunoreactive with the polyclonal anti-mPKR antibody (Section 4.4)
- d. Partial purification of protein kinases (Section 4.5)
c. Enrichment by protein size (Section 4.6)
f. Combined purification of size-fractionated and dsRNA binding proteins (Section 4.7)
g. Enrichment by ionic strength (Section 4.8)

The partial purification of pPKR and the presence of putative pPKR activity were then detected using the four main tools described in Section 4.1. Proteins present within specific bands of interest, separated on protein gels following purification using the seven different approaches, were subsequently identified using peptide sequencing via MS. The sequence data obtained as well as the potential pPKR candidates identified are described in Section 4.9.

**4.1 Detection Tools for the Partial Purification of the Putative Plant-functional Homolog of Protein Kinase R**

Four main tools were used to aid the detection of candidates of pPKR partially purified from *Arabidopsis* using the seven different approaches. Table 4.1 lists these detection tools and briefly summarises the proteins or activity detected by each tool. At least one of these tools, as appropriate, was used to determine pPKR enrichment and/or the presence of pPKR activity in each purification approach. Protein stain and immunoblot detection of proteins were performed using twin gels running concurrently within the single electrophoresis apparatus and loaded with the same samples. Phosphorimaging was performed on either the dried protein stained-gel or the immunoblot. This protocol of protein detection allowed for comparison between the three gel-based assays. Similarly, immobilised eIF2α peptide assays were performed on common samples so as to allow for correlation between gel-based and activity-based assays. In the same way as the immobilised eIF2α peptide assays, the gel-based assays shown in this research thesis represented results from at least three experiments performed on separate days and for which a similar pattern of results was obtained.

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4.2 Identification of Plant Tissues with Highest Putative Plant Protein Kinase R Activity

Foremost to undertaking the different purification approaches to enrich for pPKR, it was important to locate the plant tissue which contains the highest specific activity of eIF2α kinases, such as pPKR, and to determine whether environmental or developmental factors influenced the regulation and cellular localisation of putative pPKR activity. A better understanding of the localisation of pPKR, as well as the effect of these factors on pPKR activity and/or expression, could then be utilised to optimise the tissue sampling protocol for the purification of pPKR. In turn, this would increase the likelihood of purifying the target protein.

The use of virus-infected plant tissues was suggested as a possible method to further increase the likelihood of enriching for activated pPKR as it has previously been shown that the infection of TMV results in the activation of pPKR activity (Crum et al., 1988). However, it is also known that TMV, as well as Arabidopsis-infesting viruses Turnip mosaic virus (TuMV) and Turnip vein clearing virus, recruit the cellular PKR inhibitor p58IPK during infection to support their replication and spread (Bilgin et al., 2003). This PKR inhibitor interacts directly with the PKR protein and inhibits the activation of PKR by preventing homodimerisation. Weighing the options, it was concluded that tissues from plants infected with viruses were not favourable for the purification of pPKR as the presence of virus-activated inhibitors may significantly influence the identification and understanding of pPKR localisation and activity in plants. In addition, it is important to note that there is the presence of another eIF2α kinase, GCN2, in Arabidopsis. This plant GCN2 homolog has been demonstrated to phosphorylate eIF2α in response to a variety of stress signals (Lagiex et al., 2008; Y. Zhang et al., 2008) and the presence of its RNA transcript is detected throughout Arabidopsis (Y. Zhang et al., 2003). Thus, it is possible that eIF2α phosphorylation activity detected by the immobilised eIF2α peptide assay may include GCN2 activity as well as the potential pPKR phosphorylation activity. However, the presence of GCN2 can be distinguished via gel-based assays as the molecular weight of Arabidopsis GCN2 is 140 kDa, which is significantly larger than the postulated 68 – 70 kDa size of pPKR. A null mutant for GCN2 has also been generated by Cold Spring Harbour Laboratory, New York (http://genetrap.cshl.org and as utilised by Y. Zhang et al. (2008) for the analysis of GCN2 activity in Arabidopsis) and would have been valuable for use in my research to eliminate misinterpretation of GCN2 activity as the enriched pPKR phosphorylation activity. Unfortunately, this GCN2 mutant was not available during my
research studies and was therefore not obtained for use. Thus, tissues from uninfected wildtype *Arabidopsis* plants were used for the partial purification pPKR.

Three different environmental, developmental, and cellular factors were examined for their effects on putative pPKR activity and the results from these studies are described in Sections 4.2.1 to 4.2.3:

- a. Sensitivity to light (Section 4.2.1)
- b. Plant tissue (Section 4.2.2)
- c. Subcellular localisation (Section 4.2.3)

The immobilised eIF2α peptide assay was used to monitor the influence of these factors on the activity of putative pPKR.

4.2.1 Sensitivity to Light

Plant growth, especially in the annual plant *Arabidopsis*, can be highly sensitive to different seasonal and circadian changes and nearly every protein has been shown to be altered in expression by the presence of different temperatures and light received by the plant (Covington, Maloof, Straume, Kay, & Harmer, 2008; Mikkelsen & Thomashow, 2009). Similarly, it was essential to determine whether the activity of eIF2α kinases, such as pPKR, varied with light exposure.

The circadian cycle was assessed for its effects on putative pPKR activity. *Arabidopsis* plants were grown at a fixed day-night time length of eight hr light with 16 hr darkness. Leaf samples were taken at different timepoints during the circadian cycle and analysed for their level of eIF2α kinase activity using the immobilised eIF2α peptide assay. Three samples were taken during the eight hr light period – at one, four, and seven hr after the plant was exposed to light – and one sample was taken during the dark period – at one hr after the plant entered the dark cycle.

As shown in Figure 4.1, the level of eIF2α peptide phosphorylation observed in the plant extract increased with increasing time of exposure to light, up to seven hr. However, after the plant entered the 16 hr dark period, the level of eIF2α phosphorylation decreased within an hour (Fig. 4.1, 1 hr after exposed to dark). These results demonstrated a response of putative pPKR specific
activity to the presence of light, with specific activity being highest with an increasing time of exposure to light.

![Figure 4.1 Quantification of the effect of light exposure time periods on putative pPKR activity.](image)

An understanding of the localisation of active pPKR would also aid the purification of the protein kinase from plants. As different tissues of the plant have different functions, the amount of active pPKR that is present in the various tissues may also be distinct.

A selection of tissues was collected from *Arabidopsis* that had been exposed to four hr of light to determine the level of eIF2α phosphorylation activity in these different localisations. Leaf tissues and flower buds were collected from *Arabidopsis* plants of three age groups to generate a set of tissues from the young (three to four weeks old), adult (six weeks old), and senescent (eight to nine weeks old) plants. Developing siliques and stems were taken from the adult plant.
Figure 4.2 show the levels of eIF2α peptide phosphorylation activity that were observed in the various plant tissues. High eIF2α phosphorylation activity was detected in the flower bud tissues, with peak activity in unopened buds and the flower buds of young plants. Similarly, high levels of eIF2α kinase activity were observed in cauline leaves from young plants as compared to senescent leaf tissues from old plants, although the overall level of eIF2α peptide phosphorylation was lower in leaves than was observed in the flower bud tissues. Conversely, very low levels of eIF2α phosphorylation activity were detected in the silique, stem, and seed tissues. Overall, these results revealed that eIF2α kinase activity was highest in unopened buds and flower buds of young Arabidopsis plants.

**Figure 4.2 Quantification of eIF2α kinase activity in different plant tissues.** Bar graph showing the difference in eIF2α phosphorylation detected in different tissues of Arabidopsis plants representing three age groups (young, adult, and old). The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
4.2.3 Subcellular Localisation

In mammals, a large portion of expressed mPKR is found in the cytosol or associated with the ribosomal fraction of the cell (Jeffrey et al., 1995). Correspondingly, it has been shown that pPKR activity isolated from barley leaf tissues is detected in both of these fractions, with more activity detected in the ribosomal fraction (Langland et al., 1995). It was thus of interest to investigate the level of putative pPKR activity that was detected in these two subcellular fractions isolated from Arabidopsis.

Proteins were extracted from both leaf and flower bud tissues of young Arabidopsis plants following four hr of exposure to light and were separated into their respective cytoplasmic and ribosomal fractions as described in Section 2.7.2.

The eIF2α phosphorylation profiles observed for the Arabidopsis leaf and flower bud tissues were quite distinct from each other (Fig. 4.3). As described in Section 4.2.2 and shown in Figure 4.3, the level of eIF2α phosphorylation was higher in the flower buds than in the leaf sample. When these tissues were separated into their subcellular fractions, a higher kinase activity was detected in the cytoplasmic fraction of the leaf tissue, whereas the ribosomal fraction from flower buds displayed a greater level of eIF2α phosphorylation. In fact, a 2.2-fold increase in specific activity was observed in the ribosomal fraction of the flower bud tissues as compared to the respective total protein and cytoplasmic fractions, suggesting either a significant portion of the flower bud eIF2α kinase activity is associated with ribosomes or that the specific activity had been markedly increased by the removal of many non-eIF2α kinase related proteins during the purification. This observation of a predominantly ribosomal localised eIF2α kinase in plants parallels the subcellular profile that is detected for mPKR whereby the active protein is mostly found associated with ribosomes (Jeffrey et al., 1995). This result suggests that the detected eIF2α kinase activity is derived from pPKR. In contrast, higher levels of eIF2α phosphorylation activity were seen in the equal amount of protein from cytoplasmic fraction of leaf tissues compared to the respective total protein and ribosomal fractions. It is important to note that yeast GCN2 has been shown to also associate with ribosome subunits in yeast (Ramirez et al., 1991). However, the potential association of Arabidopsis GCN2 with ribosomes has not been investigated.
Figure 4.3 Quantification of putative pPKR activity in different subcellular fractions. Bar graph showing the difference in eIF2α phosphorylation detected in the total protein, cytoplasmic, and ribosomal fractions of Arabidopsis leaf and flower tissues. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.

4.2.4 Summary of Plant Tissues with Highest Putative Plant Protein Kinase R Activity

It is postulated that the detection of eIF2α phosphorylation would represent the presence of active eIF2α kinases, as it does for the presence of active human PKR (validation described in Section 3.3.4.2). Hence, from the results described above, the highest amount of active eIF2α kinases, such as the putative pPKR, is located in the ribosomal fraction of flower buds of young Arabidopsis plants after at least four hr of exposure of the plant to light. By sampling in the future from this particular developmental stage at this specific point of environmental response, a higher proportion of potential active pPKR protein should be present reproducibly, which in turn will allow a greater chance of purifying the putative pPKR from Arabidopsis.

However, the time required to separate the proteins into their ribosomal fraction and then perform subsequent purification steps was prohibitive, therefore unfractionated total proteins from flower buds harvested in the early morning were used for purification instead. It is noteworthy that by
growing the *Arabidopsis* plants in a growth room, the plants will be able to receive at least seven hr of light exposure while still being able to be harvested in the early morning.

### 4.3 Partial Purification of Double-stranded RNA Binding Proteins

The most fundamental and well-known property of mPKR is its ability to bind and be activated by dsRNA. Similarly, putative pPKR has been observed to possess dsRNA binding and dsRNA-dependent autophosphorylation and *trans*-phosphorylation properties (Langland et al., 1995; Langland et al., 1996). Hence, one of the first approaches to enrich for the putative pPKR was to exploit its dsRNA binding ability through the use of the dsRNA agarose binding assay developed by Langland et al. (1995).

A brief outline of the dsRNA agarose binding assay is presented in Section 4.3.1 and an analysis of the enriched proteins, as well as the approaches that were trialled to optimise this purification assay, are described and discussed in Section 4.3.2.

#### 4.3.1 Mechanism of the Double-stranded RNA Agarose Binding Assay

The dsRNA agarose binding assay purifies dsRNA binding proteins by affinity and involves the use of agarose beads which are coated with covalently linked PolyI-PolyC (generated as described in Section 2.8.1.1). Firstly, dsRNA agarose beads (100 µL) were gently mixed with clarified protein extract (2 – 10 mL) from *Arabidopsis* and this incubation allowed the agarose-attached dsRNA to capture dsRNA binding proteins, such as pPKR, from the plant extract. Unbound proteins were removed from the agarose column by three washing steps with Buffer A (as described in Section 2.8.1.2) and the resultant agarose beads and bound proteins were pelleted and incubated in the presence of 2.5 µM ATP and 200 µCi/mL $\gamma^{32}$P-ATP to allow for kinase phosphorylation activity. The affinity purified proteins were then released from the agarose beads by addition of SDS within the loading buffer and boiled for 5 min. This was followed by centrifugation and separation of the proteins by their molecular sizes on an SDS PAGE. Subsequently, the purified proteins were analysed for their total protein and protein phosphorylation profiles as well as their immunoreactivity with the anti-mPKR antibody.
4.3.2 Optimisation of the Double-stranded RNA Agarose Binding Assay and Analysis of the Resulting Enriched Proteins

Figure 4.4 represents the purification of proteins that was achieved with the dsRNA agarose binding assay. Three bands, at 30 kDa, 55 kDa, and 68 kDa, were observed to be both phosphorylated (Fig. 4.4c) and immunoreactive with the polyclonal anti-mPKR antibody (Fig. 4.4d, asterisks). However, as shown in the protein stain (Fig. 4.4b), a large number of proteins were purified by the dsRNA agarose binding assay. In Arabidopsis, there are at least 16 dsRBM-containing proteins and numerous other proteins that contain motifs, such as the RNA recognition motif, that are demonstrated or postulated to bind to dsRNA. These known and postulated dsRNA binding proteins, as well as their interacting partners, may represent a portion of the dsRNA agarose-purified proteins, but a significant fraction of the detected proteins may also represent nonspecifically bound proteins that associated with sufficient affinity to the agarose beads during the purification process.

![Figure 4.4](image)

**Figure 4.4 Partial purification of proteins using the dsRNA agarose binding assay.** Arabidopsis proteins affinity purified with the dsRNA agarose binding assay were incubated in the presence of radiolabelled ATP and assessed by Deep Purple™ protein stain for the total protein profile (a) before purification, (b) after purification, and (c) phosphorimage for the presence of phosphorylated proteins after purification, and (d) western blot for the presence of proteins immunoreactive with the polyclonal mammalian PKR antibody after purification. Asterisks (*) indicate location of bands that are both phosphorylated and immunoreactive. Protein markers indicate molecular sizes in kDa.
The dsRNA binding specificity of the proteins purified on dsRNA agarose was assessed by pre-incubation of the protein extract with varying amounts of competing dsRNA before incubation with the dsRNA agarose (Fig. 4.5). The protein stain showed that the intensity levels of at least three different sized bands (Fig. 4.5a, red asterisks) decreased significantly in the presence of increasing concentrations of free dsRNA. Conversely, the presence of increasing amounts of free ssRNA did not have any effects on the band intensities of these proteins. These bands represented proteins that were specifically bound to the dsRNA on the agarose beads and were unable to bind to the dsRNA agarose in the presence of soluble dsRNA as their dsRNA binding domains became occupied by the free dsRNA but not ssRNA. The phosphorimage (Fig. 4.5b, green asterisk) and western blot (Fig. 4.5c, green asterisk) revealed protein bands that decreased in band intensity in the presence of increasing competitor dsRNA. These are a subset of proteins that bind specifically to dsRNA and are either immuno-related to mPKR or are phosphorylated in the presence of dsRNA. A decrease in eIF2α peptide phosphorylation activity was also observed with increasing concentrations of free dsRNA (Fig. 4.6). Similarly, the presence of free ssRNA had minor effects on the eIF2α phosphorylation activity, indicating the detection of dsRNA binding protein-dependent phosphorylation.

In contrast to those proteins that bound dsRNA specifically, the intensity of many of the bands on the protein stain did not decrease with the presence of the free dsRNA, indicating that these proteins bound nonspecifically to the agarose beads. Surprisingly, the intensity level of a number of bands (Fig. 4.5a, blue asterisks) increased with the presence of increasing amounts of free dsRNA.

To increase the specific activity of the putative plant-functional homolog of PKR, a number of approaches were undertaken in an attempt to reduce the nonspecific binding of proteins to the agarose beads. Firstly, the supernatant remaining from the plant proteins incubated with dsRNA agarose (i.e., containing the unbound proteins) was recovered and used for an additional incubation step with a second round of dsRNA agarose. An analysis of the resulting purified proteins showed little difference in the protein profile between the two rounds of dsRNA agarose incubation (Fig. 4.7). In fact, there was no noticeable reduction in the amount of nonspecifically bound proteins.
Figure 4.5 Assessing the dsRNA binding specificity of dsRNA agarose-enriched proteins. *Arabidopsis* proteins affinity purified with the dsRNA agarose binding assay were examined for their dsRNA-binding specificity by the addition of increasing concentrations of competitive dsRNA or ssRNA in the presence of radiolabelled ATP. The effect on the dsRNA agarose-bound proteins were assessed by (a) Deep Purple™ protein stain for the total protein profile, (b) phosphorimage for the presence of phosphorylated proteins, and (c) western blot for the presence of proteins immunoreactive with the polyclonal anti-mammalian PKR antibody. Red asterisks (*) and blue asterisks (**) indicate location of protein bands decreasing or increasing in intensity in the presence of increasing competitive dsRNA, respectively. Green asterisks (*) indicate location of phosphorylated or immunoreactive protein bands that decreased in intensity in the presence of increasing competitive dsRNA. Concentrations (µg/mL) of competitive dsRNA or ssRNA represented by numbers above the orange or purple bars, respectively. Protein markers indicate molecular sizes in kDa.
Figure 4.6 Quantification of putative pPKR activity of dsRNA agarose-enriched proteins in the presence of competitive dsRNA. Bar graph showing the effect on eIF2α phosphorylation of dsRNA agarose-purified *Arabidopsis* proteins incubated in the presence of radiolabelled ATP with increasing concentrations of competitive dsRNA or ssRNA and washed using phosphate buffered saline. Concentrations (µg/mL) of competitive dsRNA or ssRNA represented by numbers above the orange or purple bars, respectively. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.

Figure 4.7 Protein profile of dsRNA agarose-enriched proteins following a second incubation of the proteins with dsRNA agarose. Deep Purple™ protein stains of *Arabidopsis* proteins (a) affinity purified with one dsRNA agarose binding, and (b) proteins affinity purified from the recovered supernatant with a second round of dsRNA agarose. Protein markers indicate molecular sizes in kDa.
Subsequently, a pre-clarification with agarose beads was applied before the incubation of the plant extract with the dsRNA agarose. Although a significant portion of protein bands were still shared by the two treatments, the pre-clarification removed many proteins, some of which were specific to the agarose beads treatment (Fig. 4.8). This resulted in a reduction of bound proteins in the dsRNA agarose fraction (Fig. 4.8b). Additionally, it was demonstrated that the proteins which were immunoreactive with the polyclonal anti-mPKR antibody were not removed by the pre-clarification step (Fig. 4.8d). This observation suggests that the proteins partially purified on dsRNA agarose following pre-clarification with agarose beads may be genuine dsRNA binding proteins, including the plant-functional homolog of PKR.

**Figure 4.8** Protein profile of dsRNA agarose-enriched proteins following a pre-incubation of the proteins with agarose beads only. *Arabidopsis* proteins affinity purified with agarose beads only as assessed by (a) Deep Purple™ protein stain and (c) western blot showing immunoreactive with the polyclonal mammalian PKR antibody. Proteins affinity purified from the pre-cleared supernatant (agarose beads only) followed by a second round of dsRNA agarose as assessed by (b) Deep Purple™ protein stain and (d) western blot showing immunoreactive with the polyclonal mammalian PKR antibody. Protein markers indicate molecular sizes in kDa.
4.3.3 Summary of the Double-stranded RNA Agarose Binding Assay

The development of the dsRNA agarose binding assay represented a potential approach to partially purify pPKR from Arabidopsis. The candidates of putative pPKR identified via subsequent protein sequence analysis of dsRNA agarose-enriched fractions are listed in Table 4.2. However, due to undefined parameters as well as the nonspecific binding property of the assay even after pre-clarification with agarose beads, the effectiveness of this purification approach by itself was limited by the variations and enrichment of large numbers of non-dsRNA binding proteins.

Although the pre-clarification of the plant extract with agarose beads significantly reduced the amount of proteins bound to dsRNA agarose, the number of proteins present on dsRNA agarose still remained high. As well as a general background of poorly represented proteins, more than 40 distinct protein bands, each representing at least one protein, were observed on the protein gels. This made it difficult to identify genuine dsRNA agarose binding proteins, including potential pPKR candidates.

A variation in results was obtained with each individual batch of dsRNA agarose beads that were made (dsRNA agarose beads prepared as described in Section 2.8.1.1). When the same plant extract sample was incubated with seven columns of dsRNA agarose, the resultant protein stain revealed a variation in protein profiles (Fig. 4.9a). Correspondingly, this difference in enrichment also produced slight changes in the phosphorylation profile for each batch of dsRNA agarose beads (Fig. 4.9b).

It was also questionable whether the dsRNA agarose beads were indeed affinity enriching for the putative pPKR. Figure 4.10 showed the detection of a phosphorylated 68 kDa band that was present in the supernatant, i.e., the unbound proteins, following an incubation of the protein extract with dsRNA agarose beads (Fig. 4.10b). It is not obvious whether this band represents the putative pPKR as it was not immunoreactive with the anti-mPKR antibody (data not shown), however, it does show that there is at least one phosphorylated protein, in the size range of interest, that is not captured by the dsRNA agarose beads.
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Figure 4.9 Variation in protein profile observed with the incubation of the same protein extract with individual batches of dsRNA agarose. Total *Arabidopsis* protein extract was incubated with seven individual batches of dsRNA agarose in the presence of radiolabelled ATP and assessed by (a) Deep Purple™ protein stains for the presence of affinity purified proteins and (b) phosphorimage for the presence of phosphorylated proteins. Each lane represents an individual incubation of protein extract with a single batch of dsRNA agarose. Protein markers indicate molecular sizes in kDa.

Figure 4.10 Presence of a phosphorylated 68 kDa band in supernatant following incubation of protein extract with dsRNA agarose. *Arabidopsis* protein was incubated with dsRNA agarose in the presence of radiolabelled ATP and assessed by phosphorimage for the presence of phosphorylated proteins in (a) dsRNA agarose-bound fraction and (b) remaining supernatant following dsRNA agarose incubation. Protein markers indicate molecular sizes in kDa.
4.4 Immunoprecipitation

The significance and identification of the protein bands that were immunoreactive with the polyclonal anti-mPKR antibody (as described in Section 4.3.2) were examined by immunoprecipitation.

A brief outline of the immunoprecipitation method that was used is described in Section 4.4.1 and an analysis of the enriched proteins as well as the approaches that were trialled to optimise the immunoprecipitation method for the polyclonal anti-mPKR antibody are described and discussed in Section 4.4.2.

4.4.1 Mechanism of Immunoprecipitation

Immunoprecipitation allows the purification of a protein of interest from solution using an antibody that specifically binds to that particular protein. The antibody is first incubated with the protein extract and this allows the antibody to bind to the corresponding antigen that is located on the protein of interest. The antibody/antigen complex is then pulled out of the sample using protein A- or G-coupled agarose beads and this physically isolates and concentrates the protein of interest from the rest of the proteins.

Clarified protein extract (1 mL) from the flower buds of young *Arabidopsis* plants were incubated with the rabbit anti-mPKR polyclonal antibody (diluted 1:100 in Buffer A) to allow for antibody/antigen binding. Subsequently, the antibody/protein extract was incubated with Protein A-Sepharose™ 4B Conjugate and washed with Buffer A to isolate any proteins bound to the polyclonal anti-mPKR antibody. As recommended by the supplier (Zymed Laboratories), Protein A-coupled agarose was selected over Protein G-coupled agarose as the former conjugate had a higher binding affinity for immunoglobulins derived from rabbits. The antibody-bound proteins were released by boiling in 2x SDS gel loading buffer and separated by their molecular sizes on an SDS PAGE. The purified proteins were then analysed for their total protein profile as well as their immunoreactivity with the anti-mPKR antibody.

For each immunoprecipitation assay, a negative control was included where no antibody was added in the initial incubation step. A comparison of the protein profiles achieved with or without antibody gave an indication of the proteins that were binding nonspecifically to the agarose beads.
It was noted that preimmune serum would have been a better negative control, but this was not available for use at the time of the experiment.

### 4.4.2 Analysis of Proteins Immunoprecipitated by the Anti-Mammalian Protein Kinase R Antibody

Figure 4.11 represents the proteins which were isolated from *Arabidopsis* flower bud extracts by immunoprecipitation with or without the polyclonal anti-mPKR antibody, respectively. Several proteins enriched from the antibody-incubated extract (Fig. 4.11a) had higher intensity than the negative control that used no antibody (Fig. 4.11b). However, the overall protein profiles achieved from these two assays were in fact very similar, suggesting that the antibody was unable to immunoreact with any specific *Arabidopsis* proteins and that the partially purified proteins were binding nonspecifically to Protein A or the agarose beads. The difference in band intensities may also be due to variations in the amount of Protein A or agarose beads that were present in each aliquot of sample tested. On the other hand, a 50 kDa band was detected in the western blot of the antibody-incubated extract (Fig. 4.11c, asterisk), which most likely corresponded to the presence of the heavy chain of the antibody. The absence of other bands on the western blot suggested that immunoprecipitation using the anti-mPKR antibody was unable to purify any immunoreactive proteins. These results were surprising since the same antibody was previously immunoreactive with several proteins from *Arabidopsis* extracts (Fig. 4.4 & 4.8). A number of approaches were trialled in an attempt to achieve isolation of proteins which contained antigens to the anti-mPKR antibody, i.e., potential pPKR candidate proteins.

It was postulated that the anti-mPKR antibody may only bind to the activated form of the protein of interest. Consequently, the clarified plant extract was pre-incubated with 2.5 µM ATP to allow for phosphorylation and activation of the protein to occur prior to the incubation of the protein extract with the anti-mPKR antibody and the Protein A-coupled agarose beads. However, a similar result to that shown in Figure 4.11 was observed where no significant difference in the protein profile was detected between the antibody-incubated extract and the negative control (data not shown). Likewise, only the 50 kDa heavy chain of the antibody was detected on the western blot (data not shown).
Figure 4.11 Protein profile of proteins isolated by immunoprecipitation with the anti-mPKR antibody. *Arabidopsis* proteins incubated with or without the anti-mPKR antibody, followed by the incubation with Protein A-coupled agarose and assessed by (a, b) Deep Purple™ protein stain and (c, d) western blot showing immunoreactive with the polyclonal mammalian PKR antibody. Asterisk (*) indicates location of a band that represents the heavy chain of the antibody. Protein markers indicate molecular sizes in kDa.

It was proposed that the antigen determinant for the anti-mPKR antibody may be embedded within the tertiary structure of the protein, therefore inhibiting the binding of the antibody to the epitope and preventing the isolation of proteins of interest. To examine this, proteins were first denatured by boiling for 10 min for the putative antigenic epitopes to become exposed for binding with the anti-mPKR antibody. Unfortunately, a large amount of protein precipitates formed from the denaturation process and it became difficult to separate between the precipitated proteins from the agarose beads. To combat this, 0.1% (v/v) Tween 20 was added to the protein extract prior to the protein denaturation to reduce the amount of protein precipitation. Although fewer proteins were observed to precipitate, it was still difficult to separate the proteins from the precipitant. Hence, the Protein A-coupled agarose beads were unable to be washed to remove unbound proteins that would normally be in solution and no further investigation was made to determine whether the antigen of interest was hidden in the native structure of the protein.
4.4.3 Summary of the Immunoprecipitation

Although several dsRNA agarose-enriched proteins were observed to be immunoreactive with the polyclonal anti-mPKR antibody (Fig. 4.4 & 4.8), the identification of these proteins was unsuccessful using immunoprecipitation. It was proposed that the assay was ineffective in isolating these proteins of interest as the antigen determinant may be buried in the tertiary structure of the protein. However, denaturation of the protein extract failed to resolve this issue as the presence of protein precipitates prevented the separation of Protein A-coupled agarose beads from unbound proteins. On the other hand, a pre-clearing of the protein extract with agarose beads before the antibody incubation may aid in reducing the background level of nonspecifically bound proteins. It is possible that the large amount of proteins bound nonspecifically to the agarose beads may mask the presence of the genuine antibody-bound proteins.

4.5 Partial Purification of Protein Kinases

Another known characteristic of the putative pPKR that can be used to facilitate its partial purification and identification in plants is its ability as a protein kinase to bind ATP.

The KinaseBind™ assay was utilised to partially purify protein kinases from Arabidopsis protein extracts and a brief outline of this assay and the method used is described in Section 4.5.1. An analysis of the proteins enriched with this approach is described in Section 4.5.2.

4.5.1 Mechanism of the KinaseBind™ Assay

The KinaseBind™ assay exploits the ATP binding traits of kinases, heat-shock proteins, and other ATP-binding proteins and consists of agarose beads that are coated with an immobilised γP-linked ATP. A long hydrophilic spacer is located between the agarose bead and ATP, minimising disadvantageous hydrophobic interactions while enhancing the flexibility of the ATP to interact with binding proteins. The linking of the ATP via its terminal phosphate is also essential for the binding assay as the immobilised γP-linked ligand protects it from being removed by phosphatases present in crude protein extracts. As the ligand concentration required for the binding of pPKR was not known, two forms of ATP agarose with either high (8 – 12 mM) or low (1 – 2 mM) ligand density was used. Agarose beads with no ligand attached were also included in the binding assay as a control resin for the detection of proteins binding nonspecifically.
ATP agarose (50 µL) was first equilibrated in Buffer A/DTT and gently mixed with clarified protein extract (1 mL) from the flower buds of young *Arabidopsis*. This incubation allows the binding of ATP-binding proteins, such as the putative pPKR, to the ATP agarose. Unbound proteins were removed from the ATP agarose with five successive washing steps using Buffer A/DTT (as described in Section 2.8.2) and the proteins bound to the resin were then analysed for their eIF2α peptide phosphorylation activity by assessing them either bound to the ATP agarose or eluted from the column with 10 mM competing ATP. Proteins eluted with the competing ligand were taken directly for phosphorylation analysis or passed through a Bio-Spin® column (as described in Section 2.7.4) to remove the high concentration of ATP before the assessment for kinase activity. Subsequently, the ATP-agarose bound or ATP-eluted protein samples were incubated in the presence of 2.5 µM ATP and 200 µCi/mL γ32P-ATP with eIF2α peptide beads to allow for kinase phosphorylation activity. The partially purified proteins were also separated on an SDS PAGE and analysed for their total protein and phosphorylation profiles.

### 4.5.2 Analysis of Proteins Partially Purified by the KinaseBind™ Assay

Proteins were observed to be enriched by the high and low ligand density ATP agarose, with more proteins being bound to the high than low ligand density ATP agarose (Fig. 4.12a). On the other hand, the titre of proteins eluted from the ATP agarose with competitive ATP was low and the presence of these proteins was barely detectable by Colloidal Coomassie® protein staining. Phosphorylated bands were detected in the protein fractions enriched from the high ligand density ATP agarose but not in any of the low ligand or ATP competed fractions (Fig. 4.12b). However, this observation could not be replicated with repeated experiments, and thus the accuracy of the result was questionable. In addition, the phosphorylated bands were detected in protein samples which contained 10 mM ATP. This high level of ATP does not represent the typical environment in the cell for kinase activity (Ataullakhanov & Vitvitsky, 2002).

Similarly, the levels of eIF2α peptide phosphorylation activity detected in the ATP agarose bound or ATP-eluted protein samples were inconsistent. For the particular run shown in Figure 4.13, high kinase activity was detected from ATP eluted proteins from the control resin, while the activity of eIF2α phosphorylation did not differ between the fraction of proteins bound on the control, low ligand, and high ligand density ATP agarose. Conversely, a repeat of the same assay displayed low kinase activity in the fractions of ATP competed proteins from all the different resins, but high levels of eIF2α phosphorylation activity were detected in the high ligand density ATP agarose
bound fraction (Fig. 4.14). A relatively high amount of eIF2α phosphorylation activity was also detected in the control resin-bound fraction. Furthermore, phosphorylated proteins that presumably represent autophosphorylation activity could not be removed in the presence of free ATP.

Figure 4.12 Partial purification of proteins using the KinaseBind™ assay. Arabidopsis proteins affinity purified with the KinaseBind assay were incubated in the presence of radiolabelled ATP and assessed by (a) Colloidal Coomassie® protein stain and (b) phosphorimage for the presence of purified and phosphorylated proteins in the ATP agarose-bound fraction (ATP-bound), or in fractions eluted from the column with 10 mM competitive ATP (ATP competed), or in fractions eluted from the column with 10 mM competitive ATP that were desalted into Buffer A (ATP competed & desalted). Proteins were affinity purified with either control, low (1 – 2mM) ligand density, or high (8 – 12 mM) ligand density ATP agarose. Protein markers indicate molecular sizes in kDa.
Figure 4.13 Quantification of putative pPKR activity in KinaseBind™ assay-enriched proteins, Experiment 1. Bar graph showing the eIF2α phosphorylation detected in Arabidopsis protein fractions affinity purified with the KinaseBind assay. Proteins were assayed in the ATP agarose-bound fraction (ATP-bound), or in fractions eluted from the column with 10 mM competitive ATP (ATP competed), or in fractions eluted from the column with 10 mM competitive ATP that were desalted into Buffer A (ATP competed & desalted). Proteins were affinity purified with either control, low ligand (1 – 2 mM), or high ligand (8 – 12 mM) density ATP agarose. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
Figure 4.14 Quantification of putative pPKR activity in KinaseBind™ assay-enriched proteins, Experiment 2. Bar graph showing the eIF2α phosphorylation detected in *Arabidopsis* protein fractions affinity purified with a repeated KinaseBind assay. Proteins were assayed in the ATP agarose-bound fraction (ATP-bound), or in fractions eluted from the column with 10 mM competitive ATP (ATP competed), or in fractions eluted from the column with 10 mM competitive ATP that were desalted into Buffer A (ATP competed & desalted). Proteins were affinity purified with either control, low ligand (1 – 2mM), or high ligand (8 – 12 mM) density ATP ATP agarose. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
4.5.3 Summary of the KinaseBind™ Assay

Overall, the KinaseBind™ assay was not effective in achieving partial purification of pPKR. Results obtained with individual binding assays were generally inconsistent and repeated assays often yielded different protein and phosphorylation profiles as well as nonrepeatable levels of detected eIF2α phosphorylation activity.

4.6 Enrichment by Protein Size

The separation of proteins on the basis of its native molecular size is a different enrichment approach compared to partially purifying via the affinity binding of the target protein to known ligands. It allows proteins or protein complexes to be separated into individual fractions and thereby provides an alternative approach to achieving increased enrichment of the pPKR protein.

Size exclusion chromatography was used to separate the Arabidopsis protein extracts into their respective molecular sizes. Each set of experiments included at least three repeats of the same experiment with a similar pattern of results observed between these experiments. A brief introduction of the enrichment assay and an outline of the method used are described in Section 4.6.1. An analysis of the proteins enriched with this approach is described in Section 4.6.2.

4.6.1 Mechanism of Size Exclusion Chromatography

Size exclusion chromatography is a chromatographic method which exploits the rationale that particles of different sizes filter through a stationary phase at different rates. This stationary phase consists of a column of small porous polymer beads. As the solution travels through the column, smaller particles enter into the pores and require a longer time to elute from the column. Larger particles cannot enter into as many pores and traverse faster, leading to an earlier elution from the column. As a result, the solution of particles is separated based on their individual sizes. Provided that all particles are loaded simultaneously, molecules of the same sizes are eluted together into the same fractions.

Clarified protein extract (100 µL) from the flower buds of young Arabidopsis plants were loaded onto a BioSep™-SEC-S3000 silica-based gel filtration column and allowed to flow through the column at a rate of 0.6 mL per min (as described in Section 2.8.3). Eluted proteins, as detected at
an absorbance of $A_{280}$, were collected at 15 or 30 sec intervals and subsequently incubated in the presence of 2.5 µM ATP and 200 µCi/mL $\gamma^{32}$P-ATP to allow for kinase phosphorylation. The resultant proteins were either separated by native or denaturing gel electrophoresis for the analysis of their protein and phosphorylation profiles or further assessed for the presence of eIF2$\alpha$ phosphorylation activity.

### 4.6.2 Analysis of Proteins Separated by Size Exclusion Chromatography

Figure 4.15 illustrates the elution profile attained from the separation of proteins extracted from the flower buds of young *Arabidopsis* plants. The elution of the first major peak (high molecular weight proteins or complexes) was detected 12 min after the protein extract was loaded onto the column, and the elution of several small peaks (lower molecular weight proteins or complexes) continued for approximately 15 – 23 min after loading. Finally, a large peak (very low molecular weight proteins and salts) eluted at 25 – 30 min after loading. Elution fractions were initially collected at 30 sec intervals between 13 – 22 min after loading and assessed for their native and denatured protein profiles and phosphorylation activity (Fig. 4.16 & 4.17).

A relatively low amount of proteins was detected in the fractions collected at 13 min, despite a large protein peak observed in the protein elution profile during that time period (Fig. 4.15). A higher amount of proteins was present in the elution fractions thereafter, representing protein complexes in the native protein profile (Fig. 4.16a) and individual, separated proteins (Fig. 4.17a) in the denatured protein profile. On the whole, the amount of proteins present in each elution fraction was relatively low. This may have resulted from a dilution of the original plant extract as it traversed through the chromatography column.

A high level of phosphorylation activity was detected in the elution fractions collected from 17 min 30 sec to 19 min 30 sec (Fig. 4.16b & 4.17b, lanes 10 – 14, red and bolded). These phosphorylated bands corresponded to molecular sizes of 50, 100, and 180 kDa in the native protein profile and 25 – 30, 37, and 55 kDa in the denaturing protein gel. Similarly, the level of eIF2$\alpha$ peptide phosphorylation peaked in fractions collected from 18 min to 19 min, paralleling the presence of phosphorylated proteins with kinase activity (Fig. 4.18, fractions 11 – 13, red and bolded).
Elution fractions were collected at shorter intervals of 15 sec to improve the resolution of the separated protein compared with the previous experiment described above. As phosphorylation activity was previously observed at 17 min 30 sec to 19 min 30 sec, smaller elution fractions were collected around 17 min to 21 min. A better resolution of protein separation was observed with the 15 sec elution fractions (Fig. 4.19). The same range of phosphorylated bands was seen in the denaturing protein profile, with the most intense bands appearing in the fractions collected at 18 min 30 sec to 19 min 15 sec (Fig. 4.19, lanes 7 – 10, red and bolded). The data from the native protein profile is not shown as the gel did not run well, but a similar banding pattern to that shown in Figure 4.16 was observed and these bands were also most intense in the fractions from 18 min 30 sec to 19 min 15 sec. Likewise, an increase in eIF2α peptide phosphorylation was detected in fractions collected from 18 min 15 sec to 19 min 30 sec (Fig. 4.20, fractions 6 – 11, red and bolded), and this finding correlates with the presence of phosphorylated proteins (Fig. 4.19, fractions 7 – 10, red and bolded).
Figure 4.16 Native protein profile of proteins separated by size exclusion chromatography. *Arabidopsis* proteins were separated by size exclusion chromatography according to their native molecular sizes and incubated in the presence of radiolabelled ATP. The presence of total protein and phosphorylated proteins were assessed by (a) Colloidal Coomassie®-stained native protein stain and (b) phosphorimage. **Lanes 1 – 18**, fractions eluted at 30 seconds interval starting at 13 minutes. High levels of phosphorylation activity were detected at 17 min 30 sec to 19 min 30 sec (lanes 10 – 14, red and bolded). Protein markers indicate molecular sizes in kDa.
Figure 4.17 Denatured protein profile of proteins separated by size exclusion chromatography. *Arabidopsis* proteins were separated by size exclusion chromatography according to their native molecular sizes and incubated in the presence of radiolabelled ATP. The presence of total protein and phosphorylated proteins were assessed by (a) Colloidal Coomassie®-stained denatured protein stain and (b) phosphorimage. Lanes 1 – 18, fractions eluted at 30 seconds interval starting at 13 minutes. High levels of phosphorylation activity were detected at 17 min 30 sec to 19 min 30 sec (lanes 10 – 14, red and bolded). Protein markers indicate molecular sizes in kDa.
Figure 4.18 Quantification of putative pPKR activity in proteins separated by size exclusion chromatography. Bar graph showing the eIF2α phosphorylation detected in protein fractions separated according to their native molecular sizes. Fractions 1 – 18, fractions eluted at 30 seconds intervals starting at 13 minutes; Fraction 19, Buffer A only control. High levels of eIF2α phosphorylation activity were detected at 18 min to 19 min (samples 11 – 13, red and bolded). The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
Figure 4.19 Denatured protein profile of proteins separated by size exclusion chromatography. *Arabidopsis* proteins were separated by size exclusion chromatography according to their native molecular sizes and incubated in the presence of radiolabelled ATP. The presence of total protein and phosphorylated proteins were assessed by (a) Colloidal Coomassie-stained denatured protein stain and (b) phosphorimage. Lanes 1 – 16, fractions eluted at 15 seconds interval starting at 17 minutes. High levels of phosphorylation activity were detected at 18 min 30 sec to 19 min 15 sec (lanes 7 – 10, red and bolded). Protein markers indicate molecular sizes in kDa.
Figure 4.20 Quantification of putative pPKR activity in proteins separated by size exclusion chromatography. Bar graph showing the eIF2α phosphorylation detected in protein fractions separated according to their native molecular sizes. **Fractions 1 – 16**, fractions eluted at 15 seconds intervals starting at 17 minutes; **Fraction 17**, Buffer A only control. High levels of eIF2α phosphorylation activity were detected at 18 min 15 sec to 19 min 30 sec (samples 6 – 11, red and bolded). The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
4.6.3 Summary of the Size Exclusion Chromatography

Size exclusion chromatography provided an effective separation technique for the enrichment of putative pPKR with consistent results and protein profiles attained from repeated chromatograph assays. Protein fractions with high putative pPKR activity were collected and stored for future manipulations to aid in determining the characteristics of pPKR.

4.7 Sequential Partial Purification of Size-Fractionated and Double-stranded RNA Binding Proteins

Thus far, protein fractions partially purified with the dsRNA agarose binding assay and size exclusion chromatography have shown that significant eIF2α phosphorylation activity is often linked with the presence of phosphorylated proteins. However, a comparison of their protein phosphorylation profiles demonstrated distinct protein banding patterns, with few phosphorylated proteins of similar sizes (Fig. 4.31a & b, red asterisks). Hence, it was of interest to investigate whether the combination of the two enrichment techniques would result in a higher specific activity of the putative pPKR protein.

A brief outline of the method that was used to combine the size exclusion chromatography assay with the dsRNA agarose binding assay is described in Section 4.7.1 and an analysis of the proteins enriched with this approach is described in Section 4.7.2.

4.7.1 Combination of Size Exclusion Chromatography with the Double-stranded RNA Agarose Binding Assay

Clarified protein extract (100 µL) from the flower buds of young Arabidopsis plants were first separated by size exclusion chromatography and fractions were collected in 15 sec intervals from 18 min to 19 min. The eluted proteins were then pre-clarified with agarose beads, followed by gentle mixing with dsRNA agarose beads (100 µL), washed to remove unbound proteins, and incubated with eIF2α peptide beads in the presence of 2.5 µM ATP and 200 µCi/mL γ32P-ATP to allow for kinase phosphorylation activity.
4.7.2 Analysis of Proteins Co-Enriched by Size Exclusion Chromatography and Double-stranded RNA Agarose Binding Assay

Figure 4.21 shows the detection of the expected eIF2α phosphorylation activity in the size exclusion chromatography-separated protein fractions. However, after the incubation with dsRNA agarose beads, no kinase activity was observed. Similarly, the expected phosphorylated bands were detected in the size exclusion chromatography-separated fractions, but the presence of these bands disappeared following the incubation with dsRNA agarose beads (data not shown).

![Figure 4.21 Quantification of putative pPKR activity in proteins enriched by a combined purification approach using size exclusion chromatography and dsRNA agarose binding assay.](image)

Bar graph showing the eIF2α phosphorylation detected in protein fractions separated by size exclusion chromatography (SEC) and the same fractions following an additional partial purification step with dsRNA agarose. Fractions 1 – 4, fractions eluted at 15 seconds intervals starting at 18 minutes. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
4.7.3 Summary of the Combination of Size Exclusion Chromatography with the Double-stranded RNA Agarose Binding Assay

A combination of the size exclusion chromatography assay with the dsRNA agarose binding assay was not successful in attaining further enrichment of the putative pPKR with high specific activity. In retrospect, an incubation of the dsRNA agarose with a combined pool of the size exclusion chromatography-separated protein fractions, instead of each individual fraction, may have served as a better purification protocol, whereby a higher amount of protein would be available for the subsequent dsRNA agarose purification approach. On the other hand, the long duration of the entire experiment might have also affected the survival of the target protein in its active state and thus resulted in a loss of detected eIF2α phosphorylation activity. The sustainability of putative pPKR activity over this time period can be examined by comparing the level of eIF2α phosphorylation in either the starting material or a mock-treated protein sample alongside samples from the second partial purification.

4.8 Enrichment by Ionic Strength

Another approach that was undertaken to enrich for the putative pPKR was to separate proteins based on their charge properties. It is predicted that the isoelectric points (pIs) of the putative pPKR, in its phosphorylated and unphosphorylated forms, are 8.2 and >9, respectively (Roth & He, 1994). These expected pIs correspond to the pIs measured for human PKR, where the phosphorylated and unphosphorylated forms have a pI of 7.8 – 8.4 and 9.6, respectively (E. Meurs et al., 1990).

Ion exchange chromatography was used to separate the Arabidopsis protein extracts into their respective charge densities. Each set of experiments included at least two repeats of the same experiment with a similar pattern of results observed between these experiments. A brief introduction of the purification assay and a general outline of the method used are described in Section 4.8.1. Overall, two different ion exchangers were exploited for their ability to fractionate and enrich for proteins with high eIF2α phosphorylation activity. The methods used as well as an analysis of the proteins purified with each exchanger are described in Section 4.8.2 for the anion exchanger HiTrap™ Q Sepharose HP and Section 4.8.3 for the cation exchanger HiTrap™ SP Sepharose XL. In addition, difficulties experienced with the anion Q HP exchanger and
approaches that were trialled to optimise this purification technique are discussed in Section 4.8.2.2.

4.8.1 Mechanism of Ion Exchange Chromatography

The separation of protein molecules via ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilised ion exchange groups containing the opposite charge and is based on the observation that different molecules have different charges, charge densities and distribution of charge on their surfaces. Accordingly, these differences influence the degrees of interaction that each molecule has with the oppositely charged ion exchanger and specific molecules remain bound to or are freed from the chromatograph matrix through a gradient of charge and pH conditions.

Ion exchangers are formed of an insoluble matrix, such as dextran or agarose, and consist of covalently bound charged groups that are associated with mobile counter-ions. There are two main groups of ion exchangers and the properties of these exchangers are defined by the type and strength of their charged groups. Positively charged exchangers have negatively charged counter-ions, i.e., anions, available for exchange and are called anion exchangers. Conversely, negatively charged exchangers have positively charged counter-ions, i.e., cations, and are known as cation exchangers. In terms of strength, ion exchangers such as the anion exchanger quaternary ammonium (Q) or the cation exchangers methyl sulphonate (S) and sulphopropyl (SP) contain the respective quaternary or sulphonate amino groups that make them strong ion exchangers and these exchangers remain completely ionised over a wide pH range. On the other hand, weak ion exchangers, such as the anion exchanger diethylaminoethyl (DEAE), are ionised over a narrower pH range and their exchange capacity varies much more markedly with pH. Hence, ion exchange experiments are more controllable with strong ion exchangers as the charge characteristics of the matrix are not as easily affected by changes in pH.

The process of ion exchange chromatography begins with the equilibration of the ion exchanger into charge and pH conditions which favours the binding of the desired molecule onto the chromatograph matrix. Typically, the starting buffer is the protein extraction buffer and consists of a low salt concentration. Once equilibrated, protein molecules are loaded onto the ion exchanger and adsorption occurs where solute molecules carrying the appropriate charges displace the counter-ions and reversibly bind to the ion exchange groups. Unbound molecules are subsequently
removed in the void volume and the elution of these proteins is characteristically the first peak of
the elution profile. Bound molecules are then removed from the column by changing the eluting
buffer to conditions which are unfavourable for ionic binding and this involves a change in the
ionic strength of the buffer, such as with an increasing salt gradient. As a result, adsorbed
molecules are released from the column in the order of their strengths of binding with the most
weakly bound proteins eluted first.

Clarified protein extract (1 – 5 mL) from the flower buds of young *Arabidopsis* plants were loaded
onto a pre-equilibrated HiTrap™ Q Sepharose HP (Q HP) or HiTrap™ SP Sepharose XL (SP XL)
column (prepared and performed as described in Section 2.8.4). Bound proteins were eluted using
an increasing KCl gradient of 20 mM to 500 mM and were collected as 0.5 mL fractions. Fractions
containing eluted proteins, as detected at an absorbance of A$_{280}$, were desalted as described in
Section 2.7.4 and equilibrated into Buffer A/DTT. Subsequently, the eluted proteins were
incubated in the presence of 2.5 µM ATP and 200 µCi/mL γ$^{32}$P-ATP to allow for kinase
phosphorylation and the resultant proteins were either separated by denaturing gel electrophoresis
for the analysis of their protein and phosphorylation profiles or further assessed for the presence of
eIF2α phosphorylation activity.

### 4.8.2 Anion Exchanger HiTrap™ Q Sepharose HP

The anion Q HP column (1 mL) was first pre-equilibrated in Buffer A/DTT/20 mM KCl. Clarified
protein extract (1 mL) from the flower buds of young *Arabidopsis* plants with Buffer A/DTT (at
the normal KCl concentration of 100 mM) were then injected onto the anion exchanger using a 5
mL loop and the column was washed with five column volumes of Buffer A/DTT/20 mM KCl.
Subsequently, bound proteins were eluted from the matrix with an increasing KCl gradient of
20 mM to 500 mM over 20 column volumes and collected as 0.5 mL fractions. The KCl
concentration was then raised to 1 M for five column volumes and finally reverted back to 20 mM
KCl for another ten column volumes.

### 4.8.2.1 Analysis of Proteins Separated by Anion Exchanger HiTrap™ Q Sepharose HP

Figure 4.22 portrays the elution profile of the anion Q HP exchanger from which six main elution
peaks were detected. The first peak (P1, fractions 3 – 10) represented the void volume where
unbound proteins were removed and a high proportion of proteins were observed to elute at this
stage. The elution of proteins in fractions 22 – 25 were represented by peak 2 (P2); fractions 38 – 44 by peak 3 (P3); fractions 46 – 48 by peak 4 (P4); fractions 52 – 58 by peak 5 (P5); and fractions 67 – 71 by peak 6 (P6). The green line depicts the level of predicted conductance corresponding to the buffer salt concentrations that were introduced at the specific column volumes. On the other hand, the red line depicted the actual conductance that was detected within the solution eluting from the column and a lag in conductance change, as compared to the expected conductance, was observed. This delay in measured conductance may be explained by the difference in the period of time that was required for the eluting buffer to traverse from the wirings at the loading position (predicted conductance) to the start of the column (detected conductance). Additionally, a small increase in conductance was detected immediately following the loading of the protein extract onto the ion exchanger (Fig. 4.22, asterisk). This increase was due to the loading of proteins which were extracted from *Arabidopsis* using Buffer A/DTT, a buffer which contained 100 mM KCl. As the column was pre-equilibrated with Buffer A/DTT/20 mM KCl, the difference in salt concentration resulted in an increase in conductance for a few column volumes before the conductance level returned to that observed for 20 mM KCl.

Elution fractions were desalted and equilibrated to Buffer A/DTT for subsequent kinase assays. However, due to a limit in the number of lanes on the resulting protein gels as well as a constraint in time, not all the fractions of each peak were assayed. Instead, fractions resolving closest to each peak were chosen. Owing to logistical constraints and to maximise the number of samples that was processed, only Peptide S was utilised in the detection of eIF2α peptide phosphorylation. Results from previous experiments using the immobilised eIF2α peptide assay (as seen in Chapters 3 and 4) clearly shows that the Peptide A and Beads only samples serve as statistically significant negative controls and the quantitative phosphorylation detected on Peptide S can confidently be interpreted as eIF2α phosphorylation activity.

In contrast to the presence of a large amount of protein in P5 (Fig. 4.23a), the majority of phosphorylated proteins were observed in the fractions eluted from P1 and P3 (Fig. 4.23b) and the phosphorylated bands in the P3 fractions corresponded to the denatured molecular sizes of 30, 55, and 68 kDa. Similarly, the level of eIF2α peptide phosphorylation peaked in the P3 fractions, with the highest activity detected in elution fractions 41 – 43, paralleling the presence of phosphorylated proteins with kinase activity (Fig. 4.24). In addition, an analysis of the charge conditions required for the release of proteins at P3 established that the P3 fractions eluted at approximately 150 mM salt.
Figure 4.22 Protein elution profile of proteins separated with the anion exchanger Q HP, Approach 1. Elution profile of proteins extracted from the flower buds of young Arabidopsis plants separated by ion exchange chromatography using the anion exchanger Q HP. Proteins were extracted with Buffer A/DTT and the exchanger column was pre-equilibrated with Buffer A/DTT/20 mM KCl. A 5 mL loop was used for injection of protein onto column. Red numbers above X-axis represent sample number of the collected protein fractions and protein was collected as 0.5 mL fractions. The Y-axis indicates the relative amount of protein eluted (as detected at an absorbance of A$_{280}$). Red asterisk (*) indicate detection of increased conductance level immediately following protein extract loading. Blue line, protein elution profile; green line, expected conductance level; red line, actual detected conductance level; P1, elution peak 1 (also represents void volume); P2, elution peak 2; P3, elution peak 3; P4, elution peak 4; P5, elution peak 6; P6, elution peak 6.
Figure 4.23 Denatured protein profile of proteins separated with the anion exchanger Q HP. *Arabidopsis* proteins were separated by ion exchange chromatography using the anion exchanger Q HP, eluted as 0.5 mL fractions, and incubated in the presence of radiolabelled ATP. The presence of total protein and phosphorylated proteins were assessed by (a) Colloidal Coomassie®-stained denatured protein stain and (b) phosphorimage. Numbers above image represent sample number of the assayed protein fraction. P1 – 6 represent the elution peaks which correspond to the assayed protein fractions. Protein markers indicate molecular sizes in kDa. P1, elution peak 1 (also represents void volume); P2, elution peak 2; P3, elution peak 3; P4, elution peak 4; P5, elution peak 6; P6, elution peak 6.
Figure 4.24 Quantification of putative pPKR activity in proteins separated with the anion exchanger Q HP. Bar graph showing the eIF2α phosphorylation detected in protein fractions separated by ion exchange chromatography using the anion exchanger Q HP. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numbers on the X-axis indicate sample number of the assayed protein fraction. P1 – 6 represent the elution peaks which correspond to the assayed protein fractions. P1, elution peak 1 (also represents void volume); P2, elution peak 2; P3, elution peak 3; P4, elution peak 4; P5, elution peak 6; P6, elution peak 6. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
4.8.2.2 Difficulties of the Anion Exchanger HiTrap™ Q Sepharose HP

Although the separation of proteins with high phosphorylation activity was achieved, a major setback of the ion exchange chromatography experimental procedure was that the plant proteins were extracted with a buffer containing 100 mM salt, while the ion exchanger was equilibrated with a buffer containing 20 mM salt. The difference in starting buffers resulted in an initial increase in the conductance level as discussed above and shown in Figure 4.22 (asterisk) and this alteration was measured to rise from 20 mM to around 100 – 110 mM salt. In theory, the rise in salt concentration would result in the elution into the void volume of all the proteins that are exchanged at this charge condition. Similarly, a proportion of the target protein, which eluted at 150 mM salt, could possibly be eluted into the void volume, especially if this initial increase in salt concentration escalated near or above 150 mM.

A number of approaches were undertaken to improve the experimental protocol to avoid a possible elimination of the target protein into the void volume. Firstly, the concentration of salt in the protein extract was reduced. Proteins were extracted from *Arabidopsis* using Buffer A/DTT, which contains 100 mM KCl, but the solution was subsequently diluted five-fold with Buffer A/DTT (-salt) to obtain a protein extract with a buffer solution containing only 20 mM KCl. The same ion exchange chromatography protocol was applied for loading the diluted protein extract onto the anion Q HP exchanger and a protein elution profile, similar to that achieved previously with the undiluted protein extract, was observed (Fig. 4.25). Elution fractions were taken from the six peaks, desalted, and assayed for the presence of phosphorylated bands and eIF2α peptide phosphorylation activity. However, no phosphorylated bands or eIF2α phosphorylation activity were detected (data not shown). It was postulated that the lowered salt concentration might have had an effect on the structure of the target protein, resulting in a loss of kinase activity.

The next approach trialled a change in the starting charge conditions of the ion exchanger. The buffer for the extraction of *Arabidopsis* proteins remained as the original Buffer A/DTT (containing 100 mM KCl), but the anion Q HP exchanger column was pre-equilibrated with Buffer A/DTT instead of Buffer A/DTT/20 mM KCl. Hence, the ion exchange chromatography was equilibrated and began at 100 mM salt. As shown in Figure 4.26, the six peaks were still visible in the elution profile, however, P3 and P5 were hardly distinguishable above the base line and the amount of protein present was very low. An additional elution peak (P1.5) was also observed.
between P1 and P2. No phosphorylated bands or eIF2α phosphorylation activity were detected in the fractions from each elution peak (data not shown).

The last approach used the initial ion exchange chromatography protocol where proteins were extracted with Buffer A/DTT and loaded at 100 mM salt, and the column was equilibrated with Buffer A/DTT/20 mM KCl. However, a 2 mL loop, instead of a 5 mL loop, was used to inject the protein extract onto the anion Q HP. This shorter loop was trialled as only 1 – 2 mL of plant extract was prepared for injection for each ion exchange chromatography experiment. In theory, by using the 2 mL loop, less mixing of the injected protein extract and the equilibration buffer would occur inside the loop and the probability of diluting the ion concentration of the protein extract would be reduced. An elution profile similar to that achieved with the initial ion exchange method was observed, but the detected amount of proteins in the elution peaks was significantly lower (Fig. 4.27). Moreover, by using a shorter loop, the initial increase in conductance, due to the presence of 100 mM salt in the protein extract, displayed a narrower peak (Fig. 4.27, asterisk) and was measured to reach a salt concentration of 154 mM. The resolution of P3, the peak in which phosphorylation activity was previously detected, was also broader and appeared assymetrical, suggesting that it may contain an extra elution peak. In terms of kinase activity, the presence of phosphorylated bands and eIF2α phosphorylation activity was only detected in the fractions from P1, indicating that the target protein may have been eluted in the void volume (data not shown).
Figure 4.25 Protein elution profile of proteins separated with the anion exchanger Q HP, Approach 2. Elution profile of proteins extracted from the flower buds of young *Arabidopsis* plants separated by ion exchange chromatography using the anion exchanger Q HP. Proteins were extracted with Buffer A/DTT and diluted to a buffer salt concentration of 20 mM KCl and the exchanger column was pre-equilibrated with Buffer A/DTT/20 mM KCl. A 5 mL loop was used for injection of protein onto column. Red numbers above X-axis represent sample number of the collected protein fractions and protein was collected as 0.5 mL fractions. The Y-axis indicates the relative amount of protein eluted (as detected at an absorbance of A_{280}). Blue line, protein elution profile; green line, expected conductance level; red line, actual detected conductance level; P1, elution peak 1 (also represents void volume); P2, elution peak 2; P3, elution peak 3; P4, elution peak 4; P5, elution peak 6; P6, elution peak 6.
Figure 4.26 Protein elution profile of proteins separated with the anion exchanger Q HP, Approach 3. Elution profile of proteins extracted from the flower buds of young Arabidopsis plants separated by ion exchange chromatography using the anion exchanger Q HP. Proteins were extracted with Buffer A/DTT and the exchanger column was pre-equilibrated with Buffer A/DTT. A 5 mL loop was used for injection of protein onto column. Red numbers above X-axis represent sample number of the collected protein fractions and protein was collected as 0.5 mL fractions. The Y-axis indicates the relative amount of protein eluted (as detected at an absorbance of A$_{280}$). Blue line, protein elution profile; green line, expected conductance level; red line, actual detected conductance level; P1, elution peak 1 (also represents void volume); P1.5, additional elution peak between elution peak 1 and 2; P2, elution peak 2; P3, elution peak 3; P4, elution peak 4; P5, elution peak 6; P6, elution peak 6.
Figure 4.27 Protein elution profile of proteins separated with the anion exchanger Q HP, Approach 4. Elution profile of proteins extracted from the flower buds of young Arabidopsis plants separated by ion exchange chromatography using the anion exchanger Q HP. Proteins were extracted with Buffer A/DTT and exchanger column was pre-equilibrated with Buffer A/DTT/20 mM KCl. A 2 mL loop was used for injection of protein onto column. Red numbers above X-axis represent sample number of the collected protein fractions and protein was collected as 0.5 mL fractions. The Y-axis indicates the relative amount of protein eluted (as detected at an absorbance of A$_{280}$). Red asterisk (*) indicate detection of increased conductance level immediately following protein extract loading. Blue line, protein elution profile; green line, expected conductance level; red line, actual detected conductance level; P1, elution peak 1 (also represents void volume); P2, elution peak 2; P3, elution peak 3; P4, elution peak 4; P5, elution peak 6; P6, elution peak 6.
4.8.3 Cation Exchanger HiTrap™ SP Sepharose XL

Ion exchange chromatography was performed with the cation SP XL exchanger using the two approaches tested and developed from the anion exchanger studies. As it was previously shown that diluting the protein extract to 20 mM salt resulted in a loss of phosphorylation activity and the use of a 2 mL loop did not reduce the effect of the initial increase in conductance, these options were not undertaken. Clarified protein extracts (1 mL) from the flower buds of young Arabidopsis plants were all extracted using Buffer A/DTT and were loaded onto the column at 100 mM KCl using the 5 mL loop. On the other hand, the SP XL column (1 mL) was pre-equilibrated with either Buffer A/DTT/20 mM KCl or Buffer A/DTT to assess the difference in elution profiles achieved with starting charge conditions of either 20 mM salt or 100 mM, respectively. Unbound proteins were removed with five column volumes of Buffer A/DTT/20 mM KCl or Buffer A/DTT and bound proteins were subsequently released from the matrix with an increasing KCl gradient of 20 mM or 100 mM to 500 mM over 20 column volumes. The KCl concentration was then raised to 1 M for five column volumes and finally reverted back to 20 mM KCl for another ten column volumes. Eluted proteins were collected as 0.5 mL fractions.

4.8.3.1 Analysis of Proteins Separated by Cation Exchanger HiTrap™ SP Sepharose XL

Figure 4.28 shows the elution profile that was obtained when proteins were loaded and eluted from the SP XL column that was pre-equilibrated with Buffer A/DTT/20 mM KCl. Two main elution peaks were observed. The first peak (P1, fractions 2 – 16) represented the elution of unbound proteins into the void volume and as seen with the anion exchanger studies, a high proportion of proteins were eluted at this stage. The release of bound proteins was detected in a single broad peak (P2) and these proteins were eluted into fractions 34 – 56. However, a relatively low amount of protein was detected as represented by the low peak. In addition, as the column was equilibrated in 20 mM salt, an initial increase in conductance level was detected following protein extract loading (Fig. 4.28, asterisk) and the peak of the increase was measured to reach a salt concentration of 120 – 130 mM.

Elution fractions were taken from P1 and P2, desalted, and assayed for the presence of phosphorylated bands and eIF2α peptide phosphorylation activity. However, no phosphorylated bands (data not shown) or eIF2α phosphorylation activity (Fig. 4.29) were detected, except for slightly higher (~1.6-fold) levels of eIF2α phosphorylation activity in elution fractions 3 – 6 from
P1. This detection of activity in P1 again suggested that the target protein may have been eluted in the void volume.

Clarified protein extracts were also loaded onto a cation SP XL exchanger that was pre-equilibrated with Buffer A/DTT, i.e., starting with charge conditions at 100 mM salt. A protein elution profile, comparable to that observed with a pre-equilibration of 20 mM salt, was achieved (Fig. 4.30). However, the resolution of the peaks was lower and less eluted proteins were detected. Similarly, no phosphorylated bands or eIF2α phosphorylation activity was detected in the elution fractions, except for a low level of kinase activity in the fractions from P1 (data not shown).

4.8.4 Summary of the Ion Exchange Chromatography

Ion exchange chromatography was demonstrated to separate and enrich for proteins with high eIF2α phosphorylation activity using the anion Q HP exchanger, but not with the cation SP XL exchanger. Attempts with different approaches did not optimise the technique for a more stringent purification protocol that would reduce the possibility of the elution of the putative pPKR into the void volume.

Previously, Roth and He (1994) obtained significant partial purification of putative pPKR activity from tobacco using ion exchange chromatography with a DEAE exchanger followed by a Mono S column. As the anion DEAE exchanger is a weaker ion exchanger compared to the Q column, better separation of proteins should be achieved with the Q column ion exchanger. However, it should be noted that the protein separation ability of these two anion exchangers was not compared as the DEAE exchanger was not available for trial. Nevertheless, once the anion exchange chromatography technique is optimised and is demonstrated to be repeatable, it would be of interest to analyse the separation that can be achieved by collecting the P3 fractions eluted from the anion Q HP exchanger and subsequently loading these fractions onto a cation S or SP exchanger for further purification. Furthermore, it would be of interest to use sequential enrichment with anion and cation exchange chromatography followed by size exclusion chromatography and/or affinity purification via the dsRNA agarose binding assay to attain higher enrichment of putative pPKR specific activity.
Figure 4.28 Protein elution profile of proteins separated with the cation exchanger SP XL, Approach 1. Elution profile of proteins extracted from the flower buds of young Arabidopsis plants separated by ion exchange chromatography using the cation exchanger SP XL. Proteins were extracted with Buffer A/DTT and exchanger column was pre-equilibrated with Buffer A/DTT/20 mM KCl. A 5 mL loop was used for injection of protein onto column. Red numbers above X-axis represent sample number of the collected protein fractions and protein was collected as 0.5 mL fractions. The Y-axis indicates the relative amount of protein eluted (as detected at an absorbance of A_{280}). Red asterisk (*) indicate detection of increased conductance level immediately following protein extract loading. Blue line, protein elution profile; green line, expected conductance level; red line, actual detected conductance level; P1, elution peak 1 (also represents void volume); P2, elution peak 2.
Figure 4.29 Quantification of putative pPKR activity in proteins separated with the cation exchanger SP XL. Bar graph showing the eIF2α phosphorylation detected in protein fractions separated by ion exchange chromatography using the cation exchanger SP XL. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numbers on the X-axis indicate sample number of the assayed protein fraction. At represents the load sample of Arabidopsis protein extract. P1 – 2 represent the elution peaks which correspond to the assayed protein fractions. Protein markers indicate molecular sizes in kDa. P1, elution peak 1 (also represents void volume); P2, elution peak 2. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
Figure 4.30 Protein elution profile of proteins separated with the cation exchanger SP XL, Approach 2. Elution profile of proteins extracted from the flower buds of young *Arabidopsis* plants separated by ion exchange chromatography using the cation exchanger SP XL. Proteins were extracted with Buffer A/DTT and exchanger column was pre-equilibrated with Buffer A/DTT. A 5 mL loop was used for injection of protein onto column. Red numbers above X-axis represent sample number of the collected protein fractions and protein was collected as 0.5 mL fractions. The Y-axis indicates the relative amount of protein eluted (as detected at an absorbance of A$_{280}$). Blue line, protein elution profile; green line, expected conductance level; red line, actual detected conductance level; P1, elution peak 1 (also represents void volume); P2, elution peak 2.
4.9 Analysis and Identification of Potential Plant Protein Kinase R Candidates

Following the different enrichment and separation approaches, partially purified proteins were assayed for their kinase activity and separated on protein gels for the analysis of their protein and phosphorylation profiles as well as immunoblotting for the presence of anti-mPKR antibody immunoreactivity. Bands of interest from the protein gels were then excised and prepared for trypsin digest (prepared as described in Section 2.7.13.1) and protein identification via MS analysis (performed as described in Section 2.7.13.2). These bands were selected for MS analysis by their phosphorylation activity, and/or anti-mPKR antibody immunoreactivity, and/or the correlation of high eIF2\(\alpha\) peptide phosphorylation in the protein sample.

Details of the bands that were chosen for MS analysis and the method used to analyse the peptide sequence data are described in Sections 4.9.1 and 4.9.2, respectively. The proteins which were identified by MS and their significance as potential pPKR candidates are described and discussed in Section 4.9.3.

4.9.1 Selecting Bands of Interest

Bands that were excised included phosphorylated and anti-mPKR antibody immunoreactive bands detected from the dsRNA agarose binding assay, such as the bands in Figure 4.4 (asterisks). Phosphorylated bands were also taken from the size exclusion and ion exchange chromatography assays and these bands were chosen from fractions that exhibited a high level of eIF2\(\alpha\) peptide phosphorylation activity, i.e., fraction 18 min 30 sec (Fig. 4.19b, lane 7) and fraction 43 of P3 eluted from the Q HP column (Fig. 4.23b), respectively. Additionally, the protein phosphorylation profiles of these two fractions, as well as the profile of dsRNA agarose-enriched proteins, were compared (Fig. 4.31) and the bands displaying similar molecular sizes (red and blue asterisks) were excised for MS analysis.

4.9.2 Processing Peptide Sequence Data

The excised bands of interest for protein identification were digested with trypsin and prepared for MS analysis as described in Section 2.7.13.1. The generated peptide sequence data, containing the full scan MS spectra for total ion current scans and individual MS/MS spectra for selected
precursor ions, was then searched and matched against the *Arabidopsis* sequence genome. The significance of the peptide matches was determined by their Xcorr score, Sp value and ΔCn score as well as the number of hits detected for the particular peptide per sequencing run (refer to Section 2.7.13.2 for details). Once a peptide sequence was determined as significant, the matched proteins were analysed, if known, for their encoded molecular and functional properties and these traits were used to classify them as possible pPKR candidates. ORFs were searched for the presence of known nucleic acid binding domains (such as dsRBM or RNA binding domains) or general basic domains (that may represent a novel functional RNA binding domain), and/or a protein kinase domain. Proteins with molecular sizes between 55 – 70 kDa and a high pI were also considered as potential pPKR candidates but these were not essential criteria.
4.9.3 Analysis of Proteins Identified by Mass Spectrometry of Bands of Interest

Table 4.2 records all the proteins that were matched by MS analysis and that comprised one or more of the properties which were used to identify them as possible pPKR candidates. The proteins are listed according to their different properties; their molecular sizes, pIs, and motifs as well as peptide matches are shown in the table. In addition, the likelihood of the identified protein as being correctly identified as present in the sampled fraction is ranked in terms of their significance scores as well as the number of hits for the particular peptide. A potential significance grade of 5 denotes peptide matches with high significance scores and peptide hits of two or more, while a grade of 1 indicates low significance scores with a low number of peptide hits.

A total of 124 proteins were identified as possible pPKR candidates (Table 4.2). These included 43 nucleic acid binding domain-containing proteins, 23 protein kinases, two protein kinases with nucleic acid binding domains, seven calcium-dependent protein kinases, 15 protein kinases with non-nucleic acid binding domains, and 34 non-kinase and non-nucleic acid binding domain-containing proteins. Of these MS-identified proteins, three individual or groups of proteins were of particular interest:

a. Nucleic acid binding proteins (Section 4.9.3.1)

b. DsRBM-containing protein with an upstream protein kinase of unknown function (Section 4.9.3.2)

c. Calcium-dependent protein kinases (CPKs) (Section 4.9.3.3)

4.9.3.1 Identification of Nucleic Acid Binding Proteins

Although a high proportion of the MS data originated from the sequencing of bands purified by the dsRNA agarose binding assay, unexpectedly, only one dsRBM-containing protein was identified. On the other hand, 42 other proteins were identified that contained other non-dsRBM nucleic acid binding motifs. This suggested that other nucleic acid binding domains are involved in the binding of dsRNA in plants. It is also possible that the putative pPKR protein or additional dsRBM-containing proteins may be enriched at very low amounts such that their protein sequence is masked by the abundant presence of the other specifically or nonspecifically dsRNA agarose-bound proteins.
Table 4.2 Proteins identified by mass spectrometry analysis

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<th>Description¹</th>
<th>Molecular size (aa/Da)</th>
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Proteins containing at least one kinase domain but no other domain of interest
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Proteins containing non-kinase, non-nucleic acid binding domain(s)

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† abbreviated name of gene of interest; ‡ nucleic acid binding domains: ARID, AT-Rich Interaction Domain; ELM2, Egl-27 and MTA1 homology 2; CSD, Cold-Shock Domain; AIR1, Arginine methyltransferase-Interacting protein with a RING zinc finger; dsRBM, double-stranded RNA binding motif; DUF296, unknown function; DUF630, unknown function; DUF632, leucine zipper related; KH, K homology RNA-binding domain; RRM, RNA Recognition Motif; PUR, purine-rich PPR, pentatricopeptide repeat; RING, Really Interesting New Gene; Rossmann-NAD, Rossmann-fold NAD(P)^+ binding; SBP, Squamosa-promoter Binding Protein; YTH1, Cleavage and polyadenylation specificity factor; COG5219, zinc finger-related; * kinase domains: Ser/Thr, serine/threonine kinase; Tyr, tyrosine kinase; † other domains: TPR, tetratricopeptide repeat; WWA, Von Willebrand factor type A; NT2F, nuclear transport factor 2; SMc, Structural Maintenance of Chromosomes; EF hand, calcium-binding motif; B lectin, Bulb-type mannose-specific lectin; PAN-AP Plant, Plant PAN/APLLE-like domain; LRR, leucine-rich repeats; RWG, RING finger and WD40 domain related; His, Histidyl-tRNA synthase; USP, Universal Stress Protein; ABCF-EF3, ABC transporter Elongation factor 3; ANK, ankyrin repeats; Cu-oxidase, multicopper oxidase; COG3055, uncharacterised motif; NUDIX, Nucleoside Diphosphates linked to other moieties X; PRONE, Plant-specific Rop nucleotide exchanger; SHMT, Serine-glycine hydroxymethyltransferase; * peptides identified in the mass spectrometry analysis which matched to the identified protein; ° potential significance of the identified protein determined by their Xcorr score, Sp value, ΔCn score and the number of matches for each identified peptide: 5, high significance, 4, low significance.
As mentioned previously, there are no protein encoded by the *Arabidopsis* genome which contains both a dsRBM and a Ser/Thr kinase domain. However, the MS data indicated that, of the vast number of protein kinases identified, two of these proteins were associated with both a nucleic acid binding (non-dsRBM) domain and a protein kinase domain. Considering the above postulation that another nucleic acid binding domain may be used commonly by plants to bind dsRNA, it is possible that one of these nucleic acid binding domain-associated protein kinases may potentially be pPKR.

### 4.9.3.2 Identification of a Double-stranded RNA Binding Motif-containing Protein with an Upstream Protein Kinase of Unknown Function

Although there are no genes in the *Arabidopsis* genome that encode for an ORF with both a dsRBM and a Ser/Thr kinase domain, of particular interest was the identification of a dsRBM-containing gene, RNase three-like protein 1 (At1g80650), which was discovered to be located directly downstream to a gene encoding a protein kinase of unknown function (At1g80640). The short 370 nt separation between the two genes incited the possibility that the dsRBM-containing protein and kinase may have been misannotated as two separate genes and may actually represent a single gene that encompasses both a kinase domain and a dsRBM domain. This would represent the only instance of a single protein in *Arabidopsis* that contained both a kinase and a dsRBM. If this hypothesis was correct, then the two adjacent genes (annotated as distinct genes) would be translated from a single mRNA transcript.

The hypothesis that the two genes (At1g80640 and At1g80650) may represent a single gene was investigated by amplifying *Arabidopsis* cDNA (obtained from W. Cui at Plant and Food Research) across the adjacent ends of the two genes by PCR using the primers 42563378 F 1296 (a forward primer starting 300 nt at the 3’ end of the kinase gene) and 28950719 R 171 (a reverse primer starting 171 nt at the 5’ end of the dsRBM-containing gene). If the two genes were located on the same mRNA transcript, the two primers would amplify from the kinase and dsRBM-containing protein and through the 370 nt intervening sequence to generate an 800 nt or 1200 nt amplification product from *Arabidopsis* cDNA or genomic DNA, respectively.

As shown in Figure 4.32, these postulated product sizes were detected from the amplification assay, indicating that the two “genes” were indeed located on the same mRNA transcript. Products amplified from the cDNA were then cloned and sequenced for identification (refer to Appendix A
Some clones were found to comprise the full intervening sequence, while others had lengths of exons or introns absent from the kinase and/or dsRBM-containing protein. Of note, none of the clones identified had the downstream dsRBM-containing protein existing in the same translational frame as the upstream protein kinase.

It is possible that the dsRBM-containing protein might actually be a 3’ UTR of the protein kinase and is not translated in the cell. On the other hand, alternative splicing might occur sometimes or only in some tissues whereby the mRNA transcript containing both motifs in the single translational frame is generated, or it may be ubiquitous but rare. Conversely and less likely, an internal ribosome entry site might exist between the two genes or reinitiation of translation might occur at the second ORF for the translation of the dsRBM-containing protein. The significance of this finding still remains unresolved.

**Figure 4.32 Amplification of the intervening sequence between a protein kinase of unknown function and a downstream dsRBM-containing gene.** Agarose gel image of the products generated through amplification of *Arabidopsis* complementary and genomic DNA using primers which targeted the 3’ end of a protein kinase of unknown function (At1g80640), through the intervening sequence, and to the 5’ end of a dsRBM-containing protein (At1g80650). DNA markers indicate molecular sizes in nt.

### 4.9.3.3 Identification of Calcium-dependent Protein Kinases

An interesting finding in the MS analysis was the identification of seven CPKs (Table 4.2). These included CPK4, CPK6, CPK20, CPK21, CPK27, CPK29, and CPK33. It should be noted that the sequenced peptide matching to CPK21 is also located in CPK15. However, together with potential post-translational modifications that were detected on these peptides, the Xcalibur MS analysis package (ThermoFinnigan) determined CPK21 as a more likely match for this particular peptide.
As discussed in Section 1.3.2, CPKs are specifically found only in plants and some protists, but not in animals (Harper & Harmon, 2005). They are characterised by a highly conserved Ser/Thr kinase domain, an autoinhibitory domain responsible for maintaining the kinase in an inactive state, and four EF hands which bind to the calcium ions required for the activation of the protein kinase. The functional roles for these calcium-regulated kinases have so far been attributed to environmental stress signalling, such as the response to cold, salt, and drought, and to the perception of pathogen attack in plants (Babu et al., 2008; Kobayashi et al., 2007; Ma & Berkowitz, 2007; Marathe et al., 2004; Romeis et al., 2001; Tahtiharju et al., 1997; Urao et al., 1994; Yoon et al., 1999). This response to pathogens is activated by the elevation of cytosolic calcium (Garcia-Brugger et al., 2006). As shown with the infection of rotaviruses in human intestinal epithelial cells (Brunet et al., 2000), viruses can also initiate an increase in intracellular calcium concentrations. CPKs pose as potential candidates for pPKR and should be examined further for a possible role in the antiviral defense mechanism of plants.

### 4.10 Conclusions

A number of purification approaches were exploited to separate and enrich for the putative pPKR from *Arabidopsis*. These techniques included identification of the plant tissues with the highest eIF2α kinase activity, partial purification using the dsRNA agarose binding assay, immunoprecipitation with the polyclonal anti-mPKR antibody, partial purification using the KinaseBind™ assay, and separating proteins using size exclusion and ion exchange chromatography.

The size exclusion chromatography assay was shown to be most reliable and consistent in the separation and detection of putative pPKR activity, while the dsRNA agarose binding assay and ion exchange chromatography had issues with nonspecific binding and buffer salt concentrations, respectively. However, these latter two assays as well as size exclusion chromatography were able to distinguish proteins with significant putative pPKR activity and provided partially purified proteins for the subsequent MS analysis to identify potential pPKR candidates. In addition to displaying putative pPKR-like activities, samples enriched by these three different partial purification approaches displayed distinct protein banding patterns. Thus, it is postulated that a combination of these purification techniques may provide significant enhancement in achieving a more enriched fraction of pPKR. However, due to time constraints, this was not pursued. Once the various purification approaches are further optimised, their combined effectiveness on the
enrichment of the putative pPKR protein can be determined. On the other hand, the KinaseBind™
assay was not successful in the enrichment of putative pPKR and no peptide sequencing was
performed from this assay.

A large number of protein kinases and nucleic acid binding domain-containing proteins were
identified by MS analysis. The potential for these identified proteins as pPKR candidates was
determined by analysing their similarity to the molecular properties of PKR, such as size and pI, as
well as the significance of their peptide hit. It is important to note though that the ranking of
candidates according to their molecular sizes might not be an accurate analysis for the
identification of pPKR candidates. As there is no protein encoded by the Arabidopsis genome
which contains both a dsRBM and a Ser/Thr domain in the same ORF, it is possible that pPKR
might exist in plants as two proteins, one comprised of a dsRNA binding domain and the other
containing the kinase domain. These domains may interact with each other in a cooperative way to
perform PKR-like functions. If this hypothesis is correct, the predicted sizes of the two proteins
might not fall between the 55 – 70 kDa size range that was used as one criterion to determine the
potential of an identified protein as a pPKR candidate. Observations from several experiments that
each identified from plants a 68 – 70 kDa protein (size estimated under denaturing conditions) that
exhibits PKR-like properties (Langland et al., 1995) challenge the ‘two protein’ model of pPKR.
Therefore single proteins were considered as candidates of pPKR.

Of the proteins identified by the MS analysis, the most interesting finding was the identification of
CPKs as these kinases are unique to plants and most have not been characterised. More
importantly, some of these CPKs may function in response to pathogen attack. Further research
was conducted to determine the response of pPKR activity to the presence of divalent ions, such as
calcium, and the results achieved from these studies are described in Chapter 5.
Effect of Ions and Chelators on the Activity of Putative Plant Protein Kinase R

In plants, many ions have been shown to be involved in numerous signalling and regulatory pathways including their response to plant pathogens (Garcia-Brugger et al., 2006). In particular, it is well established that the calcium ion is an important secondary messenger in plants and is involved in various signalling pathways that convey messages in response to a range of environmental and developmental stimuli as well as the presence of pathogens (Lecourieux, Ranjева, & Pugin, 2006). Specific calcium signatures are recognised by different calcium sensors and calcium-dependent protein kinases (CPKs) form one of the distinct groups of calcium-binding proteins (Harper & Harmon, 2005).

Zinc is also an essential ion for the growth and development of plants. An abundance of proteins containing a zinc finger, a motif which binds to zinc to elicit its roles in numerous cellular functions, are found in plants, with approximately 0.7 percent of Arabidopsis genes encoding for C2H2-type zinc finger proteins (Ciftci-Yilmaz & Mittler, 2008). Zinc fingers are known to bind
mainly to DNA but have also been observed to interact with both RNA and protein. In fact, the zebrafish homolog of PKR uses zinc fingers rather than a dsRBD to bind to dsRNA. More importantly, recent studies have shown that these zinc-binding proteins may be activated in response to stress and pathogens in plants (reviewed in Ciftci-Yilmaz & Mittler, 2008).

As presented in Table 4.2, MS analysis of proteins partially purified with the various pPKR purification approaches has identified both CPKs and zinc fingers. It was thus of interest to examine the effects of calcium and zinc ions, as well as the chelators of these ions, on putative pPKR activity. If the activity of pPKR is influenced by these ions and/or chelators, the identified CPKs and zinc fingers may pose as potential pPKR candidates. Moreover, results from these studies may provide evidence for the presence of another substrate or substrates, in addition to dsRNA, which has a role in the regulation of pPKR in plants.

Each set of experiments included at least three repeats of the same experiment with a similar pattern of results observed between these experiments. A brief outline of the experimental approach that was conducted is described in Section 5.1. The influence of the ions and/or chelators on putative pPKR activity was assessed on four forms of purified proteins isolated from flower bud tissues of young Arabidopsis plants and the methods used as well as the results from these studies are described in Sections 5.2 to 5.5:

a. Total plant proteins clarified by centrifugation (Section 5.2)
b. Proteins partially purified by dsRNA agarose (Section 5.3)
c. Desalted total plant proteins clarified by centrifugation (Section 5.4)
d. Proteins separated by size exclusion chromatography (Section 5.5)

5.1 Experimental approach

The influence of calcium and zinc on the activity of putative pPKR was examined in protein samples extracted directly from plant tissue or after partial purification via the different purification approaches. The effects were then determined by supplementing the protein extracts with different concentrations of the divalent ions of interest. The effects of the chelating agents ethylene glycol tetraacetic acid (EGTA) and EDTA were also assessed by supplementing the protein extracts with different concentrations of the chelators alone or in the presence of the divalent ions. These chelating agents were relevant to the research as they are both divalent ion-
binding. While the chelator EGTA has a higher binding affinity for calcium than other divalent ions, EDTA is not as specific and sequesters most divalent ions, including calcium and zinc. The ion/chelator-supplemented protein samples were then assessed for the presence of kinase activity using the immobilised eIF2α peptide assay.

5.2 Assessment of Ion Specificity of Putative Plant Protein Kinase R Activity in Total Plant Protein Samples

Plant proteins clarified by centrifugation were extracted from flower bud tissues of young *Arabidopsis* plants and assessed for protein concentration as described in Sections 2.7.1 and 2.7.5, respectively. A standard amount of protein (between 10 – 20 µg depending on the experimental protocol) was taken for each sample and supplemented with the desired concentrations of ion and/or chelator. The protein samples were then incubated with eIF2α peptide beads in the presence of 2.5 µM ATP and 200 µCi/mL γ³²P-ATP and assessed for their phosphorylation activity using the immobilised eIF2α peptide assay (as described in Sections 2.9.4).

5.2.1 Effects of Calcium and EGTA

Varying concentrations of calcium and the chelator EGTA were added to total plant protein and assessed for their effects on eIF2α phosphorylation activity. As shown in Figure 5.1, the presence of 1.1 mM calcium resulted in an increase in the phosphorylation level with 0 mM and 0.5 mM EGTA (Fig. 5.1). In the absence of added calcium, the presence of 1 mM EGTA enhanced this kinase activity almost two-fold compared with no added EGTA. Moreover, although not statistically significant as indicated by the standard errors in Figure 5.1, the combined presence of 1.1 mM calcium and 1 mM EGTA may have further increased the level of eIF2α phosphorylation (as shown in a repeat of this comparison, Figure 5.2)
Figure 5.1 Quantification of the effects of calcium and EGTA on putative pPKR activity of total plant protein extract, Experiment 1. Bar graph showing the effects of the combined presence of varying concentrations of calcium (Ca) and EGTA on eIF2α phosphorylation in proteins extracted from Arabidopsis flowers. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
Concentrations of calcium higher than 1 mM did not enhance the eIF2α phosphorylation. In fact, the phosphorylation activity was significantly inhibited in the combined presence of 11 mM or 110 mM calcium with 1 mM EGTA (Fig. 5.2). The observed levels of kinase activity were even lower than the activity detected in plant protein samples that had no additional ion/chelator supplemented.

**Figure 5.2 Quantification of the effects of calcium and EGTA on putative pPKR activity of total plant protein extract, Experiment 2.** Bar graph showing the effects of the combined presence of varying concentrations of calcium (Ca) with 1 mM EGTA on eIF2α phosphorylation in proteins extracted from Arabidopsis flowers. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.2.2 Effects of Zinc and EGTA

Total plant protein extracts were supplemented with varying concentrations of zinc and the chelator EGTA. The addition of 1.1 mM zinc resulted in a decrease in eIF2α phosphorylation, but no significant difference was observed with the combined presence of 1.1 mM zinc and 1 mM EGTA (Fig. 5.3). On the other hand, an increase of more than two-fold in eIF2α phosphorylation was observed in total extract supplemented with 11 mM zinc and 1 mM EGTA.

![Figure 5.3](image_url)

**Figure 5.3 Quantification of the effects of zinc and EGTA on putative pPKR activity of total plant protein extract.**

Bar graph showing the effects of the combined presence of varying concentrations of zinc (Zn) and EGTA on eIF2α phosphorylation in proteins extracted from *Arabidopsis* flowers. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.2.3 Effects of EGTA and EDTA with Ions

The influence of ion chelators on eIF2α phosphorylation was examined by supplementing total plant protein with either EGTA or EDTA, with or without the addition of calcium or zinc.

With no additional ions present, the phosphorylation of eIF2α increased slightly in the presence of 1 mM EGTA but not with 1 mM EDTA (Fig. 5.4). This increase in kinase activity was further enhanced by the supplementation of 1 mM calcium or 11 mM zinc. The combined presence of 11 mM zinc and 1 mM EGTA was particularly effective and a three-fold increase in eIF2α phosphorylation was observed. Conversely, the combined addition of 1 mM EDTA with 1 mM calcium or 11 mM zinc did not result in any significant alterations of the eIF2α phosphorylation activity.

![Figure 5.4 Quantification of the effects of EGTA and EDTA on putative pPKR activity of total plant protein extract.](image)

Bar graph showing the effects of the combined presence of 1 mM EGTA or 1 mM EDTA with 1 mM calcium (Ca) or 11 mM zinc (Zn) on eIF2α phosphorylation in proteins extracted from Arabidopsis flowers. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.2.4 Summary of the Ion Specificity of Putative Plant Protein Kinase R Activity in Total Plant Protein Samples

The activity of eIF2α phosphorylation in total proteins extracted from the flower buds of young Arabidopsis plants was enhanced maximally by the combined presence of 11 mM zinc and 1 mM EGTA. The presence of both calcium and EGTA also increased the kinase activity, however, calcium concentrations above 11 mM reduced eIF2α phosphorylation. On the other hand, no significant changes in the level of eIF2α phosphorylation activity were observed with the addition of EDTA, with or without the addition of calcium or zinc ions.

5.3 Assessment of Ion Specificity of Putative Plant Protein Kinase R Activity in Double-stranded RNA Agarose-Enriched Protein Samples

Previously, the effect of ions and/or chelators had been performed with plant proteins clarified by centrifugation, however we were also interested in assessing the impact of these additives on dsRNA agarose-purified extracts so as to ensure that we were indeed measuring the effects of pPKR. Hence, clarified plant protein extract from flower bud tissues of young Arabidopsis plants were incubated with dsRNA agarose as described in Section 2.8.1.2. The unbound proteins were then removed by three washing steps using Buffer A supplemented with the desired concentrations of ion and/or chelator and the resultant agarose beads were pelleted and incubated with eIF2α peptide beads in the presence of 2.5 µM ATP and 200 µCi/mL $\gamma^{32}$P-ATP and assessed for their phosphorylation activity using the immobilised eIF2α peptide assay.

5.3.1 Effects of Calcium and EGTA

Proteins bound to dsRNA agarose were washed in the presence of varying concentrations of calcium and EGTA and assessed for their eIF2α phosphorylation activity. As observed with total plant proteins (Section 5.2.1), the combined presence of 1.1 mM calcium and 1 mM EGTA resulted in more than two-fold increase in the phosphorylation of eIF2α by dsRNA agarose-purified proteins (Fig. 5.5). Correspondingly, the addition of 110 mM calcium with 1 mM EGTA resulted in a lowered kinase activity. However, this decreased activity was still higher than the activity detected in dsRNA agarose-enriched proteins that had no supplemented ion/chelator.
Figure 5.5 Quantification of the effects of calcium and EGTA on putative pPKR activity of dsRNA agarose-enriched proteins. Bar graph showing the effects of the combined presence of varying concentrations of calcium (Ca) with 1 mM EGTA on eIF2α phosphorylation in *Arabidopsis* proteins partially purified by the dsRNA agarose binding assay. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.3.2 Effects of Zinc and EGTA

Proteins bound to dsRNA agarose were washed and equilibrated with varying concentrations of zinc and EGTA and assessed for their eIF2α phosphorylation activity. The levels of eIF2α phosphorylation, in the presence of the ion zinc and chelator EGTA, were comparable to that observed in total plant protein extracts. The addition of 1 mM zinc and 1 mM EGTA had no significant effect on eIF2α phosphorylation, while the kinase activity was significantly enhanced (six-fold) with the presence of 11 mM zinc and 1 mM EGTA (Fig. 5.6).

![Figure 5.6](image-url)

**Figure 5.6 Quantification of the effects of zinc and EGTA on putative pPKR activity of dsRNA agarose-enriched proteins.** Bar graph showing the effects of the combined presence of varying concentrations of zinc (Zn) with 1 mM EGTA on eIF2α phosphorylation in *Arabidopsis* proteins partially purified by the dsRNA agarose binding assay. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
When the protein profiles of the zinc and EGTA supplemented dsRNA agarose-enriched proteins were examined, the presence of 11 mM zinc resulted in the precipitation and loss of a number of higher molecular weight proteins (Fig. 5.7, bracket). The precipitated proteins were presumably removed from the dsRNA agarose/protein mix via washing steps following ion supplementation. It is possible that the precipitation of these proteins may have increased the enrichment of the pPKR protein and thereby increased the eIF2α phosphorylation specific activity, rather than simply stimulated the activity.

<table>
<thead>
<tr>
<th>Zn (mM)</th>
<th>0</th>
<th>1.1</th>
<th>11</th>
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<tbody>
<tr>
<td>EGTA (mM)</td>
<td>0</td>
<td>1</td>
<td>1</td>
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Figure 5.7 Protein profile of dsRNA agarose-enriched proteins supplemented with zinc and EGTA. Deep Purple™ protein stains of Arabidopsis proteins partially purified by the dsRNA agarose binding assay and supplemented with varying concentrations of zinc (Zn) with 1 mM EGTA. Bracket indicates the loss of high molecular weight proteins with the presence of 11 mM zinc. Protein markers indicate molecular sizes in kDa.

5.3.3 Summary of the Ion Specificity of Putative Plant Protein Kinase R Activity in Double-stranded RNA Agarose-Enriched Protein Samples

The effects of calcium, zinc, and EGTA on the eIF2α phosphorylation activity of dsRNA agarose-enriched proteins were similar to that detected for total plant protein extracts. Comparably, the level of kinase activity was proportionally higher in the dsRNA agarose-bound fractions. However, the radioactivity used in each assay may have been in different stages of their half life and these experiments should not be compared quantitatively between each other.
5.4 Assessment of Ion Specificity of Putative Plant Protein Kinase R Activity in Desalted Total Plant Protein Samples

Although it is observed that the presence of ions (calcium and zinc) as well as dsRNA increases eIF2α phosphorylation activity in both the total protein and the dsRNA agarose-purified protein extracts, it is important to also keep in mind that these ions are probably present, at some level, in the plant itself. Hence, it was essential to remove these background salts before assessing the effects of additives in order to obtain a more accurate representation of the effect of ions and chelators on the phosphorylation activity of eIF2α by pPKR.

Clarified plant protein extracts from flower bud tissues of young Arabidopsis plants were desalted using Bio-Spin® P-6 Columns as described in Section 2.7.4. The resultant proteins were eluted in Buffer A supplemented with the desired concentrations of ion and/or chelator, incubated with eIF2α peptide beads in the presence of 2.5 µM ATP and 200 µCi/mL γ32P-ATP and assessed for their phosphorylation activity using the immobilised eIF2α peptide assay.

5.4.1 Effects of Desalting

Prior to examining the influence of ions and chelators on desalted protein extracts, it was important to analyse the effect on eIF2α phosphorylation with simply the removal of salts in the total protein extract. As shown in Figure 5.8, an increase (1.7-fold) in eIF2α phosphorylation was detected in the desalted protein sample. This suggested that some ions and salts present in the original plant protein sample may have had an inhibitory effect on the ability of the putative pPKR to phosphorylate eIF2α.
Figure 5.8 Quantification of the effects of desalting on putative pPKR activity of total plant protein extract.

Bar graph showing the effects on eIF2α phosphorylation before and after the removal of salts in proteins extracted from Arabidopsis. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.4.2 Effects of Calcium

A wider range of calcium concentrations (0.1 µM – 10 mM) than previously used in Sections 5.2 and 5.3 was analysed for their influence on eIF2α phosphorylation activity in the desalted protein samples. The presence of 0.1 µM calcium had the highest, albeit not substantially bigger (1.2-fold), effect on enhancing the eIF2α phosphorylation activity (Fig. 5.9). On the other hand, calcium concentrations above 0.01 mM did not further increase the kinase activity.

Figure 5.9 Quantification of the effects of calcium on putative pPKR activity of desalted total plant protein extract. Bar graph showing the effects of varying concentrations of calcium on eIF2α phosphorylation in desalted Arabidopsis-extracted proteins. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.4.3 Effects of Zinc

The influence of zinc on eIF2α phosphorylation activity was analysed by supplementing the desalted plant protein samples with 0.1 µM – 10 mM zinc. A slight (1.1-fold) increase in kinase activity was detected in the presence of 0.1 µM zinc (Fig. 5.10). No major difference in eIF2α phosphorylation activity was observed with a further increase in the concentration of zinc, except for a high level of phosphorylation activity in the presence of 10 mM zinc (3.4-fold above 0 mM zinc). As discussed in Section 5.3.2, this increase in kinase activity could possibly be due to the precipitation of proteins by the high concentration of zinc and therefore a higher specific activity of pPKR.

Figure 5.10 Quantification of the effects of zinc on pPKR activity of desalted total plant protein extract. Bar graph showing the effects of varying concentrations of zinc on eIF2α phosphorylation in desalted Arabidopsis-extracted proteins. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.4.4 Effects of EGTA

Desalted total plant proteins were supplemented with varying concentrations of EGTA and assessed for their eIF2α phosphorylation activity. A range of concentrations, from 0.1 µM – 5 mM EGTA, were examined for their effects on kinase activity, however, no significant changes were detected with or without the presence of EGTA (Fig. 5.11). Interestingly, this observation differed with the kinase activity obtained from EGTA-supplemented total plant protein and dsRNA agarose-purified proteins, where the presence of 1 mM EGTA significantly increased the detected level of eIF2α phosphorylation (Section 5.2.1 and 5.3.1). It was thus postulated that the observed increase in eIF2α phosphorylation in the above studies may have arisen from the chelation by EGTA of the ions present in the original plant extract, which then led to a release of their inhibition on pPKR and resulted in an increase in kinase activity. Conversely, these inhibitory ions may be absent in the desalted extract. Addition of EGTA (and other tested additives) to the desalted extracts is therefore ineffective and does not lead to an increase of kinase activity.

Figure 5.11 Quantification of the effects of EGTA on putative pPKR activity of desalted total plant protein extract. Bar graph showing the effects of varying concentrations of EGTA on eIF2α phosphorylation in desalted *Arabidopsis*-extracted proteins. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.4.5 Effects of EDTA

The effect of EDTA on eIF2α phosphorylation was examined in the desalted plant proteins by supplementing with varying concentrations of EDTA. A small (1.2-fold) increase in kinase activity was detected in the presence of 0.1 µM EDTA and this activity had a general trend that decreased slightly with increasing concentrations of EDTA (Fig. 5.12). This downward trend was only statistically significant (by comparing standard errors) when the concentration of EDTA was increased to 5 mM. Again this result differed from results observed in EDTA-supplemented total plant protein samples, where the presence of 1 mM EDTA had no effect on the level of eIF2α phosphorylation (Section 5.2.3). More interestingly, as the ions previously present in the original plant extract were removed by desalting, it was unclear what EDTA was binding to, though it is possible that EDTA may be stripping ions previously bound to proteins.

![Figure 5.12](image)

**Figure 5.12 Quantification of the effects of EDTA on putative pPKR activity of desalted total plant protein extract.** Bar graph showing the effects of varying concentrations of EDTA on eIF2α phosphorylation in desalted *Arabidopsis*-extracted proteins. Peptide beads were washed using phosphate buffered saline. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.4.6 Summary of the Ion Specificity of Putative Plant Protein Kinase R Activity in Desalted Total Plant Protein Samples

It was observed that the removal of salts from the total plant protein extract resulted in an increase in the level of eIF2α phosphorylation, suggesting a possible inhibition by ions on pPKR activity. Moreover, this increase in kinase activity was further enhanced by the presence of 0.1 µM calcium or 0.1 µM EDTA.

A downward trend in eIF2α phosphorylation was observed in the desalted proteins with increasing concentrations of EGTA or EDTA. These results differed from the enhancing effects of the chelators on total plant proteins extracts as described in Section 5.2. However, the biological environments of these two forms of purified protein extracts are clearly distinct from each other and the contradicting effects of the chelators may indicate the influence of salts which are removed in the desalted extracts but which are present in the intact plant system. When both the specific ions and chelators are present in the normal levels as found in plants, the addition of chelators may release the inhibiting effects of ions on the activity of putative pPKR or may interact with specific salts to activate putative pPKR. In contrast, when the ion balance is disrupted by desalting, these activating effects by chelators are no longer effective.

5.5 Assessment of Ion Specificity of Putative Plant Protein Kinase R Activity of Proteins Separated by Size Exclusion Chromatography

As described in Section 4.6, protein fractions separated by size exclusion chromatography displayed high eIF2α phosphorylation activity indicating the presence of enriched putative pPKR protein. Hence, these protein fractions provided a relatively enriched protein sample to determine the effect of ions and chelators on the activity of putative pPKR, with minimal influence by other proteins and ions.

Clarified plant protein extracts from flower bud tissues of young Arabidopsis plants were separated by their molecular size via a BioSep™-SEC-S3000 silica-based gel filtration column (as described in Section 2.8.3). The eluted protein fractions with high eIF2α phosphorylation activity, as detected in Figure 4.20, were then mixed with glycerol (final concentration of 10% (v/v)), frozen with liquid nitrogen and stored at -80°C for future use. Aliquots of the collected protein
were then thawed when required and supplemented with the desired concentrations of ion and/or chelator. The resultant proteins were incubated with eIF2α peptide beads in the presence of 2.5 μM ATP and 200 μCi/mL γ32P-ATP and assessed for their phosphorylation activity using the immobilised eIF2α peptide assay.

5.5.1 Assessment of Activity in Aliquots of Stored Proteins Separated by Size Exclusion Chromatography

The feasibility of using the stored size exclusion chromatography-separated protein fractions for putative pPKR activity analysis was examined by comparing their eIF2α phosphorylation activity before and after freezing. Fractions (as shown in Fig. 4.20) with low activity, i.e., proteins eluted at 17 min and 17 min 15 sec, and fractions with high activity, i.e., proteins eluted at 18 min 30 sec and 18 min 45 sec, were assayed either immediately following molecular size separation or thawed after being stored at -80°C for one day.

The phosphorylation of eIF2α did not alter significantly in the low activity fractions before or after thawing (Fig. 5.13, 17m0s and 17m15s). On the other hand, the kinase activity in the high activity fractions (Fig. 5.13, 18m30s and 18m45s) decreased by nearly a third after thawing. Although there was a loss in phosphorylation activity, the detected activity still remained significantly higher than the low activity fractions. Hence, it was possible to use these stored protein fractions for subsequent analysis of the influence of ions and/or chelators on their eIF2α phosphorylation activity.
Figure 5.13 Quantification of putative pPKR activity in fresh or stored proteins separated by size exclusion chromatography. Bar graph showing the effects of a single freeze/thawing on eIF2α phosphorylation in *Arabidopsis* proteins separated by size exclusion chromatography. Protein fractions were sampled immediately after separation (fresh) or thawed after freezing overnight at -80°C (stored). Low activity fractions represented by 17m0s and 17m15s fractions. High activity fractions represented by 18m30s and 18m45s fractions. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. For each treatment, numerical values above each bar represent the ratio of the counts per minute detected on Peptide A or Beads only relative to Peptide S following the same treatment. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S; red bar, Peptide A; green bar, Beads only.
5.5.2 Effects of Calcium

Varying concentrations of calcium was added to the protein fractions separated by size exclusion chromatography. These calcium concentrations ranged from 1 nM – 10 mM. No increase in eIF2α phosphorylation was detected in the presence of calcium (Fig. 5.14). In fact, it was observed that an increasing amount of calcium resulted in a decreasing trend in the level of kinase activity.

![Bar graph showing the effects of varying concentrations of calcium on eIF2α phosphorylation in protein fractions separated by size exclusion chromatography and which previously displayed high eIF2α phosphorylation. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.](image-url)

**Figure 5.14 Quantification of the effects of calcium on putative pPKR activity of proteins separated by size exclusion chromatography.** Bar graph showing the effects of varying concentrations of calcium on eIF2α phosphorylation in protein fractions separated by size exclusion chromatography and which previously displayed high eIF2α phosphorylation. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
5.5.3 Effects of Zinc

The influence of zinc was assessed in the protein fractions separated by size exclusion chromatography. Zinc concentrations ranging from 1 nM – 10 mM were added to the protein samples. As observed with the calcium study (Section 5.5.2), an increase in zinc concentration resulted in a downward trend in the level of eIF2α phosphorylation activity detected (Fig. 5.15). Interestingly, the presence of 10 mM zinc did not increase the specific kinase activity of pPKR as was observed in initial or desalted total plant extracts or dsRNA agarose-purified proteins supplemented with a high concentration of zinc (Sections 5.2.2, 5.4.3, and 5.3.2, respectively).

Figure 5.15 Quantification of the effects of zinc on putative pPKR activity of proteins separated by size exclusion chromatography. Bar graph showing the effects of varying concentrations of zinc on eIF2α phosphorylation in protein fractions separated by size exclusion chromatography and which previously displayed high eIF2α phosphorylation. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
5.5.4 Effects of EGTA

Protein samples separated by size exclusion chromatography were supplemented with increasing amounts of EGTA. An increase in eIF2α phosphorylation was detected with the presence of 1 nM EGTA, however, no further enhancement of the kinase activity was detected with concentrations of EGTA over that amount (Fig. 5.16). Moreover, the phosphorylation of eIF2α was significantly reduced with the addition of 5 mM EGTA.

![Figure 5.16](image-url)

**Figure 5.16 Quantification of the effects of EGTA on putative pPKR activity of proteins separated by size exclusion chromatography.** Bar graph showing the effects of varying concentrations of EGTA on eIF2α in protein fractions separated by size exclusion chromatography and which previously displayed high eIF2α phosphorylation. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
5.5.5 Effects of EDTA

The effect of EDTA on the level of eIF2α phosphorylation was examined in protein samples separated by size exclusion chromatography. An increasing trend in phosphorylation activity was detected in the presence of 1 µM – 0.1 mM EDTA (Fig. 5.17). However, as observed for EGTA (Section 5.5.4), the presence of a high amount of the chelator EDTA (10 mM) reduced the level of eIF2α phosphorylation by half in the size exclusion chromatography-separated protein samples.

Figure 5.17 Quantification of the effects of EDTA on putative pPKR activity of proteins separated by size exclusion chromatography. Bar graph showing the effects of varying concentrations of EDTA on eIF2α phosphorylation in protein fractions separated by size exclusion chromatography and which previously displayed high eIF2α phosphorylation. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
5.5.6 Effects of Calcium and EGTA

The combined effects of calcium and EGTA on the eIF2α phosphorylation activity of size exclusion chromatography-separated samples were assessed by supplementing the protein extracts with 1 nM EGTA and an increasing amount of calcium. An enhancement of kinase activity was observed with a combination of the ion and chelator with a peak in phosphorylation activity detected when supplemented with both 10 nM calcium and 1 nM EGTA (Fig. 5.18). As observed with the addition of calcium alone (Section 5.5.2 and Fig. 5.14), higher concentrations of calcium resulted in a decrease in kinase activity, even with the presence of EGTA.

![Figure 5.18](image-url)

**Figure 5.18** Quantification of the effects of calcium and EGTA on putative pPKR activity of proteins separated by size exclusion chromatography. Bar graph showing the effects of the combined presence of varying concentrations of calcium (Ca) with 0.001 µM EGTA on eIF2α phosphorylation in protein fractions separated by size exclusion chromatography and which previously displayed high eIF2α phosphorylation. The Y-axis indicates the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Blue bar, Peptide S.
5.5.7 Summary of the Ion Specificity of Putative Plant Protein Kinase R Activity of Proteins Separated by Size Exclusion Chromatography

The influence of calcium, zinc, EGTA, and EDTA on the level of eIF2α phosphorylation in protein samples separated by size exclusion chromatography differed to that observed in the desalted total proteins. Considering that each purification approach yields profiles of proteins and ions that are distinct from each other, it is possible that specific proteins, required for the ions and/or chelators to be effective, may have been present in the desalted protein extracts but excluded into another fraction by size exclusion chromatography, resulting in the conflicting results. However, it is important to note that a combination of calcium and EGTA once again resulted in an enhancement of kinase activity, although in this case, it was only a slight increase.

5.6 Conclusions

Four different types of protein extracts were examined for the effects of ions and/or chelators on their eIF2α phosphorylation activity. The results obtained from the analysis of the different protein extracts were not entirely consistent and are summarised in Table 5.1. This is not surprising as the different protein extracts may have distinct protein and ion profiles due to the various purification approaches to which they were subjected to and the presence of specific proteins or salts may have had a substantial influence on the eIF2α phosphorylation activity. This explanation is corroborated by the observation that the desalting of total protein extracts resulted in a significant increase in kinase activity of the unamended extract.

Although differing degrees of effect for each ion alone were observed in the four protein extracts examined, the combined presence of calcium and EGTA consistently enhanced the level of eIF2α phosphorylation in the protein samples, regardless of their different purification methods. It is not known why the presence of both the divalent ion and chelator is required for increasing the kinase activity and this unusual observation remains unresolved. However, these results do suggest that the activity of pPKR can be influenced, both positively and negatively, by the presence of calcium.
Table 5.1 Summary of the different ions and/or chelators assessed for their influence on the eIF2α phosphorylation activity in four different forms of Arabidopsis-enriched proteins

<table>
<thead>
<tr>
<th>Form of partially purified protein</th>
<th>Supplemented with:</th>
<th>Ion and/or chelator with highest influence on pPKR activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>calcium</td>
<td>zinc</td>
</tr>
<tr>
<td>a. Total plant proteins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>b. dsRNA agarose-enriched proteins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>c. Desalted total plant proteins</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>d. Size exclusion chromatography-separated proteins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The potential relationship between PKR and calcium is unclear. A study by Thomas, Kim, Morgan, & Hanley (1998) showed that human PKR, ectopically expressed in Xenopus oocytes, was activated in response to a depletion of intracellular calcium stores and resulted in an increase in eIF2α phosphorylation. This activation of PKR was not to induce the re-entry of calcium to replenish the depleted stores, but rather to dampen the depletion-induced calcium entry elicited by calcium-mobilising receptors, presumably to maintain calcium levels in a balanced state. However, a later report observed that PKR was not the primary kinase responsible for the calcium mobilisation-induced eIF2α phosphorylation, but rather the action of another eIF2α kinase, PERK (Kimball et al., 2001). The eIF2α kinase PERK is known to be activated by an accumulation of misfolded proteins in the lumen of the endoplasmic reticulum and is involved in the unfolded protein response (Raven & Koromilas, 2008). On the other hand, it has been shown that plant cells overcome this endoplasmic reticulum stress not by the regulation of translation through eIF2α phosphorylation, but via enhancing protein folding activity, degrading unfolded proteins, and by
regulating apoptosis (Kamauchi, Nakatani, Nakano, & Urade, 2005). Moreover, PERK has yet to be identified in plants.

The correlation between pPKR and calcium is thus far unknown, but it has been shown that the activity of putative pPKR, as detected by eIF2α phosphorylation, is activated in the presence of calcium and the chelator EGTA. These findings suggest that a calcium binding domain or a binding domain for a calcium binding chaperone protein may be located within the pPKR protein sequence and supports the notion that the putative pPKR protein may be, or is regulated by, a CPK.
Assessment of Putative Plant Protein Kinase R Activity in Transgenic *Arabidopsis thaliana*

The identification of pPKR can be assisted by examining the eIF2α phosphorylation activity in transgenic plant lines which have disruptions in or are overexpressing particular genes postulated to be involved in the pPKR regulatory pathway. Gene disruptions were obtained via T-DNA insertion lines, while overexpressions and knockdowns were achieved by *A. tumefaciens* transformation of the constructs containing the genes of interest into *Arabidopsis*. These transgenic plant lines were then assessed for changes in putative pPKR activity using the immobilised eIF2α peptide assay.
The genes of interest which were assessed for their involvement in the pPKR regulatory pathway are described in Section 6.1. The generation of the transgenic plant lines as well as the results from the studies are described in Sections 6.2 to 6.3:

a. T-DNA insertion plant lines (Section 6.2)  
b. Overexpression plant lines (Section 6.3.1)  
c. Knockdown plant lines (Section 6.3.2)

### 6.1 Genes of Interest for Assessment

The genes chosen for the transgenic *Arabidopsis* study were based on their known or postulated involvement in the pPKR regulatory pathway. These genes can be categorised in six groups and include genes which encode for dsRBM-containing proteins, CPKs, protein kinases with unknown function, other known eIF2α protein kinases, known inhibitors of PKR, and eIF2α. The possible associations of these genes with pPKR are briefly explained below.

#### 6.1.1 Double-stranded RNA Binding Motif-Containing Genes

It is recognised that mPKR contains two dsRBMs and is activated by dsRNA. The activation of pPKR activity by dsRNA has also been detected in plants (described in Section 1.3.1.2). Although there is no known protein in *Arabidopsis* that contains both a dsRBM and protein kinase domain, there are 16 genes in *Arabidopsis* which contain a dsRBM. It was therefore of interest to examine whether these genes are involved in the recognition of dsRNA by pPKR. A plant carrying a disruption for the expression of one of these genes that is indeed pPKR or is involved in the pPKR pathway would be anticipated to have reduced eIF2α phosphorylation activity.

#### 6.1.2 Calcium-dependent Protein Kinases

As observed in Chapter 5, the activity of putative pPKR is influenced by the presence of calcium ions. Moreover, a number of CPKs were identified in the MS sequencing analysis (Table 4.2). These results suggest a potential role of calcium in the regulation of pPKR and this regulation may possibly involve the function of one of the 34 CPK genes that are found in *Arabidopsis*. A plant carrying a disruption for the expression of one of these CPK genes that is indeed pPKR or is involved in the pPKR pathway would be anticipated to have reduced eIF2α phosphorylation...
activity. However, it is important to note that redundancy in function within the CPK genes may prevent a clear distinction of the effects caused by the disruption of each individual kinase.

### 6.1.3 Protein Kinases of Unknown Function

A high number of kinase domain-containing proteins were identified in the MS sequencing analysis (Table 4.2) with many posing as potential candidates of pPKR. An approach to assess their possible significance as the pPKR protein would be to analyse the effect of their disruptions on pPKR activity. A plant carrying a disruption for the expression of one of these genes that is indeed pPKR or is involved in the pPKR pathway would be anticipated to have reduced eIF2\(\alpha\) phosphorylation activity. However, again, there is the possibility of redundancy in function within the group of kinases which would prevent clear analysis of the gene disruption.

### 6.1.4 Other Known eIF2\(\alpha\) Protein Kinases

As well as PKR, three other eIF2\(\alpha\) protein kinases – HRI, GCN2, and PERK – have been recognised in mammals; however, only the GCN2 functional homolog has to date been identified in plants (Y. Zhang et al., 2003). It was of interest to investigate the effect of a disruption or overexpression of GCN2 on eIF2\(\alpha\) phosphorylation activity. If the level of dsRNA-activated eIF2\(\alpha\) phosphorylation is not affected by a disruption of this protein kinase, this would provide strong evidence that pPKR is not GCN2 and that there are indeed two eIF2\(\alpha\) protein kinases encoded by plants.

### 6.1.5 Known Inhibitors of Protein Kinase R

Three known inhibitors of PKR were chosen for the mutagenesis study – the cellular inhibitor p58\(\text{IPK}\) that has recently been identified in plants (Bilgin et al., 2003) and the *Vaccinia virus*-encoded E3L and K3L proteins. As discussed in Section 1.2.1.3.1, p58\(\text{IPK}\) and E3L prevent the activation of mPKR by inhibiting its homodimerisation and by sequestering the activating dsRNA, respectively. On the other hand, K3L acts as a pseudosubstrate of mPKR and therefore more directly inhibits eIF2\(\alpha\) phosphorylation. It is postulated that an overexpression of any of these three inhibitors will result in a decrease in eIF2\(\alpha\) phosphorylation. In turn, such a result would also provide evidence that these proteins are capable of inhibiting the activity of pPKR in plants.
6.1.6 Translation Initiation Factor eIF2α

It is well recognised that eIF2α is one of the main substrates of PKR, particularly in its antiviral role in mammals (Langland et al., 2006). Therefore, it was of interest to generate transgenic lines that overexpress the wildtype or mutant form of the eIF2α gene and to examine the phenotypes or phosphorylation activity that resides in these plants. Furthermore, generation of plants with a perturbed eIF2α protein would allow the study of the effect on viral infection in these plants.

6.2 Assessment of eIF2α Phosphorylation in Transgenic Arabidopsis thaliana Transferred DNA Insertion Plant Lines

T-DNA from A. tumefaciens Ti plasmids can be inserted into the plant genome via recombination events which results in the disruption of the target gene. This tool is widely used for genetic engineering and plant insertional mutagenesis (Azpiroz-Leehan & Feldmann, 1997). The insertion of T-DNA into the plant genome is selected via kanamycin resistance due to the presence of a neomycin phosphotransferase gene on the T-DNA. The insertion point of the T-DNA and therefore the identification of the putatively disrupted gene, and the likelihood of gene disruption, is determined by the amplification of the flanking sequences of the T-DNA border. In turn, any phenotypic or functional changes detected in the mutated plants could be correlated with the disrupted gene (Bouche & Bouchez, 2001). As the sequence of the entire Arabidopsis genome is available, Arabidopsis serves as an ideal reference plant for these insertion mutagenesis studies.

The T-DNA insertion lines used in this research study were imported as seed stocks from ABRC. Table 6.1 lists the mutant lines for T-DNA insertions in 15 of the 16 dsRBM-containing genes found in Arabidopsis. Table 6.2 lists the T-DNA insertion lines for the 34 Arabidopsis CPKs. Table 6.3 lists the T-DNA insertion lines for a protein kinase of unknown function, the eIF2α kinase GCN2, and the plant-functional homolog of the mammalian PKR inhibitor p58IPK.
<table>
<thead>
<tr>
<th>Name</th>
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<th>Description</th>
<th>T-DNA insertion line</th>
<th>Location of T-DNA inserted</th>
<th>Plant line grown?</th>
<th>If tested, any changes in eIF2α phosphorylation?</th>
<th>F probability</th>
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<tr>
<td>dsRBM1</td>
<td>AT3G62800</td>
<td>dsRNA-binding protein 4</td>
<td>SALK_000736</td>
<td>5' UTR</td>
<td>Homozygous</td>
<td>No</td>
<td>0.816</td>
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<td>dsRBM2</td>
<td>AT5G04895</td>
<td>ATP-dependent helicase</td>
<td>SALK_019865</td>
<td>Exon 10 (mid)</td>
<td>Homozygous</td>
<td>No</td>
<td>0.965</td>
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<tr>
<td>dsRBM3</td>
<td>AT5G01270</td>
<td>CTD phosphatase-like 2</td>
<td>SALK_060177</td>
<td>Exon 9 (mid)</td>
<td>Homozygous</td>
<td>No</td>
<td>0.837</td>
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<td>dsRBM4</td>
<td>AT1G09700</td>
<td>Hyponastic leaves 1</td>
<td>SALK_1057_H07</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
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<tr>
<td>dsRBM5</td>
<td>AT4G21670</td>
<td>CPL1 (FIERY 2)</td>
<td>SALK_107871</td>
<td>Promoter</td>
<td>Homozygous</td>
<td>Yes</td>
<td>0.259</td>
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<tr>
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<td>AT5G41070</td>
<td>dsRNA-binding protein 5</td>
<td>SALK_126609</td>
<td>Exon 3 (mid)</td>
<td>Homozygous</td>
<td>No</td>
<td>0.102</td>
</tr>
<tr>
<td>dsRBM7</td>
<td>AT2G28380</td>
<td>dsRNA-binding protein 5</td>
<td>SALK_012017</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
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<tr>
<td>dsRBM8</td>
<td>AT5G45150</td>
<td>RNAse three-like protein 3</td>
<td>SALK_137248</td>
<td>Exon 1 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>dsRBM9</td>
<td>AT5G20320</td>
<td>DICER-like 4</td>
<td>SAIL_510_A03</td>
<td>Exon 2 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td></td>
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<tr>
<td>dsRBM10</td>
<td>AT3G26932</td>
<td>dsRNA-binding protein 3</td>
<td>SALK_022644</td>
<td>Exon 1 (upper)</td>
<td>homozygous</td>
<td>No</td>
<td>0.028</td>
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<td>AT1G01040</td>
<td>DICER-like 1</td>
<td>SALK_081595</td>
<td>Exon 1 (upper)</td>
<td>homozygous</td>
<td>No</td>
<td>0.064</td>
</tr>
<tr>
<td>dsRBM12</td>
<td>AT3G03300</td>
<td>DICER-like 2</td>
<td>SALK_095069</td>
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<tr>
<td>dsRBM13</td>
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<td>DsRBD-containing protein</td>
<td>SALK_003406</td>
<td>3' UTR</td>
<td>homozygous</td>
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<td>dsRBM14</td>
<td>AT2G01130</td>
<td>ATP-dependent helicase</td>
<td>SALK_066261</td>
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<td>homozygous</td>
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<td>dsRBM15</td>
<td>AT3G20420</td>
<td>RNAse three-like protein 2</td>
<td>WISCDsLOX429C12</td>
<td>Exon 1 (upper)</td>
<td>Did not grow</td>
<td>-</td>
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<tr>
<td>dsRBM16</td>
<td>AT1G80650</td>
<td>RNAse three-like protein 1</td>
<td>N/A</td>
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<td>-</td>
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</tr>
</tbody>
</table>

*Abbreviated name of gene of interest, bolded name indicate gene identified previously by mass spectrometry analysis of proteins purified via pPKR purification approaches (Table 4.2); †SALK lines created by Salk Institute Genome Analysis Laboratory (Alonso et al., 2003), SAIL lines created by Syngenta Arabidopsis Insertion Library, WiscDsLox lines created by WiscDsLox lines created by Woody, Austin-Phillips, Amasino, & Krysan (2007), N/A, no insertion line available from ABRC; ‡brackets denote position of exon relative to the entire gene; §F probability calculated using GenStat to determine the statistical significance of any difference in the level of eIF2α phosphorylation detected between the mutant and wildtype plants; AGI, Arabidopsis Gene Index; UTR, untranslated region; boxes with dash (−) denotes no data available.
<table>
<thead>
<tr>
<th>Name</th>
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<th>Comments</th>
<th>T-DNA insertion line</th>
<th>Location of T-DNA inserted</th>
<th>Plant line grown?</th>
<th>If tested, any changes in elf2α phosphorylation?</th>
<th>F probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK1</td>
<td>AT5G04870</td>
<td>Stimulated by phospholipids (Binder, Harper, &amp; Sussman, 1994) Overexpression resulted in enhanced NADPH oxidase activity and oxidative burst (Xing, Wang, Malik, &amp; Miki, 2001) Localised in peroxisome membrane (Dammann et al., 2003)</td>
<td>SAIL_828_H10</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
<td>0.583</td>
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<tr>
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<td>AT3G10660</td>
<td>Localised in ER (S. X. Lu &amp; Hrabak, 2002)</td>
<td>SALK_059237</td>
<td>Exon 1 (upper)</td>
<td>Homozygous</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>CPK3</td>
<td>AT4G23650</td>
<td>Ubiquitously expressed (Hong et al., 1996) Localised in cytosol and nucleus (Dammann et al., 2003) Transcript identified in phloem (Deeken et al., 2008)</td>
<td>SALK_022862</td>
<td>Exon 1 (upper)</td>
<td>Heterozygous</td>
<td>-</td>
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<td>CPK4</td>
<td>AT4G09570</td>
<td>Identified in this research thesis by MS analysis of proteins purified via pPKR purification approaches (Table 4.2) Localised in cytosol and nucleus (Dammann et al., 2003) Positive regulator of abscisic acid signal transduction (Zhu et al., 2007)</td>
<td>SALK_122981</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
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<td>CPK5</td>
<td>AT4G35310</td>
<td></td>
<td>SAIL_657_C06</td>
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<td>-</td>
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<td>-</td>
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<td>CPK7</td>
<td>AT5G12480</td>
<td>Localised in plasma membrane (Dammann et al., 2003) Phosphorylated in response to flg22 (Benschop et al., 2007)</td>
<td>SALK_127223</td>
<td>Exon 5 (mid)</td>
<td>Heterozygous</td>
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<td>If tested, any changes in eIF2α phosphorylation?</td>
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<td>Ubiquitously expressed (Hong et al., 1996) Localised in plasma membrane</td>
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<td></td>
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<td></td>
<td></td>
<td>(Dammann et al., 2003) Phosphorylated in response to flg22 (Benschop et al., 2007)</td>
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<td>WiscDsLox241G12</td>
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<td>CPK10</td>
<td>AT1G18890</td>
<td>Induced by drought and salt stress (Urao et al., 1994) Overexpression</td>
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<td></td>
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<td>resulted in activation of stress inducible HVA1 promoter (Sheen, 1996)</td>
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<td>Phosphorylated in response to flg22 (Benschop et al., 2007)</td>
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<td>regulator of abscisic acid signal transduction (Zhu et al., 2007)</td>
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<td>Phosphorylates AtDi19 in vitro (Rodriguez Milla et al., 2006; Uno, Rodriguez Milla, Maher, &amp; Cushman, 2009)</td>
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<td>CPK12</td>
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<td>AT2G41860</td>
<td>Transcript level induced 5.29-fold upon Plum pox virus infection (17 days post inoculation) (Babu et al., 2008)</td>
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<td>AT4G21940</td>
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<td>SAIL_1284_H09</td>
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<td>Name</td>
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<td>Comments</td>
<td>T-DNA insertion line</td>
<td>Location of T-DNA inserted</td>
<td>Plant line grown?</td>
<td>If tested, any changes in eIF2α phosphorylation?</td>
<td>F probability</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>--------------------------------------------------------------------------</td>
<td>----------------------</td>
<td>-----------------------------</td>
<td>-------------------</td>
<td>-------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>CPK16</td>
<td>AT2G17890</td>
<td>Localised in plasma membrane (Dammann et al., 2003)</td>
<td>SAIL_234_E05</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK17</td>
<td>AT5G12180</td>
<td></td>
<td>SALK_026477</td>
<td>5' UTR</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK18</td>
<td>AT4G36070</td>
<td></td>
<td>SALK_061352</td>
<td>Exon 2 (upper)</td>
<td>Heterozygous</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK19</td>
<td>AT1G61950</td>
<td></td>
<td>SAIL_1215_F07</td>
<td>Exon 1 (upper)</td>
<td>Homozygous</td>
<td>Decrease</td>
<td>0.002</td>
</tr>
<tr>
<td>CPK20</td>
<td>AT2G38910</td>
<td>Identified in this research thesis by MS analysis of proteins purified via pPKR purification approaches (Table 4.2)</td>
<td>SALK_044320</td>
<td>Intron (mid)</td>
<td>Homozygous</td>
<td>No</td>
<td>0.399</td>
</tr>
<tr>
<td>CPK21</td>
<td>AT4G04720</td>
<td>Identified in this research thesis by MS analysis of proteins purified via pPKR purification approaches (Table 4.2) Localised in plasma membrane (Dammann et al., 2003)</td>
<td>SALK_029412</td>
<td>Exon 1 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK22</td>
<td>AT4G04710</td>
<td></td>
<td>SALK_136625</td>
<td>Exon 1 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK23</td>
<td>AT4G04740</td>
<td>Involved in drought and salt stress response (S. Y. Ma &amp; Wu, 2007)</td>
<td>SALK_007958</td>
<td>Intron (lower)</td>
<td>Homozygous</td>
<td>No</td>
<td>0.17</td>
</tr>
<tr>
<td>CPK24</td>
<td>AT2G31500</td>
<td></td>
<td>SALK_146353</td>
<td>Exon 2 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK25</td>
<td>AT2G35890</td>
<td></td>
<td>SAIL_664_G07</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK26</td>
<td>AT4G38230</td>
<td></td>
<td>SALK_107005</td>
<td>Exon 6 (lower)</td>
<td>Homozygous</td>
<td>No</td>
<td>0.475</td>
</tr>
<tr>
<td>CPK27</td>
<td>AT4G04700</td>
<td>Identified in this research thesis by MS analysis of proteins purified via pPKR purification approaches (Table 4.2)</td>
<td>SALK_129020</td>
<td>Promoter</td>
<td>Homozygous</td>
<td>No</td>
<td>0.829</td>
</tr>
<tr>
<td>CPK28</td>
<td>AT5G66210</td>
<td>Transcript level induced 2.63-fold upon Plum pox virus infection (17 days post inoculation) (Babu et al., 2008) Localised in plasma membrane (Dammann et al., 2003)</td>
<td>WiscDsLox264D03</td>
<td>Exon 3 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
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</table>

194
Table 6.2 continued

<table>
<thead>
<tr>
<th>Name</th>
<th>AGI</th>
<th>Comments</th>
<th>T-DNA insertion line</th>
<th>Location of T-DNA inserted</th>
<th>Plant line grown?</th>
<th>If tested, any changes in elf2α phosphorylation?</th>
<th>F probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK29</td>
<td>AT1G76040</td>
<td>Identified in this research thesis by MS analysis of proteins purified via pPKR purification approaches (Table 4.2) Transcript level induced 6.26-fold upon Cucumber mosaic virus strain Y infection (18 days post inoculation) (Marathe et al., 2004)</td>
<td>SAIL_51_C09</td>
<td>5'UTR</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK30</td>
<td>AT1G74740</td>
<td>Overexpression resulted in activation of stress inducible HVA1 promoter (Sheen, 1996)</td>
<td>SALK_036638</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK31</td>
<td>AT4G04695</td>
<td></td>
<td>SALK_076400</td>
<td>Promoter</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK32</td>
<td>AT3G57530</td>
<td>Transcript level induced 3.32-fold upon Plum pox virus infection (17 days post inoculation) (Babu et al., 2008)</td>
<td>SALK_112664</td>
<td>Promoter</td>
<td>Heterozygous</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK33</td>
<td>AT1G50700</td>
<td>Identified in this research thesis by MS analysis of proteins purified via pPKR purification approaches (Table 4.2)</td>
<td>SALK_059467</td>
<td>Exon 1 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPK34</td>
<td>AT5G19360</td>
<td></td>
<td>SALK_040609</td>
<td>Exon 1 (upper)</td>
<td>Did not grow</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* abbreviated name of gene of interest, bolded names indicate genes identified previously by mass spectrometry (MS) analysis of proteins purified via pPKR purification approaches (Table 4.2); SALK lines created by Salk Institute Genome Analysis Laboratory (Alonso et al., 2003), SAIL lines created by Syngenta Arabidopsis Insertion Library, WiscDslox lines created by WiscDslox lines created by Woody et al. (2007); brackets denote position of exon relative to the entire gene; $^d$ F probability calculated using GenStat to determine the statistical significance of any difference in the level of elf2α phosphorylation detected between the mutant and wildtype plants; AGI, *Arabidopsis* Gene Index; ER, endoplasmic reticulum; flg22, 22 aa sequence of the general elicitor flagellin; AtDi19, *Arabidopsis* dehydration-induced 19; UTR, untranslated region; boxes with dash (-) denotes no data available.
Table 6.3 Transgenic Arabidopsis thaliana T-DNA insertion lines of a protein kinase of unknown function, GCN2, and p58IPK obtained from ABRC

<table>
<thead>
<tr>
<th>Name</th>
<th>AGI</th>
<th>Description</th>
<th>T-DNA insertion line</th>
<th>Location of T-DNA inserted</th>
<th>Plant line grown?</th>
<th>If tested, any changes in eIF2α phosphorylation?</th>
<th>F probability$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein kinase of unknown function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G80640</td>
<td>AT1G80640</td>
<td>Protein kinase family protein</td>
<td>SALK_144214</td>
<td>Exon 7 (lower)$^c$</td>
<td>Homozygous</td>
<td>No</td>
<td>0.023</td>
</tr>
<tr>
<td><strong>Other eIF2α kinase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCN2</td>
<td>AT3G59410</td>
<td>Protein kinase family protein</td>
<td>SAIL_793_H07</td>
<td>Promoter</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Inhibitor of PKR (p58IPK)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPK</td>
<td>AT5G03160</td>
<td>DNAJ heat shock N-terminal domain-containing protein</td>
<td>SALK_145851</td>
<td>Promoter</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ abbreviated name of gene of interest; $^b$ SALK lines created by Salk Institute Genome Analysis Laboratory (Alonso et al., 2003), SAIL line created by Syngenta Arabidopsis Insertion Library; $^c$ brackets denote position of exon relative to the entire gene; $^d$ F probability calculated using GenStat to determine the statistical significance of any difference in the level of eIF2α phosphorylation detected between the mutant and wildtype plants; AGI, Arabidopsis Gene Index; boxes with dash (-) denotes no data available.
6.2.1 Growth and Analysis of the Transferred DNA Insertion Plant Lines

The ABRC-imported seed stocks for the T-DNA insertion lines were first stored at 4°C to simulate vernalisation. The seeds were then surface sterilised, grown on Plant Basal Medium (prepared as described in Section 2.10.2) and maintained on Plant Basal Medium supplemented with 100 mg/L kanamycin to select for transgenic plants (as described in Section 2.10.5). Once root development occurred, the seedlings were transferred to soil and allowed to establish growth. Seeds were collected from these plants and replanted on Plant Basal Medium supplemented with 50 mg/L kanamycin. In the instance where all planted seedlings survived on the antibiotic-supplemented medium, the resultant plant line was determined as homozygous for the T-DNA insert. For other lines, another round of kanamycin resistance testing was conducted on the next generation of seedlings to obtain homozygous lines.

The homozygous mutant plant lines were grown in individual pots until flowering was established and the flower buds of young transgenic plants were collected for the detection of putative pPKR activity using the immobilised eIF2α peptide assay. Typically, one flower bud was taken per plant, extracted in 50 µL Buffer A, and its protein concentration determined. The same amount of protein, usually 10 – 20 µg, was taken from each sample within any given experiment and incubated with the eIF2α peptide beads in the presence of 2.5 µM ATP and 200 µCi/mL γ32P-ATP to allow for kinase activity. The level of eIF2α phosphorylation in the transgenic lines was either assessed for individual plants or up to three plants from the same line and was compared with the activity detected in flower buds taken from three wildtype Arabidopsis plants. Each set of experiments included at least three repeats of the same experiment with a similar pattern of results observed between these experiments. The significance of any difference in phosphorylation level detected between the mutant and wildtype plants was determined by calculating the F probability, using the statistical programme Genstat, via the residual maximum likelihood method for fitting linear mixed models. The difference in phosphorylation levels was considered as statistically significant when the calculated F probability fell below 0.01. The results of experiments for each plant line are summarised in Tables 6.1 to 6.3.
6.2.2 Results of eIF2α Phosphorylation in Transgenic Arabidopsis thaliana Transferred DNA Insertion Plant Lines

Of the 16 dsRBM-containing genes in Arabidopsis, only 15 had available T-DNA insertion lines (Table 6.1). Unfortunately, a T-DNA insertion line was unavailable for the dsRBM-containing gene, RNase three-like protein 1, that was identified by MS analysis of the proteins purified via the pPKR purification approaches (Table 4.2). Of the 15 T-DNA insertion lines that were available, ten (dsRNA-binding protein 4, ATP-dependent helicase, CTD phosphatase-like 2, CPL1 (FIERY 2), dsRNA-binding protein 5, dsRNA-binding protein 3, DICER-like 1, DICER-like 2, DsRBD-containing protein, and ATP-dependent helicase) were self-crossed to a homozygous state for the T-DNA insertion, while five (Hyponastic leaves 1, dsRNA-binding protein 2, RNase three-like protein 3, DICER-like 4, and RNase three-like protein 2) did not grow. The ten homozygous lines were tested for a change in eIF2α phosphorylation activity, but no significant differences in kinase activity were detected in these transgenic lines. Moreover, no apparent abnormal phenotypes were observed in these mutant plants.

Of the 34 CPK-containing genes in Arabidopsis, all 34 were available as CPK T-DNA transgenic lines (Table 6.2). Only eight homozygous lines (CPK2, CPK10, CPK13, CPK19, CPK20, CPK23, CPK26, and CPK27) were able to be generated for the T-DNA mutant and these included two (CPK20 and CPK27) of the seven CPKs which were identified in the MS analysis of proteins purified using the pPKR purification approaches (Table 4.2). Heterozygous lines were generated for six other CPKs (CPK3, CPK7, CPK12, CPK14, CPK18, and CPK32). No homozygous lines were able to be generated for the six heterozygous plants and these plants were not tested for their eIF2α phosphorylation activity as the wildtype allele was still present for the gene of interest. Unfortunately, five (CPK4, CPK6, CPK21, CPK29 and CPK33) out of the seven CPKs that were identified in the MS analysis (Table 4.2) were unable to be generated to a homozygous or heterozygous state and their kinase activity could not be assessed for a difference which could have provided further evidence, together with the MS analysis sequencing, for the significance of these CPKs as potential pPKR candidates.

Of the eight homozygous CPK T-DNA insertion plant lines that were generated and tested for a change in eIF2α phosphorylation, seven, including the two CPK lines (CPK20 and CPK27) that were identified in the MS analysis, did not display any significant differences in kinase activity or phenotype as compared to wildtype. However, one of the CPK T-DNA insertion lines, CPK19,
showed a decrease in eIF2α phosphorylation activity. Seeds were collected from the CPK19 plant line and sown on Plant Basal Medium supplemented with 50 mg/L kanamycin to grow the next generation of homozygous sub-lines of the CPK19 T-DNA transgenic plant. Ten sub-lines (grown from seeds of initial homozygous plant) were planted, but only two, CPK19b and CPK19d, grew. Plants from these sub-lines showed a decrease in pPKR activity as compared to wildtype plants (Fig. 6.1a & c). When the mean phosphorylation levels of the CPK19b and CPK19d lines were compared to mean levels of wildtype plants, an F probability value of 0.002 and 0.007, respectively, was calculated (Fig. 6.1b & d). These values indicated that the reductions in phosphorylation were statistically significant. A noticeable change in phenotype was also observed in the CPK19 mutant plants (Fig. 6.2). These plants displayed slowed growth, a stunted main stem, and increased branching. However, these phenotypes were not seen in all the plants derived from the CPK19b and CPK19d mutant lines and ranged from severely stunted to mildly affected and some plants appeared to look like wildtype plants (Fig. 6.3).

No difference in eIF2α phosphorylation or phenotype was observed in the T-DNA insertion line of the protein kinase of unknown function (At1g80640) (Table 6.3). In addition, numerous attempts to grow the GCN2 and p58IPK T-DNA insertion lines failed. Thus, it was not possible to use these lines to further validate the detected kinase activity as pPKR.
Figure 6.1 Quantification of putative pPKR activity in transgenic Arabidopsis T-DNA lines CPK19b and CPK19d. Bar graphs showing the eIF2α phosphorylation detected in three wildtype Arabidopsis plants compared with five transgenic Arabidopsis plants each from two sub-lines containing a T-DNA insert in the calcium-dependent protein kinase 19 gene. Two lines of transgenic Arabidopsis CPK19 T-DNA plants were assessed. Comparison of the individual phosphorylation activity detected in (a) wildtype vs CPK19b T-DNA plants and (c) wildtype vs CPK19d T-DNA plants. Comparison of the combined mean phosphorylation activity in (b) wildtype vs CPK19b T-DNA plants and (d) wildtype vs CPK19d T-DNA plants. F probability (F) indicates the significance of their difference. The Y-axis measures the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Peptide S, blue bar.
Figure 6.2 Phenotypes of calcium-dependent protein kinase (CPK) 19 T-DNA insertion lines. Comparison of the phenotypes observed in wildtype Arabidopsis and transgenic Arabidopsis plants containing a T-DNA insert in the calcium-dependent protein kinase 19 gene.
Figure 6.3 Range of phenotypes observed for calcium-dependent protein kinase (CPK) 19 T-DNA insertion line CPK19d. Comparison of the range of phenotypes observed in transgenic *Arabidopsis* plants containing a T-DNA insert in the calcium-dependent protein kinase 19 gene.
6.2.3 Summary of eIF2α Phosphorylation in Transgenic Arabidopsis thaliana Transferred DNA Insertion Plant Lines

Overall, a total of 52 T-DNA seed stocks were imported and 19 of these were grown into plant lines homozygous for the T-DNA insert (Table 6.4). The 19 homozygous plant lines were analysed for their eIF2α phosphorylation activity and one line (CPK19) displayed kinase activity that was statistically different from the wildtype plants.

Table 6.4 Summary of the transgenic Arabidopsis thaliana T-DNA insertion lines obtained and grown for the analysis of their eIF2α phosphorylation activity

<table>
<thead>
<tr>
<th>T-DNA insertion lines²</th>
<th>Seed stock imported?</th>
<th>Number of lines grown?</th>
<th>Number of lines tested?</th>
<th>If tested, any changes in eIF2α phosphorylation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsRBM-containing genes (16)</td>
<td>15 out of 16</td>
<td>10 homozygous lines (5 did not grow)</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>Calcium-dependent protein kinases (34)</td>
<td>34 out of 34</td>
<td>8 homozygous lines (6 heterozygous) (20 did not grow)</td>
<td>8</td>
<td>1 line (CPK19) had decrease in activity</td>
</tr>
<tr>
<td>Protein kinase of unknown function (1)</td>
<td>1 out of 1</td>
<td>1 homozygous line</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>eIF2α kinase GCN2 (1)</td>
<td>1 out of 1</td>
<td>1 did not grow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inhibitor of PKR p58IPK (1)</td>
<td>1 out of 1</td>
<td>1 did not grow</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

² brackets indicate the number of genes present in Arabidopsis that matches the category of interest; dsRBM, dsRNA binding motif; boxes with dash (-) denotes no data available

6.3 Assessment of eIF2α Phosphorylation in Transgenic Overexpressed and Knockdown Arabidopsis thaliana Plant Lines

Stably transformed transgenic plants were generated by introducing genes of interest into Arabidopsis by A. tumefaciens-mediated transformation using the floral dip method as described in Section 2.10.3. The bacterial and nucleic acid manipulation protocols used for generating the overexpression gene and hairpin constructs are described in Sections 2.5 and 2.6 and the oligonucleotide primers used for cloning and sequence verification are listed in Table 2.2 and 2.3.
6.3.1 Generation of Overexpression Constructs for *Arabidopsis thaliana* Transformation

The overexpression constructs used for plant transformations were generated via the following cloning strategy. Firstly, the cDNA of each gene was amplified, inserted into a cloning plasmid via TA Cloning®, released and integrated between the 35S promoter and ocs3’ of the shuttle vector pART7 (Fig. 2.2) via their unique restriction enzyme sites that was compatible with the sites within the multiple cloning site of pART7. The 35S-gene-ocs3’ sequence was then removed as a NotI fragment and introduced into the binary vector, pART27 (Fig. 2.3) between the left and right border sequence of the *A. tumefaciens*-derived T-DNA. The resultant expression vector containing the gene of interest was transformed into *A. tumefaciens* and used for the transformation of *Arabidopsis* plants.

The expression constructs for the overexpression of the following genes of interest were generated or attempted to be generated and the cloning strategies are described in Sections 6.3.1.1 to 6.3.1.3:

- a. Mouse PKR, *Arabidopsis* p58IPK, and *Vaccinia virus* E3L and K3L (Section 6.3.1.1)
- b. *Arabidopsis* GCN2 (Section 6.3.1.2)
- c. *Arabidopsis* eIF2α wildtype (Section 6.3.1.3.1)
- d. *Arabidopsis* eIF2α mutants (Section 6.3.1.3.2)

The growth and generation of these transgenic *Arabidopsis* plant lines are described and discussed in Section 6.3.1.4.

6.3.1.1 Expression Constructs for the Overexpression of Mouse Protein Kinase R, *Arabidopsis thaliana* p58IPK and *Vaccinia virus* E3L and K3L

The pART27 overexpression constructs of mouse PKR, p58IPK, E3L, and K3L were obtained from previous research studies (Tang, 2006) performed at Plant and Food Research and The University of Auckland.
**6.3.1.2 Generation of Expression Construct for the Overexpression of Arabidopsis thaliana GCN2**

The pART27 overexpression construct of GCN2 was generated as part of this research thesis and is briefly described in the following section (refer to Appendix B for full description).

The GCN2 gene (At3g59410) was amplified by PCR from *Arabidopsis* cDNA (obtained from W. Cui at Plant and Food Research) using the primers At GCN2 5’ EcoRI NdeI and At GCN2 3’ BglII. A ~3.7 kb product was generated with restriction enzyme sites of EcoRI and NdeI at the 5’ end and BglII at the 3’ end. The amplified GCN2 gene was then inserted into pCR®2.1-TOPO® and released and ligated into pART7 via the EcoRI sites located in pCR®2.1-TOPO®. The pART7-GCN2 clone with the correct restriction enzyme digestion profile and verified DNA sequence was selected and digested with NotI to release the 35S-GCN2-ocs3’ fragment which was then ligated into the binary vector pART27. Putative pART27-GCN2 clones were selected and screened via restriction enzyme digests with four different enzymes to confirm the insertion of the 35S-GCN2-ocs3’ fragment into pART27. As anticipated for the correct clone, digestion with BamHI released two bands of ~0.9 and ~4.7 kb; HindIII released three bands of ~0.05, ~1.2, and ~2.2 kb; XbaI released a ~1.3 kb band; and BglII released three bands of ~0.7, ~1.2, and ~2.7 kb. The resultant pART27-GCN2 clone was transformed into *A. tumefaciens* and prepared for transformation into *Arabidopsis*.

**6.3.1.3 Generation of Expression Constructs for the Overexpression of Arabidopsis thaliana elf2α wildtype and mutants**

The *Arabidopsis* elf2α wildtype (Elf2A WT, At5g05470) and three elf2α mutants were generated for overexpression in *Arabidopsis*. All three mutants contain a point mutation at nt 162 which results in a loss of the SacI restriction enzyme site and a missense mutation at codon 56 where the Ser residue normally phosphorylated by PKR is modified to an Ala residue (elf2A S56A), an Asp residue (elf2A S56D), or a Glu residue (elf2A S56E). The former modification results in a nonphosphorylatable form of elf2α, while the latter two mutations mimic the phosphorylated form of elf2α.
6.3.1.3.1 Generation of Expression Construct for Overexpression of Arabidopsis thaliana eIF2α wildtype

The pART27 overexpression construct of eIF2A WT was generated as part of this research thesis and is briefly described in the following section (refer to Appendix C for full description).

The eIF2A WT gene was amplified by PCR from Arabidopsis cDNA (obtained from W. Cui at Plant and Food Research) using the primers At eIF2A F EcoRI NcoI and At eIF2A R BamHI. A ~1.0 kb product was generated with restriction enzyme sites of EcoRI and NcoI at the 5’ end and BamHI at the 3’ end. The amplified eIF2A WT gene was then inserted into pCR® 2.1-TOPO® and released and ligated into pART7 via the primer added EcoRI and BamHI ends. The pART7-eIF2A WT clone with the correct restriction enzyme digestion profile and verified DNA sequence was selected and digested with NotI to release the 35S-eIF2A WT-ocs3’ fragment for ligation into the binary vector pART27. Putative pART27-eIF2A WT clones were selected and screened via restriction enzyme digests with two different enzymes to confirm the insertion of the 35S-eIF2A WT-ocs3’ fragment into pART27. The digestion with HindIII released a ~0.6 kb band; while SacI released two bands of ~1.5 and ~1.7 kb. The resultant pART27-eIF2A WT clone was transformed into A. tumefaciens and prepared for transformation into Arabidopsis for the generation of stably transformed eIF2A WT overexpression transgenic lines.

6.3.1.3.2 Generation of Expression Constructs for Overexpression of Arabidopsis thaliana eIF2α mutants

The pART27 overexpression constructs of eIF2A S56A and S56D/E were generated (or attempted to be generated) as part of this research thesis and are briefly described in the following section (refer to Appendix D for full description).

The eIF2α mutants were each generated via a three-step cloning strategy. The first half of the eIF2α gene was amplified by PCR from pCR® 2.1-TOPO®-eIF2A WT using the primers At eIF2A F EcoRI NcoI and At R 181-148 noSacI S56A for generating eIF2A S56A; and the primers At eIF2A F EcoRI NcoI and At R 181-148 noSacI S56D/E for generating eIF2A S56D and S56E. A ~0.18 kb product was generated with the first amplification step. The second half of the eIF2α gene was then amplified by PCR from pCR® 2.1-TOPO®-eIF2A WT using the primers At eIF2A F 170-197 and At eIF2A R BamHI and a ~0.9 kb product was generated. The third PCR reaction
was performed with the primers At eIF2A F EcoR I NcoI and At eIF2A R BamHI using a mixture of the two amplified products as template. The overlapping ends of the two products created a template to obtain a full-length eIF2α gene product which would contain the different mutations at codon 56.

The resulting eIF2α mutants were inserted into pCR®2.1-TOPO® and screened for the presence of an eIF2A S56A, S56D, or S56E mutant. Unfortunately, numerous attempts failed to generate a pCR®2.1-TOPO®-eIF2A S56E clone. However, as the eIF2A S56D and S56E constructs both mimic the phosphorylated form of eIF2α, it was not critical to have generated both of these expression constructs. The same cloning protocol, as per the cloning of eIF2A WT, was then applied for the integration of eIF2A S56A and S56D into pART27. Putative pART27-eIF2A S56A and S56D clones were then verified by the digestion with HindIII which released a ~0.6 kb band and SacI which released two bands of ~1.5 and ~1.7 kb. The resultant pART27-eIF2A S56A and S56D clones were transformed into A. tumefaciens and prepared for transformation into Arabidopsis.

6.3.1.4 Growth of the Transgenic Overexpression Arabidopsis thaliana Plant Lines

Except for mouse PKR and eIF2A S56E, all other constructs were successfully transformed into A. tumefaciens for the transformation of Arabidopsis using the floral dip method. Table 6.5 lists the genes for which floral dipping was performed in an attempt to generate stably transformed overexpression plants.

While the eIF2A S56E construct was unable to be generated, the pART27-mouse PKR expression construct was available, but, even with repeated attempts, no colonies appeared on the antibiotic-supplemented LB plates after electro-transformation of the construct into A. tumefaciens. The GCN2 and eIF2A WT, S56A and S56D constructs were successfully generated but due to the limited time available, they were not transformed into Arabidopsis.
Table 6.5 List of genes for the generation of transgenic overexpression Arabidopsis lines

<table>
<thead>
<tr>
<th>Gene to be overexpressed</th>
<th>Name</th>
<th>Cloned into pART27?</th>
<th>Floral dipped?</th>
<th>Stably transformed Arabidopsis?</th>
<th>Changes in eIF2α phosphorylation?</th>
<th>F probability&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mus musculus PKR</td>
<td>Mouse PKR</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabidopsis pS8&lt;sup&gt;PKR&lt;/sup&gt;</td>
<td>IPK</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Decrease</td>
<td>0.01</td>
</tr>
<tr>
<td>Vaccinia virus E3L</td>
<td>E3L</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>-</td>
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<tr>
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<td>Yes</td>
<td>No</td>
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<td>-</td>
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<tr>
<td>Arabidopsis GCN2</td>
<td>GCN2</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<td>-</td>
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<tr>
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<td>eIF2A WT</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> F probability calculated using GenStat to determine the statistical significance of any difference in the level of eIF2α phosphorylation detected between the mutant and wildtype plants; boxes with dash (-) denotes no data available

Three Arabidopsis plants were floral dipped (as described in Section 2.10.3.2) for each of the overexpression constructs IPK, E3L, and K3L. Dipped plants were allowed to mature and seeds were collected. The collected seeds were stored at 4°C to simulate vernalisation, surface sterilised, and then grown and maintained on Plant Basal Medium supplemented with 100 mg/L kanamycin to select for transgenic plants (as described in Sections 2.10.2 and 2.10.5). Once root development occurred, the seedlings were transferred to soil and allowed to establish growth. As these were overexpression plants, the phenotype was likely to be dominant and therefore, it was not necessary to develop homozygous lines before the plants could be analysed for their eIF2α phosphorylation activity.

The transgenic lines were grown until flowering established and the inflorescences were collected for the detection of pPKR activity using the immobilised eIF2α peptide assay. Samples for the analysis of eIF2α phosphorylation were taken as per the protocol used for the T-DNA insertion lines (as described in Section 6.2.1). The specific details and results observed for each plant line are found in Table 6.5.
6.3.2 Transgenic *Arabidopsis thaliana* Knockdown Plant Lines

Generation of stably transformed transgenic knockdown plants was attempted by producing constructs which contained a hairpin of the gene of interest and introducing these constructs into *Arabidopsis* by *A. tumefaciens*-mediated transformation using the floral dip method (as described in Section 2.10.3). The hairpin constructs would simulate the presence of dsRNA and result in the activation of PTGS and the specific mRNA degradation of the genes that the hairpins are derived from. In turn, the transcript levels of the targeted genes would be reduced and lead to a knockdown of the genes of interest.

The cloning strategy and the different approaches that were applied for the generation of the hairpin constructs are described in Appendix E. Table 6.6 lists the genes and the corresponding primers used for which stably transformed knockdown plants were attempted to be generated. However, due to various setbacks experienced in the cloning of these genes, the hairpin constructs were only able to be cloned into the pART7 construct (as described in Appendix E Section E4) and no transgenic plant lines were able to be grown and developed for the detection of eIF2α phosphorylation and phenotype analysis.

### Table 6.6 List of genes for the generation of transgenic knockdown *Arabidopsis* lines and the corresponding oligonucleotide primers used

<table>
<thead>
<tr>
<th>Gene to be knocked down</th>
<th>Construct name</th>
<th>Sense primers</th>
<th>Antisense primers</th>
</tr>
</thead>
</table>
| *Arabidopsis* p58<sup>35S</sup> | IPK hairpin | At IPK hp1 5’ XhoI  
At IPK hp1 3’ BglII | At IPK hp2 5’ BamH1  
At IPK hp2 3’ XbaI |
| *Arabidopsis* GCN2 | GCN2 hairpin | At GCN2 hp1 5’ XhoI  
At GCN2 hp1 3’ BglII | At GCN2 hp2 5’ BamH1  
At GCN2 hp2 3’ XbaI |

6.3.3 Results of eIF2α Phosphorylation in Transgenic Overexpressed and Knockdown *Arabidopsis thaliana* Plant Lines

Of the nine overexpression constructs and two knockdown constructs that were to be generated, only three overexpression constructs, IPK, E3L, and K3L, were used for floral dip transformation of *Arabidopsis*.

Seeds collected from *Arabidopsis* transformed with the overexpression constructs were grown on Plant Basal Medium supplemented with 100 mg/L kanamycin. Only successfully transformed
plants were able to be maintained in this antibiotic-supplemented medium. No stably transformed Arabidopsis were generated for the overexpression of K3L and although one stably transformed Arabidopsis line for the overexpression of E3L was generated, its flower buds were small (as shown in Fig. 6.7) and were not sampled for the detection of kinase activity. Seven stably transformed Arabidopsis lines were generated for the overexpression of p58\textsuperscript{IPK} and these plants developed suitable inflorescences for the analysis of eIF2α phosphorylation activity.

Of the seven stably transformed first generation (T\textsubscript{1}) p58\textsuperscript{IPK} overexpressed Arabidopsis lines, three lines, IPK6.1.3.1, IPK6.1.3.2, and IPK6.1.3.3, displayed a decrease in eIF2α phosphorylation activity as compared to wildtype plants (Fig. 6.4a). Mean phosphorylation levels from these plants as compared to the mean levels of the wildtype plants showed a statistically significant decrease in activity with an F probability value of 0.01 (Fig. 6.4b). In addition, a noticeable change in phenotype was observed in these three p58\textsuperscript{IPK} overexpression lines which had decreased eIF2α phosphorylation (Fig. 6.5). These plants displayed slower growth, a stunted main stem, and increased branching. However, as noted with the CPK T-DNA insertion lines (Section 6.2.2), a range of phenotypes was observed among individual T\textsubscript{1} plants within this overexpression line (Fig. 6.6). These variations in phenotype and activity level between transgenic lines may be due to the presence of different expression levels from the p58\textsuperscript{IPK} gene caused by the insertion locus or the insertion of the construct into a gene important for pPKR activity.

It was interesting to note that the phenotypes observed in the IPK overexpressed plants were similar to that observed in the CPK19 T-DNA insertion lines (Fig. 6.7). A slowed growth and stunted main stem was detected in these plants and increased branching was even more severe than in the CPK19 T-DNA insertion lines. A stunted phenotype was also observed in the E3L overexpressed plants (Fig. 6.7). Their inflorescences were undersized and malformed and could not be taken for the analysis of their eIF2α phosphorylation activity. On the other hand, these plants displayed a more prominent growth of the central stem than the CPK19 T-DNA insertion and IPK overexpression lines.
Figure 6.4 Quantification of putative pPKR activity in T1 transgenic *Arabidopsis* overexpression lines IPK6.1.3.1, IPK6.1.3.2, and IPK6.1.3.3. Bar graphs showing the eIF2α phosphorylation detected in three wildtype *Arabidopsis* plants compared with three T1 transgenic *Arabidopsis* plants overexpressing for the *Arabidopsis* p58<sup>IPK</sup> gene. (a) Comparison of the individual phosphorylation activity detected in wildtype vs three T1 p58<sup>IPK</sup> overexpression plants. (b) Comparison of the combined phosphorylation activity detected in wildtype vs the p58<sup>IPK</sup> overexpression plants. F probability (F) indicates the significance of their difference. The y-axis measures the amount of radioactivity (counts per minute) present in each peptide bead sample. Numerical values (blue) above each bar represent actual counts per minute detected in the Peptide S beads sample. Peptide S, blue bar.
**Figure 6.5 Phenotypes of T$_1$ p58$^{IPK}$ overexpression transgenic Arabidopsis line.** Comparison of the phenotypes observed in wildtype Arabidopsis and T$_1$ transgenic Arabidopsis plants overexpressing for the Arabidopsis p58$^{IPK}$ gene.
Figure 6.6 Range of phenotypes observed for the T$_1$ p58$^{IPK}$ overexpression transgenic *Arabidopsis* line. Comparison of the range of phenotypes observed in T$_1$ transgenic *Arabidopsis* plants overexpressing for the *Arabidopsis* p58$^{IPK}$ gene.
Figure 6.7 Phenotypes of transgenic *Arabidopsis* T-DNA line CPK19d and p58<sup>IPK</sup> and E3L overexpression lines. Comparison of the phenotypes observed in a T-DNA insertion transgenic *Arabidopsis* line containing a T-DNA insert in the calcium-dependent protein kinase 19 gene and two overexpression transgenic *Arabidopsis* lines overexpressing for the *Arabidopsis* p58<sup>IPK</sup> gene and *Vaccinia virus* E3L gene. Image for the E3L overexpression line is magnified 2.5-fold compared to images of the CPK19 T-DNA and p58<sup>IPK</sup> overexpression lines.
6.3.4 Summary of eIF2α Phosphorylation in Transgenic Overexpressed and Knockdown Arabidopsis thaliana Plant Lines

Constructs of seven of the nine genes of interest for overexpression were obtained or cloned into pART7. Of the seven constructs, three were transformed into Arabidopsis, but only two of these, p58\textsuperscript{IPK} and E3L, were stably transformed and successfully grown for further activity and/or phenotype analysis. A statistically significant decrease in eIF2α phosphorylation activity was detected in the p58\textsuperscript{IPK} overexpression plant lines IPK6.1.3.1, IPK6.1.3.2, and IPK6.1.3.3. The p58\textsuperscript{IPK} and E3L overexpression lines displayed a similar stunting and/or increased branching as observed in the CPK19 T-DNA insertion line. Theoretically the expression of the inhibitors results in an inhibition of pPKR and the CPK19 T-DNA insertion results in the knockout of CPK19 expression. Hence, these results point to the possibility that CPK19 may be pPKR or is involved in the pPKR regulatory pathway.

Various setbacks were experienced in the cloning of the Arabidopsis p58\textsuperscript{IPK} and GCN2 hairpins for the generation of the transgenic knockdown lines. However, the final approach using GenScript-synthesised hairpins (as described in Appendix E4) was effective in achieving pART7 clones. Once the hairpins are successfully cloned into pART27, they can be introduced into Arabidopsis via A. tumefaciens-mediated transformation and the effect of the p58\textsuperscript{IPK} and GCN2 gene knockdowns on eIF2α phosphorylation can be examined.

6.4 Conclusions

A total of 21 transgenic Arabidopsis plant lines were analysed for their eIF2α phosphorylation activity and/or phenotype changes. A statistically significant decrease in kinase activity was detected in the CPK19 T-DNA line and the p58\textsuperscript{IPK} overexpression line as compared to wildtype Arabidopsis. In addition, a similar change in phenotype was detected in the CPK19 T-DNA line and the p58\textsuperscript{IPK} and E3L overexpression lines, suggesting that CPK19 is a potential candidate as the plant-functional homolog of PKR or possibly has a role in the pPKR regulatory pathway.

The assessment of the T-DNA, overexpression, and knockdown transgenic lines that have yet to be generated will be important to further uncover genes that are involved in the pPKR pathway. Moreover, the generation of the knockdown plant lines will be valuable as a validation for the immobilised eIF2α peptide assay to confirm that it indeed detects for putative pPKR activity. Of
Chapter Six – Assessment of Putative Plant Protein Kinase R Activity in Transgenic *Arabidopsis thaliana*

the four eIF2α kinases that have been characterised in mammals, only one, GCN2, has thus far been identified in plants. Hence, if eIF2α phosphorylation is still detected in the GCN2 knockdown plant lines, especially in the presence of dsRNA, this would validate that the assay is detecting for putative pPKR activity and not an additional activity of GCN2. Recently, a null mutant for *Arabidopsis* GCN2 has been generated (as utilised by Y. Zhang et al., 2008) which can also function in the same way as knockdown plant lines to distinguish the pPKR and GCN2 contributions of the detected eIF2α phosphorylation activity.

It is also important to note that redundancy in function may be present within the genes that have or will be tested. Such redundancy might mask the effects of disrupting a particular gene. An initial experiment which can determine the possibility of redundancy would be to generate a transgenic plant line which has disruptions in both the CPK19 and GCN2 genes. If eIF2α phosphorylation is no longer detected in this plant line, it is anticipated that no redundancy in function is present for these genes of interest.
7

In Silico Analysis of Calcium-dependent Protein Kinase CPK19 for Protein Kinase R-like Properties

Although a large number of proteins was identified as potential pPKR candidates via MS analysis of enriched pPKR fractions (Table 4.2), findings on the influence of divalent ions, especially calcium, and the analysis of transgenic Arabidopsis plant lines, present CPK19 as the best candidate, to date, for the plant-functional homolog of PKR. This proposed role for CPK19 is supported by a decrease of eIF2α phosphorylation activity in the CPK19 T-DNA insertion line (Figure 6.1) as well as the observation of similar phenotypic changes in this line and plant lines that overexpress either of the two inhibitors of PKR, p58IPK or E3L (Figure 6.7). However, a significant peptide match of this protein from the MS analysis was not obtained in this study. Therefore, the results observed in the transgenic Arabidopsis lines cannot be associated with the sequencing data to provide supporting biochemical evidence for the identification of the putative
pPKR. However, a calcium-dependent kinase that can phosphorylate eIF2α is in keeping with the activation of eIF2α kinase activity by calcium (as assessed in Chapter 5).

Without additional biochemical results, it is possible to use an *in silico* approach to study the protein sequence and structure of CPK19 for PKR-like properties as well as its possible ability to function in PKR-like activities. Findings from this analysis will provide further evidence for the potential of CPK19 as the putative plant-functional homolog of PKR.

The similarities and differences of protein sequence and domains between CPK19 and the other members of the CPK family and human PKR were determined using sequence comparisons and alignments with Geneious (Biomatters) and Basic Alignment Search Tool (BLAST). Protein statistics were calculated using Sequence Manipulation Suite (SMS). Secondary structures were predicted using Parallel Protein Information Analysis system (PAPIA).

### 7.1 Overview of *In Silico* Analysis of Calcium-dependent Protein Kinase CPK19

CPKs are characterised by four distinct domains – a variable N-terminal domain, a Ser/Thr protein kinase domain, a short autoinhibitory domain, and a calmodulin-like domain (CaM-LD) that is comprised of four calcium binding EF hands with sequences similar to calmodulin (Fig. 1.19). Correspondingly, these motifs are found in CPK19. An analysis of the sequence and/or structure of these domains for PKR-like properties is described in Sections 7.2 to 7.4:

- a. Ser/Thr kinase domain (Section 7.2)
- b. CaM-LD (Section 7.3)
- c. N-terminal domain (Section 7.4)

A comparison of the protein sequence of CPK19 with human PKR is described in Section 7.5.

Of note, the protein sequence and structure of the short 38 aa autoinhibitory domain of CPK19 did not display specific residues of interest, such as an abundance of phosphorylatable or basic residues, or particular motifs of interest, such as sequence or protein structure with PKR-like properties. Hence, the assessment of this autoinhibitory domain of CPK19 is not discussed.
On the whole, a number of PKR-like attributes are identified in CPK19, including the characteristic eIF2α kinase motif, similarities with a dsRBM of human PKR, and a possible dsRNA binding motif. These PKR-like features are present respectively in the kinase domain, EF hand, and variable N-terminal domain of CPK19. Figure 7.1 provides a summary illustration of these PKR-like features.

**Figure 7.1 Summary of the PKR-like features of CPK19.** Schematic diagram of the domains of CPK19 consisting of a variable N-terminus (grey box), Ser/Thr kinase domain (light blue box), autoinhibitory domain (J, red box), and a calmodulin-like domain (CaM-LD, orange box) with four EF hands (EFh1-4, blue boxes). The variable N-terminus comprise of a dsRNA binding domain-like secondary structure and is composed of two α-helices (α, red line) and a β-sheet (β, blue line). Basic residues (Arg, Lys, and His) that are located in the N-terminus are denoted by orange dots. A motif characteristic of animal eIF2α kinase (eIF2α kinase-like motif, green line) is located in the Ser/Thr kinase domain. A black dot in the Ser/Thr kinase domain denotes a Tyr residue that is predicted to be phosphorlated in CPK19 (at Tyr257) and corresponds to Thr466 in human PKR. A blue dot in the Ser/Thr kinase domain denotes Thr303 that is a conserved Thr residue critical for eIF2α binding in human PKR (Thr487 in human PKR). The CaM-LD is similar in sequence to calmodulin but two residue substitutions from Asp (D) and Asn (N) to Ser (S) in EFh1 and EFh3, respectively (purple dots) are identified in CPK19. A motif similar to the second dsRBM of human PKR (dsRBM2-like motif, purple line) is also located in EFh1 of CPK19.
Chapter Seven – *In Silico* Analysis of Calcium-dependent Protein Kinase CPK19 for Protein Kinase R-like Properties

7.2 Analysis of the Serine/Threonine Protein Kinase Domain

As expected, the Ser/Thr kinase domains present in the CPK family are generally conserved, particularly in the activation loops where the ATP binding pockets are located. Similarly, common residues important for the function of the kinase domain are conserved between the kinase domains of CPK19 and human PKR. Importantly, a highly conserved region (LFIQMEFCD) characteristic of animal eIF2α kinases (Cai & Williams, 1998 and described in Section 1.2.1.1) is observed in a similar context in CPK19, albeit with several aa substitutions (Fig. 7.2, underlined, and illustrated in Fig. 7.1). This sequence motif is also located in all of the kinases of the CPK family and as such does not distinguish CPK19 from the other CPK family members. This motif is however also present in the *Arabidopsis* GCN2 (Fig. 7.2, At GCN2), the only characterised eIF2α kinase identified to date in plants (Y. Zhang et al., 2003).

![Figure 7.2 Conserved sequence motif characteristic of eIF2α kinases is also located in CPK19. Sequence alignment of two human eIF2α kinases PKR and GCN2, *Danio rerio* PKZ, and *Arabidopsis thaliana* (At) GCN2 and CPK19. A sequence similar to the conserved motif sequence LFIQMEFCD (underlined in red) that is characteristic of eIF2α kinases is present in CPK19 with a few amino acid substitutions. Degree of similarity between the sequences is denoted by black (high), grey (medium), and white (low) highlights. Numbers denote the amino acid location from which the sequence was extracted from.](image)

7.3 Analysis of the Calmodulin-like Domain

When the *Arabidopsis* calmodulin protein (At3g43810) was aligned with the EF hands located in the CaM-LD of the CPKs, the assessed EF hands were generally similar to calmodulin (40.9% pairwise identity), particularly in the calcium binding regions consisting of the conserved Asp and Glu (50% to 75% pairwise identity). However, substitutions of two residues from Asp and Asn to Ser were detected in the calcium binding sites of the first and third EF hands (EFh1 and EFh3) of CPK19, respectively (Fig. 7.3, orange boxes, and illustrated in Fig. 7.1). Interestingly, the Ser substitution in EFh1 of CPK19 is a predicted phosphorylation site (as predicted by SMS). The binding of positively charged calcium ions typically requires the presence of negatively charged
residues, such as Asp and Glu, which may be mimicked by a phosphorylated Ser residue. Thus, as long as the Ser residue is indeed phosphorylated, EFh1 should theoretically also be able to bind to calcium ions. This suggests a possible activation of CPK19 by phosphorylation to regulate the effects of calcium-related signalling.

Upon further examination, 22 members (including CPK19) of the 34 members of the CPK family were identified with the EFh1 substitution while 24 members carry the EFh3 substitution. Overall, 18 CPKs, including CPK19, carry both of the aa substitutions.

When the CaM-LD of CPK19 was compared by BLAST with the dsRBMs of human PKR, a shared motif was identified between EFh1 and the second dsRBM of the PKR protein (Fig. 7.4). This similarity between a well characterised RNA binding motif and EFh1 suggests that EFh1 may have possible nucleic acid binding capacity in addition to its calcium binding function.

Figure 7.3 Conserved regions between calmodulin and the calmodulin-like domain of CPK19. Sequence alignment of the Arabidopsis thaliana calmodulin protein with the calmodulin-like domain of CPK19. Four EF hands (EFh1-4) are located in the sequences and the predicted calcium binding sites are denoted by red lines. The substitutions of two residues in calmodulin from Asp and Asn to Ser are portrayed by the orange boxes. Degree of similarity between the sequences is denoted by black (high), grey (medium), and white (low) highlights. Numbers denote the amino acid location from which the sequence was extracted from.

Figure 7.4 Conserved regions between EFh1 of CPK19 and the second dsRBM of human PKR. Sequence alignment of the first EF hand (EFh1) of Arabidopsis thaliana CPK19 with the second dsRNA binding motif (dsRBM2) of human PKR. Degree of similarity between the sequences is denoted by black (high), grey (medium), and white (low) highlights. Numbers denote the amino acid location from which the sequence was extracted from.
Chapter Seven – In Silico Analysis of Calcium-dependent Protein Kinase CPK19 for Protein Kinase R-like Properties

7.4 Analysis of the N-terminal Domain

As shown in Figure 7.5, the N-terminal domains (the region directly terminal to the kinase domain) of the 34 members of the CPK family are variable both in sequence and in length. These sequences ranged from 20 aa (CPK12) to 184 aa (CPK2) with CPK19 comprising the eighth longest N-terminus with 96 aa. Other characteristics were also compared between the N-terminal domains of these CPKs (Table 7.1). On the whole, the N-termini of CPKs were relatively rich in the basic residues Arg, Lys, and His, with these residues comprising 9.9% (CPK17) to 27.4% (CPK16) of the N-terminal sequences. On the other hand, the pI values of the N-termini of CPKs were more variable and spanned a broader range of 5.55 (CPK17) to 12.41 (CPK24). CPK19 was ranked tenth highest in both features with an N-terminal region that was composed of 20.8% of basic residues with a pI of 10.59. In terms of secondary structure, 16 of the CPKs, including CPK19, were predicted to contain one or more α-helices in their N-termini, and of these, only two – CPK19 and CPK25 – comprised of a N-terminal region that was longer than 65 aa (the minimal length of a dsRNA binding domain as shown in Table 7.2) with an appropriate charge to bind nucleic acids, i.e., consists of more than 20% basic residues, and had a pI greater than 8 (Table 7.1, shaded orange).

Although the protein sequence of CPK19 is not annotated as a dsRNA binding domain, nor align with the nucleic acid binding domains of other known dsRNA binding proteins (such as those listed in Table 7.2), the basic properties of its N-terminal suggest that it may possibly bind to dsRNA. Table 7.2 compares the properties of the CPK19 N-terminus with a wide range of known and postulated dsRNA binding domains of seven proteins. These proteins each comprise of a motif which represents a distinct form of a dsRNA binding domain. These compared dsRNA binding domains include dsRBM1 of human PKR (Kaufman, 2002); the Za domain of Carassius auratus PKZ (Rothenburg et al., 2005); zinc fingers 1-3 of Xenopus laevis dsRBP-ZFa (Finerty & Bass, 1999); a suppressor of silencing p19 from Tomato bushy stunt virus (Xia, Zhu, Zhu, & Zhou, 2009); the N-terminal of NS1A from Influenza A virus (Min & Krug, 2006); the C-terminal of VP35 from Ebola virus (Leung et al., 2009); and the N-terminal of B2 from Flock house virus (Lingel, Simon, Izaurrealde, & Sattler, 2005).
Figure 7.5 The N-terminal domains of the 34 *Arabidopsis thaliana* calcium-dependent protein kinases (CPKs). Sequence alignment of the N-terminal domains of *Arabidopsis* CPKs 1-34. CPK2 has the longest N-terminal region while CPK12 has the shortest. The sequences have been aligned to conserved non-polar residues (highlighted in black) using gaps (dashes). Numbers denote the amino acid location from which the sequence was extracted from.
Table 7.1 Comparison of the N-terminal domains of the 34 Arabidopsis thaliana calcium-dependent protein kinases (CPKs)

<table>
<thead>
<tr>
<th>Protein</th>
<th># of residues</th>
<th># of basic residues</th>
<th>Isoelectric point</th>
<th># of α-helices</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK1</td>
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<td>10</td>
<td>12.9</td>
<td>4</td>
</tr>
<tr>
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<td>106</td>
<td>12</td>
<td>16.4</td>
<td>8</td>
</tr>
<tr>
<td>CPK17</td>
<td>71</td>
<td>2</td>
<td>16.5</td>
<td>2</td>
</tr>
<tr>
<td>CPK18</td>
<td>69</td>
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<td>1</td>
</tr>
<tr>
<td>CPK19</td>
<td>96</td>
<td>5</td>
<td>16.7</td>
<td>3</td>
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</tr>
<tr>
<td>CPK21</td>
<td>78</td>
<td>4</td>
<td>16.8</td>
<td>4</td>
</tr>
<tr>
<td>CPK22</td>
<td>34</td>
<td>0</td>
<td>16.8</td>
<td>3</td>
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</tr>
<tr>
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<td>22</td>
<td>1</td>
<td>16.8</td>
<td>2</td>
</tr>
<tr>
<td>CPK27</td>
<td>26</td>
<td>1</td>
<td>16.8</td>
<td>5</td>
</tr>
<tr>
<td>CPK28</td>
<td>60</td>
<td>6</td>
<td>16.8</td>
<td>3</td>
</tr>
<tr>
<td>CPK29</td>
<td>110</td>
<td>3</td>
<td>16.8</td>
<td>4</td>
</tr>
<tr>
<td>CPK30</td>
<td>57</td>
<td>5</td>
<td>16.8</td>
<td>2</td>
</tr>
<tr>
<td>CPK31</td>
<td>26</td>
<td>1</td>
<td>16.8</td>
<td>5</td>
</tr>
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<td>CPK32</td>
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</tr>
<tr>
<td>CPK33</td>
<td>71</td>
<td>5</td>
<td>16.8</td>
<td>2</td>
</tr>
<tr>
<td>CPK34</td>
<td>66</td>
<td>4</td>
<td>16.8</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) for their corresponding AGI, please refer to Table 6.2; \(^b\) of the N-terminal domain of the CPKs (region directly terminal to the kinase domain); \(^c\) as predicted by PAPIA; \(^d\) does not contain an α-helical region; orange shaded boxes denote CPKs which contains an N-terminal that is longer than 65 aa, with a pi over 8, and has two or more predicted α-helices in its secondary structure; protein statistics calculated by SMS.
Table 7.2 Comparison of the N-terminal domain of CPK19 with known or postulated dsRNA binding regions of specific proteins

<table>
<thead>
<tr>
<th>dsRNA binding region</th>
<th>Protein a</th>
<th># of residues b</th>
<th># of basic residues b</th>
<th>Isoelectric point b</th>
<th># of α-helices c</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsRBM1</td>
<td>PKR (aa 10-78)</td>
<td>69</td>
<td>5</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Zα domain</td>
<td>PKZ (aa 1-66)</td>
<td>66</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Zinc fingers 1-3</td>
<td>dsRBP-ZFa (aa 34-192)</td>
<td>159</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Pair of tryptophans</td>
<td>p19 (Trp39 &amp;Trp42)</td>
<td>172 (entire protein)</td>
<td>8</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>N-terminal</td>
<td>NS1A (aa 1-73)</td>
<td>73</td>
<td>14</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>C-terminal</td>
<td>VP35 (aa 221-340)</td>
<td>120</td>
<td>7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>N-terminal</td>
<td>B2 (aa 1-72)</td>
<td>72</td>
<td>7</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>N-terminal?</td>
<td>CPK19 (aa 1-96)</td>
<td>96</td>
<td>5</td>
<td>13</td>
<td>2</td>
</tr>
</tbody>
</table>

a origin and location (in bracket) of dsRNA binding regions from the following proteins: PKR, Homo sapiens protein kinase R; PKZ, Carassius auratus protein kinase Z; dsRBP-ZFa, Xenopus laevis dsRNA binding protein-zinc finger A; p19, Tomato bushy stunt virus-encoded p19 protein; NS1A, Influenza A virus-encoded non-structural protein 1; VP35; Ebola virus-encoded viral protein 35; B2, Flock house virus-encoded B2 protein; CPK19, Arabidopsis thaliana calcium-dependent protein kinase 19; b of dsRNA binding region as specified; c as predicted by PAPIA; protein statistics calculated by SMS

The majority of the dsRNA binding domains listed in Table 7.2 form secondary structures which consist of α-helical regions and, where known, the dsRNA is postulated to bind to basic residues Arg and Lys which are located in these structures. It is noteworthy that p19 binds dsRNA in a distinct manner. Rather than the presence of basic residues that bind the length of dsRNA, p19 binds like a bracket at the dsRNA ends via two hydrophobic residues (Trp39 and Trp42) held apart by the secondary structure of the entire protein (Xia et al., 2009). Hence, the entire p19 protein sequence was taken for comparison with the CPK19 N-terminus.

In general, the basic residue composition, pI, and predicted secondary structure of the CPK19 N-terminus were relatively similar to the dsRNA binding regions of the compared proteins (Table 7.2 and 7.3), with the exception of p19 which had a much lower pI. However, as mentioned above, the p19 protein binds dsRNA in a distinct manner such that little of its composition is specifically involved in binding dsRNA, but is required instead to form the secondary structure which positions the two dsRNA-interacting Trp residues (Xia et al., 2009).
Table 7.3 Comparison of the sequence and predicted secondary structure of the CPK19 N-terminus with known or postulated dsRNA binding regions of specific proteins

<table>
<thead>
<tr>
<th>PKR (dsRBM1)</th>
<th>PKZ (zα domain)</th>
<th>dsRBP-ZFa (zinc fingers 1-3)</th>
<th>P19</th>
</tr>
</thead>
<tbody>
<tr>
<td>R K K H R R R R K K K</td>
<td>R K R K K K R H R R R K K</td>
<td>K K H R K H R R R K K K H</td>
<td>R R K K R</td>
</tr>
<tr>
<td>FMKELNTYRQKQGVLKYQELPN</td>
<td>MSAETQMEKRIIDFLRNGKSLTIA</td>
<td>TQCKVCSAVLSESQKLHYSQSRKHA</td>
<td>MERRAIQGNDAREQANSERWDGGGG</td>
</tr>
<tr>
<td>NTVI IDGREFPEEGRSEKKEA</td>
<td>NTKSLTIAEIGLSTNVHLN</td>
<td>NKYQSNQVFSNEKPPV</td>
<td>NFQGCNATN</td>
</tr>
<tr>
<td>NNK LAVELNKE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
</tr>
<tr>
<td>PKZ (zα domain)</td>
<td>dsRBP-ZFa (zinc fingers 1-3)</td>
<td>P19</td>
<td></td>
</tr>
<tr>
<td>RK R K K H R R R K K K</td>
<td>K K H R K H R R R K K</td>
<td>R R K K R</td>
<td></td>
</tr>
<tr>
<td>MSAETQMEKRIIDFLRNGKSLTIAEIGLSTNVHLN</td>
<td>TQCKVCSAVLSESQKLHYSQSRKHA</td>
<td>MERRAIQGNDAREQANSERWDGGGG</td>
<td></td>
</tr>
<tr>
<td>NTKSLTIAEIGLSTNVHLN</td>
<td>NKYQSNQVFSNEKPPV</td>
<td>NFQGCNATN</td>
<td></td>
</tr>
<tr>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td></td>
</tr>
<tr>
<td>PKZ (zα domain)</td>
<td>dsRBP-ZFa (zinc fingers 1-3)</td>
<td>P19</td>
<td></td>
</tr>
<tr>
<td>RK R K K H R R R K K K</td>
<td>K K H R K H R R R K K</td>
<td>R R K K R</td>
<td></td>
</tr>
<tr>
<td>MSAETQMEKRIIDFLRNGKSLTIAEIGLSTNVHLN</td>
<td>TQCKVCSAVLSESQKLHYSQSRKHA</td>
<td>MERRAIQGNDAREQANSERWDGGGG</td>
<td></td>
</tr>
<tr>
<td>NTKSLTIAEIGLSTNVHLN</td>
<td>NKYQSNQVFSNEKPPV</td>
<td>NFQGCNATN</td>
<td></td>
</tr>
<tr>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td>HHHHHHHHHHHHHHHHCCCCEEEE</td>
<td></td>
</tr>
<tr>
<td>PKZ (zα domain)</td>
<td>dsRBP-ZFa (zinc fingers 1-3)</td>
<td>P19</td>
<td></td>
</tr>
<tr>
<td>RK R K K H R R R K K K</td>
<td>K K H R K H R R R K K</td>
<td>R R K K R</td>
<td></td>
</tr>
<tr>
<td>MSAETQMEKRIIDFLRNGKSLTIAEIGLSTNVHLN</td>
<td>TQCKVCSAVLSESQKLHYSQSRKHA</td>
<td>MERRAIQGNDAREQANSERWDGGGG</td>
<td></td>
</tr>
<tr>
<td>NTKSLTIAEIGLSTNVHLN</td>
<td>NKYQSNQVFSNEKPPV</td>
<td>NFQGCNATN</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td><strong>Table 7.4 continued</strong></td>
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</tr>
<tr>
<td>-------------------------</td>
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<tr>
<td><strong>NS1A (N-terminal)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>H RK R RR R R K H K K K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDSNTVSSFQVDCFLWHIRKQVVDQELSDAPFLDRRLRDRQRSRLRGNLTGGLDIKAATHVGKQIVERIKKES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VP35 (C-terminal)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K R H H K K H KR H RR R K R K R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKDLRNIYDHLPFGTAFHQLVQVICLKGDSNSLDIHAEFQASLAEGDSPQCALIQTITKRVPQDAAFPVIHISRGRDIPRACQKSLRPVPSPKIDRGWVCVFQLOD</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>K K GKTGLIKI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B2 (N-terminal)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K K R RR R H K K R K</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPSKLALIQELPRIQTAVEAMGMSYQDPNNVRELDIHLACLNKAKLTVMSTSSLKPSVAYLEGK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CPK19 (N-terminal)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K K K K R R R R K K H K K K R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGGCLCINLKKVVKPTIDISEQNTEVKSREITPKEQPRQRQQPAPRAKFQIVVQPHKLLPLPQFOEKQLNHQKSTLQQQPEPILGRPFEDIKE</td>
<td></td>
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</tr>
<tr>
<td>CCHHCHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHEEECCCCCCCCCCCCCCCCCCHHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DsRNA binding proteins and their known or postulated dsRNA binding regions (*italics*) as listed in Table 7.3; bold letters denote protein sequence of interest; orange letters above protein sequences denote the position of basic residues Arg (R), Lys (K), and His (H); coloured letters below protein sequences denote secondary structures as predicted by PAPIA, representing α-helical (H), coiled-coil (C), and extended β-sheet (E) structures; grey highlights denote regions of predicted α-helical structures; red highlights denote the pair of Trp residues required by p19 to form the dsRNA binding bracket.
The comparison of the location of basic regions as well as the secondary structures of the representative dsRNA binding regions and the N-terminus of CPK19 further highlights the similarities between these regions (Table 7.3). Each of the dsRNA binding domains spans at least two α-helical structures, including at least one basic residue in each, and an extended β-sheet (albeit very short in the case of PKZ and B2). Likewise, the N-terminal of CPK19 contains two α-helices encompassing several basic residues, mainly Lys, in each and an extended β-sheet. These similarities of CPK to other dsRNA binding domains support the possible ability of the CPK19 N-terminal to bind nucleic acids, such as dsRNA, and thus the identification of CPK19 as the plant-functional homolog of PKR.

7.5 Comparison with Human Protein Kinase R

In addition to the comparison of CPK19 with other members of the CPK family and with the dsRNA binding regions of several proteins of interest, it was valuable to compare the CPK19 protein directly with mPKR. As mentioned above, the CPK19 protein sequence aligned with two regions of human PKR. Firstly, residues important for function were conserved between the kinase regions of the two proteins including a highly conserved region characteristic of eIF2α kinases (Fig. 7.2). Secondly, the EFh1 of CPK19 displayed sequence alignment with the dsRBM2 of human PKR (Fig. 7.4). By contrast, the CPK19 N-terminus that I postulate to act as a dsRNA binding region of CPK19, does not have high aa alignment with the dsRBMs of PKR. However, as discussed above, other recognised dsRNA binding domains, such as those listed in Table 7.2, also do not have protein sequences that align but instead, have similar secondary structures and aa compositions that distinguish them as dsRNA binding regions. Correspondingly, these similarities in secondary structures and aa composition are observed in the CPK19 N-terminal region.

Table 7.4 further demonstrates the similarities and differences in molecular properties that exist specifically between human PKR and the potential plant-functional homolog of PKR CPK19. In general, human PKR and CPK19 are very similar in their molecular properties. The two proteins comprise the same number of residues and are very similar in their predicted molecular size. Their overall composition of residues is also comparable, although PKR has slightly less Glu than CPK19. However, as the negatively charged residues Asp and Glu are required for the binding of calcium, it is reasonable that CPK19 has a higher constitution of these residues than PKR. The overall pI of human PKR is more basic than CPK19, but a comparison of the dsRBMs or basic aa region of PKR with the N-terminal region of CPK19, as well as a comparison of their
corresponding kinase regions, revealed similar pIs between the two proteins in these specific regions. The CaM-LD of CPK19 has a very acidic pI (4.10), analogous to the pI of calmodulin which is calculated to be 3.88.

Table 7.4 Comparison of the molecular properties between human PKR and CPK19

<table>
<thead>
<tr>
<th>Properties</th>
<th>Human PKR</th>
<th>CPK19</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA length</td>
<td>2696 bp</td>
<td>1661 bp</td>
</tr>
<tr>
<td>Molecular size</td>
<td>62.1 kDa / 551 aa</td>
<td>62.9 kDa / 551 aa</td>
</tr>
<tr>
<td>Amino acids compositions</td>
<td>Arg: 4.72% Lys: 10.71% His: 1.81% Asp: 6.35% Glu: 7.99%</td>
<td>Arg: 5.08% Lys: 9.8% His: 1.81% Asp: 5.81% Glu: 9.44%</td>
</tr>
<tr>
<td>Motifs (aa position)</td>
<td>dsRBM1 (aa 10-78) dsRBM2 (aa 101-168) Basic aa region (aa 232-261) Ser/Thr Kinase (aa 266-534)</td>
<td>N-terminal region (aa 1-96) Ser/Thr Kinase (aa 97-357) Autoinhibitory region (aa 358-404) CaM-LD (aa 405-537)</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>Overall: 8.63</td>
<td>Overall: 7.03</td>
</tr>
<tr>
<td></td>
<td>dsRBM1: 9.03 dsRBM2: 9.74 Basic aa region: 10.98</td>
<td>N-terminal region: 10.59</td>
</tr>
<tr>
<td></td>
<td>Ser/Thr kinase: 7.54</td>
<td>Ser/Thr kinase: 7.10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Autoinhibitory region: 10.57 CaM-LD: 4.10</td>
</tr>
<tr>
<td>Number of predicted phosphorylation sites</td>
<td>Ser: 32 Thr: 12 Tyr: 7</td>
<td>Ser: 14 Thr: 4 Tyr: 5</td>
</tr>
</tbody>
</table>

Human PKR, Homo sapiens protein kinase R; CPK19, Arabidopsis thaliana calcium-dependent protein kinase 19; dsRBM, dsRNA binding motif; CaM-LD, calmodulin-like domain; protein statistics calculated by SMS

Even though the two proteins are similar in molecular size, the mRNA length of human PKR (2696 bp) and CPK19 (1661 bp), as stated by the National Centre for Biotechnology Information (NCBI), are rather different (Table 7.4). Upon investigation, the annotated mRNA of CPK19 was found to begin directly at the starting codon of the gene without any 5’ UTR, and the mRNA only contain a short 3’ UTR of 4 bp. To date, only one mRNA has been cloned for the CPK19 gene, thus it is unconfirmed whether the 1661 bp mRNA represents the full length transcript of CPK19. As mentioned in Section 1.3.1.4, a 2500 bp transcript is detected by northern blot analyses using mouse or human PKR cDNA as probes against tobacco leaf and wheat germ tissues and this transcript is suggested to encompass the mRNA of pPKR (Langland et al., 1995). Additionally, a potential carrot homolog of Arabidopsis CPK19, Daucus carota PK431, has been identified via sequence homology between 47 nt of the variable N-terminal region of both genes, and the mRNA
of this carrot CPK is predicted to be 2000 – 2200 bp (Suen & Choi, 1991). These observations suggest that the full length transcript of CPK19 may encompass more than 1661 bp. It is also interesting to note that although sequence comparison using BLAST failed to identify any sequence homology between the human PKR and CPK19 transcripts, an alignment of the two mRNA sequences using Geneious revealed pairwise identity of 50.5%.

Another feature that distinguishes the human PKR and CPK19 proteins is the number of predicted phosphorylation sites (Table 7.4). Overall, PKR has a much higher number of phosphorylated Ser and Thr than CPK19 (32 Ser and 12 Thr for human PKR compared to 14 Ser and 4 Thr for CPK19). This may suggest a higher reliance of human PKR on phosphorylation for activation while CPK19 may be activated by both phosphorylation and calcium binding. Of note, the Ser residue that substitutes Asp in EFh1, is predicted to be phosphorylated and may mimic the acidic residue required to bind calcium. Additionally, a Thr residue located in human PKR (Thr487) that is critical for eIF2α binding (Dey et al., 2005) is conserved in CPK19 at position 303 (illustrated in Fig. 7.1, blue dot). Another Thr residue (Thr466) that is functionally relevant for the autophosphorylation activation and substrate recognition of human PKR (Romano et al., 1998; Dey et al., 2005) is not conserved in CPK19, but a corresponding Tyr residue at position 257, that is also predicted to be phosphorylated, is present instead (illustrated in Fig. 7.1, black dot).

7.6 Conclusions

Overall, the CPK19 protein exhibits similarity (52%) to the other members of the CPK family, particularly at the Ser/Thr kinase domain (62% similarity) and CaM-LD (50% similarity). However, within the CPK family, only the N-termini of CPK19 and CPK25 are longer than 65 aa (the minimal length of a dsRNA binding domain as shown in Table 7.2), consist of more than 20% basic residues, and have a pI greater than 8. Moreover, only CPK19 comprises the α-helices and extended β-sheet common to the dsRNA binding domains. Thus, the CPK19 N-terminus has basic properties and secondary structures that are similar to other known dsRNA binding domains and comprises a possible dsRNA binding region. The potential of a novel dsRNA binding domain in pPKR is not improbable as PKZ, the fish homolog of PKR, also utilises a dsRNA binding domain which differs from the dsRBM that is typically located in mammalian and chicken PKR proteins (Rothenburg et al., 2005). It is possible that CPK19 may represent a PKR protein that has co-evolved as an antiviral protein and utilises a distinct dsRNA binding domain for the same or similar function. The comparison of CPK19 with human PKR also reveals similar molecular
properties of the two proteins, particularly in their molecular size, aa composition, and predicted pl. These findings provide *in silico* support for CPK19 as the candidate protein of the plant-functional homolog of PKR.
8.1 General Conclusions and Discussion

Plants are challenged by a variety of viruses and a number of defense systems have evolved in these potential host organisms that protect them against virus infection or reduce the severity of the infection. Innate antiviral defense systems, such as the $R$ gene-activated hypersensitive response, systemic acquired resistance, and PTGS, have been recognised in plants as mechanisms which prevent or reduce the virus infection from propagating throughout the organism (Hull, 2002; Lindbo & Dougherty, 1992). The hypothesis of this doctoral research is that there is another antiviral defense mechanism that also exists in plants; the mechanism of translational inhibition. Translational inhibition is utilised by, and most studied in, mammals and relies on the phosphorylation of the translation initiation factor eIF2$\alpha$ by the dsRNA activated protein kinase PKR. Phosphorylation of eIF2$\alpha$ results in the global inhibition of translation initiation in the host cell and subsequently halts the invasion of the virus. Candidates for the plant-functional homolog of PKR have been sought as the first step in testing the hypothesis of a novel plant antiviral defense mechanism involving translation inhibition by pPKR.
In the past 15 years, there has been mounting evidence that a functional homolog of PKR is present in plants (Langland et al., 1995; Langland et al., 1996). This doctoral research aimed at identifying pPKR via a proteomics approach using *Arabidopsis*, whereby candidate proteins are first purified, as determined by the correlation of its activity, and then identified by peptide sequencing and comparison to the entire *Arabidopsis* genome sequence.

### 8.1.1 Detection of eIF2α Kinase Activity in Plants

The immobilised eIF2α peptide assay was initially developed with my assistance by another student, Ms Sushma Prakash (2006), to allow the rapid detection and quantification of eIF2α kinase phosphorylation activity *in vitro*. As part of my doctoral research, the assay was refined and validated for reliable detection of eIF2α phosphorylation activity. The refined immobilised eIF2α peptide assay was then utilised in combination with immunoblotting and phosphorimaging to detect the *Arabidopsis* tissue with the highest level of eIF2α phosphorylation activity and to detect the presence of putative pPKR protein through multiple purification approaches. Flower buds from young *Arabidopsis* plants following at least four hr of light exposure were observed to have the highest eIF2α phosphorylation activity and these were subsequently used for pPKR enrichment and characterisation.

Although it has been demonstrated that the immobilised eIF2α peptide assay reliably detects for phosphorylation activity that is kinase-dependent, dsRNA-dependent, and PKR-related, it is important to also validate that the detected kinase activity is pPKR-derived and not of GCN2. The activity of these two plant eIF2α kinases can be differentiated via the use of transgenic *Arabidopsis* lines which have a disruption in plant GCN2, or overexpression lines which overexpress inhibitors of PKR, such as p58IPK, E3L, and K3L. P58IPK blocks the dimerisation and activation of PKR (Tan et al., 1998) and has not been demonstrated to affect GCN2 activity. E3L sequesters dsRNAs to prevent PKR activation (Davies et al., 1993) and is also not known to inhibit the activation of GCN2. K3L, on the other hand, is able to affect the activity of both pPKR and GCN2 as it acts as their pseudosubstrate and inhibits the phosphorylation ability of eIF2α kinases.

In this doctoral research, the p58IPK overexpression line was generated and assessed and the anticipated decrease in eIF2α phosphorylation activity was observed (as described in Section 6.3.3). This indicates a change to phosphorylation activity due to an inhibition of the putative pPKR and not GCN2. The generation of the remaining transgenic lines, especially GCN2 mutant
lines, will provide further evidence for the detection of specific pPKR activity using the immobilised eIF2α peptide assay (discussed in Section 8.2.1).

### 8.1.2 Partial Purification of Potential Candidates of Plant Protein Kinase R

Various techniques including the dsRNA agarose binding assay, immunoprecipitation, KinaseBind™ assay, size exclusion chromatography, and ion exchange chromatography were evaluated to partially purify pPKR from *Arabidopsis* flowers. Overall, the presence of eIF2α phosphorylation activity was detected most consistently in protein fractions separated by size exclusion chromatography. Enriched proteins of interest which correlated with the presence of eIF2α phosphorylation activity were subsequently sequenced using MS and compared to the *Arabidopsis* genome. A number of RNA binding proteins and protein kinases were revealed which posed as potential pPKR candidates. Most notably, seven calcium-dependent protein kinases (CPK4, CPK6, CPK20, CPK21, CPK27, CPK29, and CPK33) were identified. However, most of the proteins identified in the MS analysis were represented only by the presence of a single peptide hit and identical proteins were rarely detected between analyses using the same or different partially purified extracts, even though similar sized bands were selected for protein identification. Interestingly, the potential pPKR candidate CPK19 was not identified by the MS analysis. A number of reasons may explain the inability of the MS analysis to repeatedly distinguish a protein with high significance, particularly in identifying CPK19 as a potential pPKR candidate:

**a. Low abundance of the putative pPKR protein**

It is possible that the pPKR protein, as with mPKR (Jeffrey et al., 1995), is present at low abundance and is masked by the presence of other higher abundance proteins. With the notion that CPK19 may be pPKR, this rationale of low abundance is supported by the fact that the CPK19 transcript has only been cloned once (as stated in The Arabidopsis Information Resource (TAIR)). A transcript expression pattern study in *Arabidopsis* also support a low abundance of CPK19 as the level of CPK19 expression was undetectable when surveyed in roots, shoots, and pollen (Harper, Breton, & Harmon, 2004). Furthermore, the Affymetrix *Arabidopsis* microarray RNA profiling data (Genevestigator; Hruz, Natora, & Agrawal, 2008) reveals that the transcript of CPK19 was detected only when more than 22 000 probe sets (ATH1 GeneChip) were used, and not when only 8000 probe sets of highly expressed genes (AG GeneChip), were used. While 7388 *Arabidopsis* transcripts are targeted by probe sets from both of these microarrays, the ATH1 GeneChip is more representative and covers 16 216 transcripts more than the AG GeneChip,
Figure 8.1 RNA profiling of CPK19 as detected by the Affymetrix *Arabidopsis* ATH1 microarray. The presence of the CPK19 transcript in various tissues of *Arabidopsis*: A, germinated seed; B, seedling; C, young rosette; D, developed rosette; E, bolting stem; F, unopened flower bud; G, flower bud from young plants; H, flower bud and developing siliques from adult plants; I, matured siliques from old plants. The Y-axis indicates the signal intensity as measured by the microarray. This value is normalised to a target signal value of 1000 and assumes an approximately constant total abundance of mRNA. Blue box denotes region of plant tissues which were assessed for their eIF2α phosphorylation activity in this doctoral research. (Modified from Genevestigator; Hruz et al., 2008)

including poorly expressed genes (Hennig, Menges, Murray, & Gruissem, 2003). On the whole, the detected level of CPK19 transcript was relatively low across a range of *Arabidopsis* tissues (signal intensity of 22 – 42 as illustrated in Fig. 8.1) compared to the transcript of another CPK such as CPK1 (signal intensity of 500 – 950) (Genevestigator; Hruz et al., 2008). However, this RNA profile of CPK19 is consistent with the presence of eIF2α phosphorylation activity detected in this doctoral research (Section 4.2.2). High eIF2α kinase activity was detected in the flower buds of young *Arabidopsis* plants compared to flowers, leaves, siliques, and stem tissues that were taken from older plants. Similarly, a higher level of CPK19 transcript is detected in the flower buds of young plants than in the flowers and developing siliques of older plants (Fig. 8.1, blue box). However, additional plant developmental stages were also tested by the Affymetrix ATH1 array, and even higher levels of the CPK19 transcript were observed in the germinated seed,
seedling, as well as mature siliques, as compared to flowers. This suggests that eIF2α phosphorylation activity may also be higher in these tissues and it is possible that an enhanced enrichment of putative pPKR may be achieved in these tissues. In comparison, the transcript of *Arabidopsis* GCN2 is also abundant in seedlings and matured siliques and a high level of GCN2 transcript is detected in unopened buds and buds from young plants (Fig. 1.5). This similarity in RNA profiles between GCN2 and CPK19 again suggests the need to utilise transgenic plant lines disrupted for the GCN2 gene to determine whether the high eIF2α kinase activity detected in the flower buds of *Arabidopsis* is derived from activity of the putative pPKR. Nevertheless, the observations of low CPK19 transcript levels corroborate the suggestion that the putative pPKR protein may be at low abundance and therefore its detection may be masked by the presence of higher abundance proteins.

**b. Detected phosphorylation activity may not be correlated with protein abundance**

It is possible that the presence of eIF2α phosphorylation activity may not directly correlate with the amount of pPKR protein that is present, especially since experiments on the various *Arabidopsis* tissues (Section 4.2.2) were performed without the addition of exogenous dsRNA and therefore assayed for the presence of activated eIF2α kinases. Specific tissues, developmental stages, or treatments of *Arabidopsis* may have higher concentrations of pPKR protein than those with high putative pPKR activity, i.e. activated pPKR. In mammals, the translation of PKR is induced by IFN that is produced initially in viral-infected cells (Balachandran & Barber, 2007). Similarly, an increase in pPKR may occur after initial virus challenge or virus infection of plant cells. Identification of a source of pPKR that has greater abundance than in flower buds would assist the purification and identification of the putative pPKR protein using MS analysis.

**c. Inability to separate putative pPKR protein into a single protein band**

The various purification approaches used to enrich for putative pPKR were unable to optimally separate the pPKR protein to a single protein band on a gel. On the other hand, if only one band had been achieved following enrichment, it may still comprise of several co-migrating proteins. Co-migration of proteins following separation by SDS PAGE may essentially mask the visualisation of pPKR. A more enriched protein fraction with high specific activity is required to reduce this possibility and to increase the likelihood of obtaining high quality MS sequencing data.

The inability of the MS analysis to identify the putative pPKR candidate CPK19 as well as other potential candidates of pPKR with high significance clearly highlights the need to improve the
enrichment techniques to achieve a higher yield of putative pPKR protein. A number of approaches can be trialled to enhance the enrichment of putative pPKR and these will be discussed in Section 8.2.2. It is also likely that the pPKR protein may be present in low levels or may require a particular stimulus to increase its expression. Hence, it will be important to assess the level of eIF2α phosphorylation in additional tissues or in response to different treatments to further enhance the enrichment of putative pPKR protein.

8.1.3 Evidence for the Calcium-dependent Protein Kinase CPK19 as the Putative Plant-functional Homolog of Protein Kinase R

CPK19 was not one of the seven CPKs that were identified in the MS analysis of enriched pPKR fractions, but several lines of evidence support the potential of CPK19 as the putative plant-functional homolog of PKR. Firstly, the phosphorylation of eIF2α was observed to increase in the presence of calcium. Interestingly, this kinase activity also increased when the specific chelator of calcium, EGTA, was present. It is as yet unknown why both calcium and EGTA are required to be present to enhance eIF2α kinase activity the most, but these findings support the influence of calcium on eIF2α phosphorylation in plants. Secondly, a statistically significant decrease in eIF2α kinase activity was detected in the T-DNA insertion line of CPK19. This change in eIF2α phosphorylation was not detected in the 18 other T-DNA insertion lines that were generated (comprised of ten dsRBM-containing genes, seven other CPK-containing genes, and one other protein kinase gene). Additionally, as mentioned above, the transgenic line overexpressing the PKR inhibitor p58IPK had a similar reduction in eIF2α phosphorylation activity. A comparable phenotypic change was also observed in the CPK19 T-DNA insertion line, the p58IPK overexpression line as well as in the line overexpressing E3L. These findings reinforce the contention that the detected changes in eIF2α phosphorylation are putative pPKR activity rather than the activity of other eIF2α kinases such as GCN2, and suggest a role for CPK19 in the regulatory pathway of pPKR. Finally, in silico analysis of the protein sequence and structure of CPK19 identify motifs and structures in CPK19 that may have the ability to function in PKR-like activities (as summarised in Figure 7.1). The analysis of the Ser/Thr kinase domain of CPK19 reveals an eIF2α kinase-like motif as well as the presence of two residues that correspond to critical residues in human PKR that is essential for substrate recognition and eIF2α interaction. Interestingly, two negatively charged residues located in the CaM-LD of CPK19, which is important for calcium binding, are substituted with a Ser residue. A phosphorylated Ser residue is known to mimic a negatively charged state which may then permit calcium binding. Hence, it is
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postulated that the binding of calcium by EFh1 and EFh3 of CPK19 may be influenced by phosphorylation, perhaps autophosphorylation. Sequences similar to dsRBM2 of human PKR are also identified in the first EF hand of CPK19. Additionally, the N-terminus of CPK19 displays secondary structure that is similar to that recognised in other known dsRNA binding domains, suggesting a possible dsRNA binding role for this region. It is not surprising that pPKR may utilise a nucleic acid binding domain that differs to the dsRBM identified in mPKR. PKZ, the fish homolog of PKR, is known to contain a nucleic acid binding domain which specifically recognises Z-DNA forming sequences. As many fish viruses form Z-DNA intermediates, it is suggested that a motif specific for this type of nucleic acid would serve as a better detection of virus infection than the use of a dsRBM such as that in mPKR (Rothenburg et al., 2005). Similarly, pPKR may also use a distinct nucleic acid binding domain, such as the postulated dsRNA binding domain located in the N-terminus of CPK19, to detect specific dsRNA forms that arise during the infection of viruses in plants.

8.1.4 Are eIF2α Phosphorylation and CPK19 Part of a Novel Antiviral Defense Mechanism in Plants?

To date, the postulated role of eIF2α phosphorylation in response to viral infection in plants is still subject to debate. Similarly, the possible association of the detected eIF2α phosphorylation activity by the putative pPKR candidate, CPK19, with antiviral defense in plants is also unknown.

It was previously established that virus-infected plants were unsuitable for the initial enrichment of pPKR in conjunction with the detection of eIF2α phosphorylation activity as two plant viruses are known to recruit the cellular PKR inhibitor p58IpK, presumably to inhibit eIF2α phosphorylation, and thus support their replication and successful infection (Bilgin et al., 2003). The activation of this plant inhibitor would potentially conceal the detection of pPKR activity and possible changes to eIF2α phosphorylation in response to viral infection. However, with a number of tools in hand such as the pPKR candidate CPK19 gene, the validated immobilised eIF2α peptide assay which detects for eIF2α phosphorylation activity, and the generation of the transgenic Arabidopsis line which is disrupted for the p58IpK gene, the response of CPK19 and eIF2α phosphorylation to the infection of viruses in plants can be examined.

In the following sections, the observed and suggested association of eIF2α phosphorylation in plant defense against viral infection is reviewed (Section 8.1.4.1). The possible relationship
between eIF2α phosphorylation, CPK19, and virus infection is then discussed, as well as approaches which may determine the connection between them (Section 8.1.4.2).

8.1.4.1 The Involvement of eIF2α Phosphorylation in Plant Antiviral Defense

An increase in eIF2α phosphorylation was identified by J. Hu & Roth (1991) in tobacco protoplasts 12 hr after synchronised TMV infection. Bilgin et al. (2003) also observed an increase in phosphorylated eIF2α in transgenic Arabidopsis plants in response to TMV infection. On the other hand, a study by Y. Zhang et al. (2008) failed to detect eIF2α phosphorylation in Arabidopsis when infected with Turnip yellow mosaic virus (TYMV) or Turnip crinkle virus (TCV). It is suggested that the contrasting findings of these studies may have resulted from the different experimental conditions and analyses performed in each study in response to virus infection.

In the study by Bilgin et al. (2003), p58IPK-silenced plants were used that presumably could no longer inhibit kinase activity through the recruitment of p58IPK by virus-encoded proteins (such as the p50 portion of TMV replicase or the cytoplasmic inclusion protein of Tobacco etch virus). Bilgin et al. (2003) observed that eIF2α phosphorylation was undetected in infected plants which were not silenced for the p58IPK gene. This is possibly because a short-lived increase in kinase activity, as observed in newly TMV-infected cells by J. Hu & Roth (1991), lasts only a number of hr and is then inhibited once virus-encoded proteins recruit the plant-encoded inhibition mechanism. This may also explain the lack of eIF2α phosphorylation following systemic TYMV and TCV infection of Arabidopsis (Y. Zhang et al., 2008). Plant viruses may act in the same manner as animal viruses by using methods to counteract PKR activation or eIF2α phosphorylation to facilitate their replication and infection (reviewed in Jacobs & Langland, 1996).

The timepoints at which samples were taken for the detection of phosphorylated eIF2α in response to viral infection were not mentioned in the study by Y. Zhang et al. (2008), but Bilgin et al. (2003) reported that strong eIF2α phosphorylation was not observed in leaf tissue until at least eight to 12 days after infection. In leaf tissue, synchronised virus-induced detection of phosphorylated eIF2α cannot be limited to a single timepoint as is performed for protoplasts (J. Hu & Roth, 1991). Perhaps a critical number of newly infected cells are required to be infected before the increase of phosphorylation eIF2α could be detected by the immuno-detection method used by Bilgin et al. (2003). Once TMV establishes its infection in the cell, it spreads its infection intercellularly by the transport of its viral genome with the aid of a single virus-encoded movement protein, p30 (Deom,
Oliver, & Beachy, 1987; Kawakami et al., 1999). The ensuing replication of viral RNA that occurs in the subsequent cells potentially activates pPKR and it is postulated that a wave of phosphorylated eIF2α would be detected at different timepoints in the successive adjacent cells away from the infection centre. Further studies, particularly those that could detect the real time activation of pPKR and/or eIF2α phosphorylation, will be required to pinpoint the significance of pPKR and eIF2α phosphorylation in the defense of plants against viral infection.

8.1.4.2 The Potential Relationship Between eIF2α Phosphorylation, CPK19, and a Novel Antiviral Defense Mechanism in Plants

The transgenic p58IPK T-DNA insertion line provides an effective means to assess the potential changes to eIF2α phosphorylation that may occur in response to viral infection, without the influence of a functional cellular PKR inhibitor. In turn, the potential relationship that may exist between eIF2α phosphorylation and pPKR together with a novel plant defense against virus infection may be explored.

Currently, five viruses already established in New Zealand (Pearson, Clover, Guy, Fletcher, & Beever, 2006) have been successfully infected into Arabidopsis (S. Lilly, personal communication). These include TMV, TuMV, TYMV, Tomato spotted wilt virus, and Cauliflower mosaic virus. At least two of these viruses, TMV and TuMV, are known to recruit p58IPK for virulence (Bilgin et al., 2003). On infection by any of the five viruses into transgenic Arabidopsis containing a T-DNA insert in the p58IPK gene, an increase in eIF2α phosphorylation would be anticipated. Conversely, a comparable loss of eIF2α phosphorylation would be expected in virus infected transgenic lines that carry a T-DNA insert in the putative pPKR candidate, CPK19 gene.

It is notable that human GCN2 has been shown to be activated by viral RNA (Berlanga et al., 2006). However, this activation is limited to a specific secondary structure within the 5’ terminus of Sindbis virus and is not a sequence-independent activation by dsRNA as is observed for mPKR. To date, an activation of Arabidopsis GCN2 by virus infection has not been demonstrated in plants. It will be important to assess and compare the effect of virus infection in specific transgenic plant lines to reveal and differentiate between potential virus-induced GCN2-activated and/or CPK19-activated eIF2α phosphorylation. The effect of virus infection on eIF2α phosphorylation can first be examined in plant lines that have a disruption in CPK19, GCN2, or p58IPK. Observations from these lines can then be compared with the level of eIF2α phosphorylation upon virus infection in
hybrid lines which have a disruption in both CPK19 and GCN2, or lines which have a disruption of either of these two potential eIF2α kinases plus a disrupted p58IPK. A further decrease in the eIF2α phosphorylation levels in these hybrid lines compared with the single gene disruptions will suggest the ability of both CPK19 and GCN2 in the phosphorylation of eIF2α in response to virus infection, and a potential interaction between these kinases with p58IPK. Finally, a transgenic line that harbours disruptions in CPK19, GCN2 and p58IPK may also be assessed. The observation of a total reduction of eIF2α phosphorylation in this triple mutant plant line upon virus infection will suggest CPK19 and GCN2 as the only eIF2α kinases in plants that are activated by the presence of viruses.

Animal viruses exploit many strategies to counteract the antiviral defense responses by translational inhibition (as described in Section 1.2.1.3.1). Likewise, in addition to recruiting the cellular inhibitor p58IPK (Bilgin et al., 2003), plant viruses may also encode for the synthesis of short RNAs and additional proteins to interfere with potential pPKR-induced eIF2α phosphorylation. Such potential functions for counteracting a proposed pPKR-mediated antiviral mechanism in plants are largely unknown. To determine the function of virulence factors of plant viruses that are proposed anti-pPKR proteins or RNAs, such as the cylindrical inclusion protein of TuMV (Jenner et al., 2000), they may be delivered as wildtype or mutated forms in either the context of the virus or overexpressed alone. While a wildtype form of such a virulence factor delivered in its natural context within a virus may support successful infection of the host plant, a mutated form may not be able to inhibit the pPKR pathway. Moreover, in the absence of such a pPKR inhibitor, the virus may activate pPKR kinase activity, hyper-eIF2α phosphorylation and initiate cell death defences as observed by Bilgin et al. (2003). Delivery of such virulence factors alone, either as wildtype or mutated forms, may further elucidate the function and required features of such proteins or RNAs.

Whether eIF2α phosphorylation has a role in plants against virus infection is not yet proven. An activation of eIF2α phosphorylation was observed in the studies by J. Hu & Roth (1991) and Bilgin et al. (2003) in response to the infection of TMV, while the study by J. Zhang et al. (2008) failed to detect this kinase phosphorylation. However, it is postulated that viral-activated phosphorylation may have been masked by virus counterdefenses. A predicted spread of eIF2α phosphorylation upon virus infection could be assessed using in situ hybridisation with antibodies that recognise total eIF2α protein or only the phosphorylated form of eIF2α. Inoculated tissues could be sampled at various timepoints after viral infection and the presence and localisation of
total and phosphorylated eIF2α protein can be determined. To avoid the potential influence of pPKR inhibitors, viruses lacking potential virulence factors may be generated to infect hybrid transgenic *Arabidopsis* lines that harbour a T-DNA insert in GCN2, CPK19 or p58IPK. Results from these studies will provide data for the postulated spread of eIF2α phosphorylation in relation to the cell-to-cell movement of the infecting virus. These findings will be important in determining the role of both eIF2α phosphorylation and the putative candidate plant-functional homolog PKR, CPK19, in response to virus infection.

### 8.2 Future Directions

Evidence from this doctoral research identifies CPK19 as the putative plant-functional homolog of PKR. However, as this kinase was not identified in the MS analysis of enriched pPKR fractions, additional experimental evidence would be valuable in determining the significance of CPK19 as the putative pPKR. Moreover, alternative approaches can be developed which may further improve the detection of eIF2α phosphorylation activity as well as enhance the enrichment of the putative pPKR from plants. These approaches as well as experiments which may define the CPK19 as the putative pPKR are described in Sections 8.2.1 to 8.2.4:

a. Further validation for the detection of putative pPKR activity and alternative approaches to quantify eIF2α phosphorylation activity (Section 8.2.1)

b. Improvements to purification techniques for the enrichment of putative pPKR protein (Section 8.2.2)

c. *In vitro* analysis of CPK19 via expression of the CPK19 protein (Section 8.2.3)

d. *In planta* analysis of CPK19 localisation and possible redundancy in function (Section 8.2.4)

Additionally, it is postulated that eIF2α kinases may have a role in the regulation of preferentially translated mRNAs via upstream ORFs. Future approaches to examine the possible role of the eIF2α kinase pPKR in this regulation mechanism are discussed in Section 8.2.5.

### 8.2.1 Validation and Alternative Approaches for the Detection of eIF2α Kinase Activity

It has been demonstrated in this doctoral research that the immobilised eIF2α peptide assay reliably detects the phosphorylation activity of eIF2α kinases. Additionally, the assay has been
validated to detect for putative pPKR activity and aids in determining the presence of putative pPKR in enriched pPKR fractions via correlation of protein abundance with detected eIF2α phosphorylation activity. It is possible to further validate this assay for the detection of putative pPKR activity using transgenic *Arabidopsis* lines that have a disruption in or overexpress plant GCN2 or inhibitors of PKR such as p58IPK, E3L, and K3L. Table 8.1 lists the various plant lines that can be assessed in future studies and the changes in kinase activity that would be anticipated in these lines as compared to wildtype *Arabidopsis*. If indeed the anticipated findings are detected, these findings would provide further validation for the immobilised eIF2α peptide assay as a rapid detection tool for the activity of eIF2α kinase. The assessment of these transgenic plant lines will also be valuable in distinguishing between eIF2α phosphorylation activity that is potentially activated by GCN2 or the putative pPKR.

**Table 8.1** List of transgenic *Arabidopsis thaliana* lines which could be assessed to further validate the detection of putative pPKR phosphorylation activity by the immobilised eIF2α peptide assay

<table>
<thead>
<tr>
<th>Transgenic <em>Arabidopsis</em> lines</th>
<th>Anticipated change in detected eIF2α phosphorylation activity as compared to wildtype <em>Arabidopsis</em></th>
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<tbody>
<tr>
<td><strong>T-DNA insertion lines</strong></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em> GCN2</td>
<td>Decrease or no change</td>
</tr>
<tr>
<td><em>Arabidopsis</em> p58IPK</td>
<td>Increase</td>
</tr>
<tr>
<td><strong>Overexpression lines</strong></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em> GCN2</td>
<td>Increase (but not activated by or purified on dsRNA)</td>
</tr>
<tr>
<td>PKR inhibitors, e.g., p58IPK, E3L, and K3L</td>
<td>Decrease (decrease already observed in p58IPK overexpression line)</td>
</tr>
<tr>
<td><strong>Knockout lines (using RNA interference)</strong></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em> GCN2</td>
<td>Decrease or no change</td>
</tr>
<tr>
<td><em>Arabidopsis</em> p58IPK</td>
<td>Increase</td>
</tr>
<tr>
<td><strong>Genetrap line (generated by Cold Spring Harbour Laboratory)</strong></td>
<td></td>
</tr>
<tr>
<td><em>Arabidopsis</em> GCN2 null mutant GT8359</td>
<td>Decrease or no change</td>
</tr>
</tbody>
</table>

The practicality of the immobilised eIF2α peptide assay can also be improved by substituting the use of radioactivity with fluorescent markers (Xiaoming et al., 2008). This would reduce the risks and limitations of radioactivity and achieve a safer and more convenient environment for undertaking this detection assay.

Alternatively, the eIF2α phosphorylation activity of pPKR can be detected using enzyme-linked immunosorbent assay (ELISA). This diagnostic tool allows the detection of the antigen of interest
in a protein sample and relies on the use of an antibody which has specificity for that particular antigen. For *in vitro* quantification of eIF2α phosphorylation activity in a protein sample, extracts from *Arabidopsis* can be incubated with ATP to allow for kinase activity over a given time and be assessed for the amount of eIF2α phosphorylation by comparing ELISA readings with an antibody that recognises the phosphorylated form of eIF2α and an antibody that recognises total eIF2α, i.e., both phosphorylated and unphosphorylated states of eIF2α. The eIF2α phosphorylated by pPKR, or other eIF2α kinases, would thus be quantified relative to total eIF2α protein abundance. In addition, these antibodies would be valuable in determining the *in vivo* activity of pPKR, or other eIF2α kinases, by distinguishing between the amounts of modified and unmodified forms of eIF2α in plants, such as in different tissues or in the presence of various virus infections.

The development of an ELISA system to detect eIF2α phosphorylation is advantageous in its power to allow a higher throughput of samples as well as the ability to compare between experiments. To date, the number of samples that can be assessed per immobilised eIF2α peptide assay is limited to 22 samples due to the number of tubes that can be placed onto the Magnetic Particle Concentrator and the time needed to process each sample. On the other hand, ELISA can be performed with a 96-well plate, which effectively augments the number of samples that can be assessed per experiment. Moreover, when using the immobilised eIF2α peptide assay, results from different assays may only be indirectly compared with each other according to the trends observed in each experiment due to the short half life of the radioactive phosphate. By contrast, ELISA standards can be included for each antibody to allow for the comparison of results between different experiments.

### 8.2.2 Approaches to Improve the Enrichment of Putative Plant Protein Kinase R

A number of different purification techniques were undertaken in this doctoral research to enrich for the putative pPKR protein. It was recognised, though, that partially purified proteins were unable to be optimally separated into a single protein band on a gel and the enriched fractions exist mainly as a combined mix of proteins. A more purified protein fraction would thus be necessary to obtain better quality MS sequencing data that may reveal potential putative pPKR candidates with higher significance for their peptide identification.

A plausible approach to achieve a higher yield and a more enriched fraction of pPKR protein is by combining the existing protein purification protocols. As compared on Figure 4.31, a small
number of similar sized bands were common in the phosphorylation profiles of proteins partially purified and separated using dsRNA agarose, size exclusion chromatography, and ion exchange chromatography. Perhaps by performing these purification techniques in succession, a more highly enriched fraction of pPKR protein may be achieved for MS analysis. An initial attempt to combine the dsRNA agarose binding assay and size exclusion chromatography has failed to attain a better enrichment of pPKR specific activity (Section 4.7). However, in retrospect, aspects of the protocol, such as the use of a combined pool instead of individual size exclusion chromatography-separated protein fractions, could have improved the purification technique as a higher amount of proteins enriched from the first purification step would be available for the subsequent dsRNA agarose binding approach and a higher level of the putative pPKR protein may possibly be present.

The supplementation of plant extracts with high concentrations of zinc may also achieve an increased enrichment and yield of the putative pPKR protein. As observed in Figure 5.7, the presence of 11 mM zinc resulted in the loss of a large number of higher molecular proteins while achieving an increase in specific eIF2α kinase activity (Fig. 5.6). The addition of zinc to plant extracts and the resulting precipitation of proteins may serve as an effective first step in removing unwanted proteins while enriching for putative pPKR protein. This may then provide a more enriched fraction for performing subsequent pPKR partial purification approaches.

There are additional tools available which could be utilised to partially purify the putative pPKR protein from Arabidopsis. As with the previous approaches, these tools may take advantage of the known or postulated characteristics of pPKR. A predicted feature of pPKR which remains to be exploited is its autophosphorylation activity. Differing from the KinaseBind™ assay which affinity purifies for kinases, there are systems which enrich for phosphoproteins, i.e., proteins that are phosphorylated. An example of this is the immobilised metal affinity chromatography which utilises a metal sepharose medium to partially purify for phosphorylated proteins. While the nonphosphorylated proteins and other contaminants are eluted into the flow-through, phosphorylated proteins bind specifically to the metal on the column and are thus affinity purified. This enrichment tool has been successful in purifying from Arabidopsis phosphopeptides with putative roles in RNA metabolism (de la Fuente van Bentem et al., 2006).

The yeast two-hybrid system may also be useful to enrich for the putative pPKR protein via its interaction with protein inhibitors or its substrates. Presently, it is known that the plant-functional homolog of the mammalian PKR inhibitor, p58IPK, has been trialled as bait for pPKR purification,
albeit without success (S. P. Dinesh-Kumar, personal communication); the identification of pPKR prey has not been published. A substrate that has tight affinity for PKR and thus may be more suitable than other substrates as baits for pPKR is the Vaccinia virus-encoded K3L (Carroll, Elroy-Stein, Moss, & Jagus, 1993). As mentioned in Section 1.2.1.3.1.3, this PKR inhibitor displays homology to the phosphorylation site of eIF2α and acts as a pseudosubstrate of PKR. The K3L protein binds tightly to the catalytic cleft of PKR, with binding affinity higher than eIF2α, and remains stably bound to PKR. Hence, K3L would theoretically be a better substrate for capturing and purifying for pPKR than phosphorylatable kinase substrates such as eIF2α or histones.

8.2.3 In Vitro Expression of the Calcium-dependent Protein Kinase CPK19

An effective approach to determine the potential of CPK19 as a putative pPKR candidate will be to express the protein kinase in a heterologous biological system and examine for characteristics which have been observed or are postulated to exist in pPKR, for instance, the ability to bind dsRNA, to dimerise, to autophosphorylate, and to phosphorylate known PKR substrates, such as eIF2α. The detection of these characteristics in the expressed CPK19 protein will further distinguish this kinase as the potential plant-function homolog of PKR.

An essential first step to expressing CPK19 will be to identify a suitable heterologous biological system which provides reliable and effective protein expression in the absence of a wildtype plant or other PKR protein originating from the overexpressing organism. This is essential for distinguishing the activity of the endogenous PKR from the recombinant PKR protein. Additionally, expressed putative pPKR may potentially be activated by dsRNA present in the expression system. This would result in translational inhibition due to the phosphorylation of eIF2α by the putative pPKR and may pose as lethal for the expression system, such as that observed in the yeast system (Ung, Cao, Lu, Ozato, & Dever, 2001). Hence, it will be valuable to use an expression system that does not phosphorylate the expressed protein, such as a prokaryotic system or a eukaryotic expression system in the presence of a co-expressed protein phosphatase. An alternative option will be to use an expression system which overexpresses an eIF2α carrying a nonphosphorylatable Ala in place of the Ser51phosphorylation site. The toxic effects of expressed PKR were demonstrated to be alleviated in strains of yeast which overexpressed this mutated eIF2α protein (Ung et al., 2001). The expression of an unphosphorylated CPK19 would be important in understanding the activation and phosphorylation activity of this putative pPKR protein. On the other hand, the expression of the phosphorylated form of CPK19 would also be
useful in determining possible toxicity effects of this protein when activated. It is noted that a phosphorylated form of PKR has been difficult to overexpress (Xu, Wang, Lee, & Williams, 2004).

The IMPACT™-CN System, which expresses the gene of interest in *E. coli* and allows the protein to be purified by its intein tag in a single chromatographic step, has thus far been successfully used for the expression of human PKR by Conn (2003) and by myself (Section 3.3.4.1). The expressed PKR protein was shown to display the dsRNA binding, autophosphorylating, and trans-phosphorylating properties of PKR, indicating that a functional nonphosphorylated protein was expressed. This system therefore provides an ideal expression technique for producing large amounts of the CPK19 protein for *in vitro* analysis. Similarly, eukaryotic systems, such as the baculovirus (Murhammer, 1991) or yeast expression systems (Curak, Rohde, & Stagljar, 2009), can also be assessed for their ability to express a functional phosphorylated form of the CPK19 protein.

Once expressed and purified to homogeneity, the overexpressed CPK19 protein can be examined for PKR-like properties. The ability to bind dsRNA can be investigated using gel retardation assays, while autophosphorylation activity can be assessed with radiolabelled ATP and phosphorimaging, potentially using dsRNA as the activation molecule, and trans-phosphorylation activity can be quantified using the immobilised eIF2α peptide assay (or an adapted version of this assay as described in Section 8.2.1). On the other hand, the eIF2α protein can be cloned and expressed and the ability of CPK19 to phosphorylate eIF2α can be assessed using phosphorimaging or ELISA. Similarly, histone proteins, a known substrate of mPKR, can be expressed or obtained, and possible trans-phosphorylation by CPK19 can be detected using phosphorimaging. As observed by Syam Prakash & Jayabaskaran (2006), two CPK isoforms in chickpea have been shown to phosphorylate histone III. N-terminal truncated forms of the CPK19 protein can also be expressed to determine the likelihood of the N-terminal region as a dsRNA binding motif as well as the possibility of an autoinhibitory and dsRNA-activated role of the N-terminal region in the regulation of CPK19 activation. In mammals, the dsRBD of mPKR keeps the Ser/Thr kinase domain in its inactivated form until the binding of dsRNA to the dsRBD which results in a conformational change and activation of the kinase (Kaufman, 2002; S Nanduri et al., 2000). The effect of PKR inhibitors, such as p58IPK, E3L, and K3L, on CPK19 activity can also be assayed. The overexpression of p58IPK in *Arabidopsis* has already been shown to inhibit the phosphorylation of eIF2α (Section 6.3.3), hence if CPK19 is indeed pPKR, its activation and phosphorylation activity is anticipated to be inhibited either directly or indirectly by these PKR
inhibitors. A straightforward approach to assess the effects of these inhibitors will be to express and incubate these proteins with expressed CPK19, and evaluate for changes in the ability of CPK19 to phosphorylate eIF2\(\alpha\) using the immobilised eIF2\(\alpha\) peptide assay in the presence or absence of PKR inhibitors. Findings from these \textit{in vitro} studies will be crucial in determining whether CPK19 has properties similar to PKR and will also provide additional support for CPK19 as the potential plant-functional homolog of PKR.

8.2.4 \textit{In Planta} Analysis of Calcium-dependent Protein Kinase CPK19

The use of transgenic plant lines has proven effective during this doctoral research for revealing genes of interest which affect eIF2\(\alpha\) phosphorylation activity. The phosphorylation of eIF2\(\alpha\) was reduced by the disruption of the CPK19 gene, identifying this gene as a potential pPKR candidate or a protein involved in a regulatory pathway of pPKR. The overexpression of plant p58\textsuperscript{IPK} also decreased eIF2\(\alpha\) phosphorylation, verifying the inhibition effect of this protein on the activity of pPKR. However, a number of transgenic plant lines with altered gene expression (disruptions in or overexpression) of specific genes postulated to be involved in the pPKR regulatory pathway could still be generated to dissect the pPKR pathway in plants. By assessing the eIF2\(\alpha\) phosphorylation activity in these plant lines with altered gene expression, it may be possible to identify additional genes, other than CPK19, which may have a role in the pPKR pathway. The transgenic \textit{Arabidopsis} lines that are suggested to determine the possible pPKR involvement of CPK19 and/or other potential genes involved in the pPKR pathway are discussed in Section 8.2.4.1.

As previous results have demonstrated, the detection of eIF2\(\alpha\) phosphorylation is highest in the flower buds of young \textit{Arabidopsis} plants (Section 4.2.2). It will be of interest to determine if the localisation and accumulation of CPK19 corresponds to the localisation of detected kinase activity. The possible approaches that can be utilised to investigate the transcription and expression of CPK19 \textit{in planta} are discussed in Section 8.2.4.2.

8.2.4.1 Further Generation of Transgenic \textit{Arabidopsis} thaliana

Of the 21 transgenic plant lines that were generated and assessed, no difference in eIF2\(\alpha\) phosphorylation, as compared to wildtype \textit{Arabidopsis}, was detected in 19 of the plant lines. Directly interpreting these findings, it can be assumed that the affected genes are not involved in the pPKR pathway. However, it is also possible that the potential roles of these genes in pPKR
activity may have been masked by incomplete disruption or overexpression of the gene of interest and/or complementation by other functional eIF2α kinases.

A key experiment to determine the potential redundancy of pPKR activity by other genes will be to assess the level of eIF2α phosphorylation in transgenic plants that are disrupted for both the putative pPKR candidate CPK19 and GCN2. If eIF2α is no longer phosphorylated in these plant lines, redundancy in pPKR activity is unlikely to be present. This finding would therefore reveal whether there are only two eIF2α kinases, pPKR and GCN2, in Arabidopsis. On the other hand, if eIF2α phosphorylation is still detected in transgenic plants with disrupted CPK19 and GCN2, it is possible that other genes, perhaps other CPKs, may complement CPK19 or GCN2 in the eIF2α phosphorylation pathway.

Table 8.2 lists the specific transgenic lines that may be crossed to generate hybrid plant lines which contain disruptions or overexpression in two or more genes. Once generated, these transgenic lines can be assessed for the possible role of the altered gene in complementing eIF2α kinase activity in plants. The hybrid plant lines may also provide additional support for CPK19 as the putative plant-functional homolog of PKR.

Only eight out of the 34 possible CPK T-DNA insertion lines have so far been generated into plant lines homozygous for the T-DNA insert. The possible effects of the disruption on eIF2α phosphorylation activity for the remaining 26 CPK genes are yet unknown. Importantly, these 26 untested T-DNA insertion lines carry inserts within the encoded proteins of five (CPK4, CPK6, CPK21, CPK 29 and CPK33) of the seven CPKs which were identified by MS analysis of partially purified pPKR (Table 4.2). Homozygous lines for the remaining 26 CPK T-DNA insertion lines should be generated to determine the effect of these gene disruptions and to establish whether other CPK genes may also have roles in the pPKR regulatory pathway.

Once the eIF2α phosphorylation activity of all the CPK genes are determined, it will be necessary to cross each CPK T-DNA insertion line with the others to distinguish any possible redundancies between the CPK genes. Specifically, it will be important to select any additional CPK T-DNA insertion lines which have reduced eIF2α phosphorylation activity to cross with the CPK19 T-DNA insertion line. If both genes are involved in the pPKR pathway, an augmented decrease in kinase activity may be anticipated. Additionally, transgenic Arabidopsis which overexpresses CPK19 (and any CPK genes which demonstrate possible roles in the pPKR pathway) should be
generated to assess the effect of the overexpressed genes on eIF2α phosphorylation activity. The overexpression of the CPK19 gene is anticipated to result in an increase of eIF2α phosphorylation activity and this effect may be augmented if two or more candidate pPKR genes are overexpressed. These CPK T-DNA insertion and overexpression studies will be crucial in determining the potential of CPK19 as being the single gene or a gene within a group of genes involved in the pPKR regulatory pathway.

Table 8.2 List of transgenic Arabidopsis thaliana lines that can be crossed and the anticipated change in kinase activity and phenotype in these hybrids as compared to wildtype Arabidopsis thaliana

<table>
<thead>
<tr>
<th>Transgenic Arabidopsis line 1</th>
<th>Transgenic Arabidopsis line 2</th>
<th>Anticipated change in detected eIF2α phosphorylation activity and phenotype as compared to wildtype Arabidopsis thaliana</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPKn T-DNA</td>
<td>x CPKn T-DNA</td>
<td>No change or possible alteration of phosphorylation and phenotype</td>
</tr>
<tr>
<td>CPKn T-DNA (except CPK19)</td>
<td>Wildtype</td>
<td>No change or possible alteration of phosphorylation and phenotype</td>
</tr>
<tr>
<td>CPKn T-DNA (except CPK19)</td>
<td>CPKn T-DNA (except CPK19)</td>
<td>No change or possible alteration of phosphorylation and phenotype</td>
</tr>
<tr>
<td>CPK19 T-DNA or OE x CPKn T-DNA or OE</td>
<td></td>
<td>Decrease in phosphorylation and altered phenotype (observed in Section 6.2.2)</td>
</tr>
<tr>
<td>CPK19 T-DNA</td>
<td>Wildtype</td>
<td>Increase in phosphorylation and possible alteration of phenotype</td>
</tr>
<tr>
<td>CPK19 OE</td>
<td>Wildtype</td>
<td>Decrease in phosphorylation and altered phenotype (as compared to CPK19 T-DNA alone)</td>
</tr>
<tr>
<td>CPK19 T-DNA</td>
<td>CPK19 OE</td>
<td>Increase/Decrease in phosphorylation and possible alteration of phenotype</td>
</tr>
<tr>
<td>Wildtype</td>
<td>CPK19 T-DNA (with observed altered phosphorylation)</td>
<td>Decrease/increase in phosphorylation and possible alteration of phenotype</td>
</tr>
<tr>
<td>Wildtype</td>
<td>CPK19 T-DNA (with observed altered phosphorylation)</td>
<td>Increase/decrease in phosphorylation and possible alteration of phenotype</td>
</tr>
<tr>
<td>CPK19 T-DNA</td>
<td>CPK19 OE (with observed altered phosphorylation)</td>
<td>Decrease and altered phenotype or possible rescue of CPK19-induced decrease of phosphorylation and phenotype</td>
</tr>
<tr>
<td>Wildtype</td>
<td>CPK19 T-DNA (with observed altered phosphorylation)</td>
<td>Decrease and altered phenotype or possible rescue of CPK19-induced decrease of phosphorylation and phenotype</td>
</tr>
<tr>
<td>Wildtype</td>
<td>p58IPK T-DNA (with observed altered phosphorylation)</td>
<td>Decrease and altered phenotype or possible rescue of CPK19-induced decrease of phosphorylation and phenotype</td>
</tr>
<tr>
<td>CPK19 T-DNA</td>
<td>Wildtype</td>
<td>Described above</td>
</tr>
<tr>
<td>CPK19 OE</td>
<td>Wildtype</td>
<td>Described above</td>
</tr>
<tr>
<td>Wildtype</td>
<td>p58IPK T-DNA</td>
<td>Increase in phosphorylation in response to virus infection with no effect on plant growth and development (observed by Bilgin et al., 2003)</td>
</tr>
<tr>
<td>Wildtype</td>
<td>p58IPK OE</td>
<td>Increase in phosphorylation in response to virus infection with no effect on plant growth and development (observed in Section 6.3.3)</td>
</tr>
</tbody>
</table>
To further verify the significance of CPK19 as the putative plant-functional homolog of PKR, the disruption or overexpression of CPK19 can be assessed in combination with the inactivation or overexpression of each of the PKR inhibitors – p58IPK, E3L, or K3L. If CPK19 is indeed the only plant-functional homolog of PKR, a hybrid plant line comprising inactivated CPK19 and the overexpression of any of the PKR inhibitors would be anticipated to result in no further decrease in eIF2α phosphorylation. Conversely, if more than one functional homolog of PKR exists in plants, the crossing of plants homozygous for an inactivated CPK19 with an overexpressed inhibitor of PKR would be anticipated to further reduce eIF2α phosphorylation. In this case, once the other plant-functional homologs of PKR are identified, it will be useful to generate transgenic plant lines that comprise disruption or overexpression of all of these CPKs with the inactivation of
p58\textsuperscript{IPK} or the overexpression of p58\textsuperscript{IPK}, E3L, or K3L. Findings from these transgenic plant studies would provide more information on the roles of CPKs in the pPKR pathway and whether functional complementations of this kinase activity is present.

8.2.4.2 In Planta Localisation of the Calcium-dependent Protein Kinase CPK19

The transcript of CPK19 is revealed by the Affymetrix Arabidopsis ATH1 array as being most abundant in seedlings and mature siliques of Arabidopsis (Figure 8.1). The localisation and accumulation of the CPK19 transcript can be further verified by cloning the gene for the green fluorescent protein (GFP) under the promoter of CPK19. In this way, the expression of GFP can be utilised as a marker for CPK19 expression pattern throughout the plant and during its development (Ehrhardt, 2003). These data could then be verified by quantitative reverse transcription PCR of the CPK19 transcript. In addition, studies could be made to determine if CPK19 transcription increases in the presence of viral infection. However, the presence of the CPK19 transcript will not necessarily correlate with the presence of expressed CPK19 protein (Ebert et al., 2008). Hence, it will be important to also link the GFP gene to the CPK19 ORF, transform and express this construct in planta, and examine the accumulation of this marker protein as an indication of the accumulation and localisation of the CPK19 protein. Additionally, antibodies could be generated against CPK19 and utilised together with immunoblotting to determine the presence of CPK19 in the different plant tissues and cellular components. A comparable expression of CPK19 with the detection of eIF2\textalpha phosphorylation activity would provide significant evidence for CPK19 as the potential plant-functional homolog of PKR.

8.2.5 The Involvement of eIF2\textalpha Phosphorylation in the Preferential Translation of Upstream Open Reading Frames

As described in Section 1.2.1.4.4, PKR is postulated to be involved in the regulation of preferentially translated mRNAs in response to stress signals. This particular mechanism involves initiation of translation at upstream ORFs and occurs due to the low availability of eIF2-GTP caused by eIF2\textalpha phosphorylation. Correspondingly, pPKR may have a similar role in plants in addition to antiviral defense. Upstream ORFs are present in around 20 – 30% of plant mRNAs (Hayden & Jorgensen, 2007) yet few have been identified for functional activity and the roles of a majority of these upstream ORFs are largely unknown.
It is possible to examine the potential control of translation from upstream ORFs via eIF2α phosphorylation by assessing their protein products in transgenic Arabidopsis overexpressing mutant forms of eIF2α. Once generated, three types of eIF2α overexpression plants will be available for analysis. These include transgenic Arabidopsis overexpressing the wildtype eIF2α (eIF2A WT), an unphosphorylatable eIF2α (eIF2A S56A), or a mimic of phosphorylated eIF2α (eIF2A S56D). Table 8.3 lists the level of translation from upstream ORFs that may be anticipated in response to the presence of the different forms of eIF2α, providing that eIF2α phosphorylation has a role in controlling their preferential translation.

<table>
<thead>
<tr>
<th>Transgenic Arabidopsis overexpression line</th>
<th>Anticipated level of translated products from upstream ORFs as compared to wildtype Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype eIF2α (eIF2A WT)</td>
<td>No change</td>
</tr>
<tr>
<td>Unphosphorylatable eIF2α (eIF2A S56A)</td>
<td>No change or lowered frequency</td>
</tr>
<tr>
<td>Mimic of phosphorylated eIF2α (eIF2A S56D)</td>
<td>Increase</td>
</tr>
</tbody>
</table>

Normal or lowered levels of translation from upstream ORFs would be anticipated only when the wildtype or unphosphorylatable forms of eIF2α are present. In the presence of the consistently phosphorylated form of eIF2α, higher translation activity from these upstream ORFs should be observed.

The level of translation from upstream ORFs can also be examined in CPK19 T-DNA insertion or overexpression lines, which would mimic either a lack of or an abundance of phosphorylated eIF2α, respectively. The effects on translation from upstream ORFs in these transgenic lines would be anticipated to be similar to that observed for an overexpression of the unphosphorylatable eIF2α (eIF2A S56A) or the phosphorylated eIF2α (eIF2A S56D), respectively. In turn, results from these studies will be valuable in determining the potential role of eIF2α kinases, such as pPKR, in the control of preferential translation from specific mRNAs as well as their effects in the presence of virus infection, especially during the early stages of plant cell infection.
8.3 Final Conclusions

The findings from this doctoral research illustrate the presence of PKR-like activity in plants and identify the calcium-dependent protein kinase CPK19 as the most likely candidate for the plant-functional homolog of PKR. Transgenic *Arabidopsis* lines generated in this doctoral research have been effective in revealing genes which may be involved in the pPKR regulatory pathway and a disruption of the CPK19 gene resulted in a loss of eIF2α phosphorylation activity. This eIF2α phosphorylation was also inhibited by the overexpression of the cellular PKR inhibitor p58IPK. Although it is yet uncertain whether eIF2α phosphorylation is used by plants as a defense mechanism against virus infection, further studies using transgenic *Arabidopsis* lines, particularly a p58IPK T-DNA insertion line, will be important in expanding our ability to examine the relationship between eIF2α phosphorylation and the pPKR candidate CPK19. This will also provide a platform to investigate the potential of a novel antiviral defense system in plants.
Appendix A

Identification of Amplification Products Generated from Polymerase Chain Reaction of Arabidopsis thaliana Complementary DNA Using Primers 42563378 F 1296 and 28950719 R 171

Subsequent to amplification by PCR from Arabidopsis cDNA (obtained from W. Cui at Plant and Food Research) using the primers 42563378 F 1296 and 28950719 R 171 (as described in Section 4.9.3.2), the amplified products were A-tailed and ligated into pCR®2.1-TOPO® via TA Cloning®. The resultant ligated products were electro-transformed into E. coli and plated on LB plates supplemented with 100 mg/L ampicillin and 1 mg X-gal. White colonies indicated the presence of an insert and these clones were selected for further screening by PCR using the primers M13 Forward and M13 Reverse (Table 2.3).

Figure A1 represents the various sizes of the amplified products that were cloned into pCR®2.1-TOPO®. The last lane illustrates the amplification of an empty pCR®2.1-TOPO®, which generated a 200 nt product. The insert sizes of the clones ranged from 100 – 800 nt and indicated that a number of mRNA transcripts were made for these genes, encompassing both the kinase and dsRBM-containing sequences, as well as the intervening sequence between the two genes. This observation was further validated by DNA sequencing where different sequences were obtained, each containing various portions of the two genes together with different lengths of the intervening region.

![Figure A1 Amplification products of inserts cloned into pCR®2.1-TOPO®. Agarose gel image of the amplification products generated by PCR from the ligated pCR®2.1-TOPO® clones with the primers M13 Forward and M13 Reverse. Lanes 1-13 illustrates the amplification of different insert sizes in each individual clone. Lane 14 is the amplification of an empty pCR®2.1-TOPO®. DNA markers indicate molecular sizes in nt.](image-url)
Appendices

Appendix B

Cloning of *Arabidopsis thaliana* GCN2

The GCN2 gene (At3g59410) was amplified by PCR from *Arabidopsis* cDNA (obtained from W. Cui at Plant and Food Research) using the primers At GCN2 5’ EcoRI NdeI and At GCN2 3’ BglII. A ~3.7 kb product was generated with restriction enzyme sites of *Eco*RI and *Nde*I at the 5’ end and *Bgl*II at the 3’ end. The PCR product was then A-tailed and ligated into pCR®2.1-TOPO® via TA Cloning®. The resultant ligated products were electro-transformed into *E. coli* and plated on LB plates supplemented with 100 mg/L ampicillin and 1 mg X-gal. Putative pCR®2.1-TOPO®-GCN2 white colonies were selected and screened via restriction enzyme digests with *Eco*RI or *Bgl*II, which would release either a ~3.7 kb band or a ~2.7 kb band, respectively. The clones displaying the correct restriction enzyme digest profiles were then analysed by DNA sequencing to further verify that the correct *Arabidopsis* GCN2 cDNA sequence had been amplified and cloned.

A pCR®2.1-TOPO®-GCN2 clone that contained the correct sequence for GCN2 was digested with *Eco*RI to release the GCN2 gene from the plasmid. Although the primers At GCN2 5’ *Eco*RI *Nde*I and At GCN2 3’ *Bgl*II added *Eco*RI and *Bgl*II ends to the GCN2 gene, the *Eco*RI sites (located in pCR®2.1-TOPO®) were used to release the gene from the vector instead of *Eco*RI and *Bgl*II, as another *Bgl*II site was located in the GCN2 gene. The digested fragment was separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The shuttle vector, pART7, was also digested with *Eco*RI to allow the ligation of the released GCN2 sequence into the linearised vector. Before the ligation reaction, 5 µg of the linearised vector was first incubated with 2.5 U of antarctic alkaline phosphatase for 15 min at 37°C to reduce the amount of self-ligated plasmid. The alkaline phosphatase was then deactivated by incubating the reaction at 65°C for 5 min and the treated vector was used for the ligation reaction with the *Eco*RI-cut GCN2 fragment. Ligated products were transformed into *E. coli* and plated on LB plates supplemented with 100 mg/L ampicillin. Putative pART7-GCN2 colonies were selected and screened via restriction enzyme digests with *Bgl*II or *Hind*III, that released from the correct pART7-GCN2 clone either a ~0.9 kb band or three bands with the sizes of ~0.05, ~1.2, and ~2.2 kb, respectively.
The pART7-GCN2 clone with the correct restriction enzyme digestion profile was selected and digested with \textit{Not}I to release the 35S-GCN2-ocs3’ fragment for ligation into the binary vector pART27. The digested fragment was separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The pART27 vector was also linearised with \textit{Not}I and treated with antarctic alkaline phosphatase to prevent self-ligation. The resulting fragment and vector were mixed and ligated and the products were transformed into \textit{E. coli} and plated on LB plates supplemented with 100 mg/L spectinomycin and 1 mg X-gal. Putative pART27-GCN2 white colonies were selected and screened via restriction enzyme digests with four different enzymes to confirm the insertion of the 35S-GCN2-ocs3’ fragment into pART27. As anticipated for the correct clone, the digestion with \textit{Bam}HI released two bands of ~0.9 and ~4.7 kb; \textit{Hind}III released three bands of ~0.05, ~1.2, and ~2.2 kb; \textit{Xba}I released a ~1.3 kb band; and \textit{Bgl}II released three bands of ~0.7, ~1.2, and ~2.7 kb.

Once verified, the pART27-GCN2 clone was transformed into \textit{A. tumefaciens} and plated on LB plates supplemented with 100 mg/L spectinomycin and 100 mg/L rifampicin. The resultant \textit{A. tumefaciens}-transformed colony was grown and prepared for transformation into \textit{Arabidopsis} for the generation of stably transformed GCN2 overexpression transgenic lines.
Appendices

Appendix C

Cloning of Arabidopsis thaliana eIF2α wildtype

The Arabidopsis eIF2α wildtype (eIF2A WT, At5g05470) was amplified by PCR from Arabidopsis cDNA (obtained from W. Cui at Plant and Food Research) using the primers At eIF2A F EcoRI NcoI and At eIF2A R BamHI. A ~1.0 kb product was generated with restriction enzyme sites of EcoRI and NcoI at the 5’ end and BamHI at the 3’ end. The PCR product was then A-tailed and ligated into pCR®2.1-TOPO® via TA Cloning®. The resultant ligated products were electro-transformed into E. coli and plated on LB plates supplemented with 100 mg/L ampicillin and 1 mg X-gal. Putative pCR®2.1-TOPO®-eIF2A WT white colonies were selected and screened via restriction enzyme digestion with EcoRI or HindIII. The EcoRI digest released a ~1.0 kb band, while the HindIII digest released a ~0.6 band and either a ~0.3 or ~0.1 kb band, depending on the orientation of the inserted gene. The clones displaying the correct restriction enzyme digest profiles were analysed by DNA sequencing to further verify that the correct Arabidopsis eIF2A WT cDNA sequence had been amplified and cloned.

The selected pCR®2.1-TOPO®-eIF2A WT clone was digested with EcoRI and BamHI to release the eIF2α gene from the plasmid. The digested fragment was separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The shuttle vector, pART7, was also digested with EcoRI and BamHI to allow the ligation of the released eIF2α sequence into the linearised vector. Ligated products were then transformed into E. coli and plated on LB plates supplemented with 100 mg/L ampicillin. Putative pART7-eIF2A WT colonies were selected and screened via restriction enzyme digests with XbaI or HindIII, which released either a ~1.0 kb band or a ~0.6 kb band, respectively.

The pART7-eIF2A WT clone with the correct restriction enzyme digestion profile was selected and digested with NotI to release the 35S-eIF2A WT-ocs3’ fragment for ligation into the binary vector pART27. The digested fragment was separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The pART27 vector was also linearised with NotI and treated with antarctic alkaline phosphatase to prevent self-ligation. The resulting fragment and vector were mixed and ligated and the products were transformed into E. coli and plated on LB plates supplemented with 100 mg/L spectinomycin.
and 1 mg X-gal. Putative pART27-eIF2A WT white colonies were selected and screened via restriction enzyme digests with two different enzymes to confirm the insertion of the 35S-eIF2A WT-ocs3’ fragment into pART27. The digestion with HindIII released a ~0.6 kb band; while SacI released two bands of ~1.5 and ~1.7 kb.

Once verified, the pART27-eIF2A WT clone was transformed into *A. tumefaciens* and plated on LB plates supplemented with 100 mg/L spectinomycin and 100 mg/L rifampicin. The resultant *A. tumefaciens*-transformed colony was grown and prepared for transformation into *Arabidopsis* for the generation of stably transformed eIFα wildtype overexpression transgenic lines.
Appendix D

Cloning of Arabidopsis thaliana eIF2α mutants

The eIF2α mutants were each generated via a three-step cloning strategy. To begin with, the first half of the eIF2α gene was amplified by PCR from pCR®2.1-TOPO®-eIF2A WT using the primers At eIF2A F EcoRI NcoI and At R 181-148 noSacI S56A for generating eIF2A S56A; and the primers At eIF2A F EcoR1 NcoI and At R 181-148 noSacI S56D/E for generating eIF2A S56D and eIF2A S56E. As the reverse primer At eIF2A R 181-148 noSacI S56D/E is a degenerate primer containing an M at nt 168, the codon GAT can be generated which codes for an Asp residue (eIF2A S56D). On the other hand, the codon GAG can also be generated with the degenerate primer and this represents the Glu residue (eIF2A S56E). A ~0.18 kb product was generated with the first amplification step.

The second half of the eIF2α gene was amplified by PCR from pCR®2.1-TOPO®-eIF2A WT using the primers At eIF2A F 170-197 and At eIF2A R BamHI and a ~0.9 kb product was generated. The third PCR reaction was performed with the primers At eIF2A F EcoRI NcoI and At eIF2A R BamHI using a mixture of the two amplified products from the first and second PCR reaction as template. As the two products have overlapping ends, it was possible to use these fragments as template and obtain a full-length eIF2α gene product which would contain the different mutations at codon 56. The resulting final PCR product was A-tailed and ligated into pCR®2.1-TOPO® via TA Cloning®. The ligated products were electro-transformed into E. coli and plated on LB plates supplemented with 100 mg/L ampicillin and 1 mg X-gal. Putative pCR®2.1-TOPO®-eIF2A mutants were selected and screened via restriction enzyme digests with EcoRI, SacI, and XhoI where necessary. The digestion with EcoRI released a ~1.0 kb band and verified that the full-length eIF2α gene was cloned into pCR®2.1-TOPO®. The digestion with SacI did not linearise the vector or release any bands as the eIF2α mutants lack the SacI restriction enzyme site. The digestion with XhoI distinguished between the S56D/E products and indicated the presence of eIF2A S56E as the newly generated GAG codon, representing the Glu residue, which also creates a XhoI restriction enzyme site. Hence, XhoI-digested clones would release a ~0.2 or ~0.9 kb band, depending on the orientation of the inserted gene. The clones displaying the correct restriction enzyme digest profiles were then analysed by DNA sequencing to further verify that the correct Arabidopsis eIF2α wildtype cDNA sequence had been amplified and cloned.
Unfortunately, numerous attempts failed to generate a pCR®2.1-TOPO®-eIF2A S56E clone. However, as the eIF2A S56D and S56E constructs both mimic the phosphorylated form of eIF2α, it was not critical to have generated both expression constructs.

The selected pCR®2.1-TOPO®-eIF2A S56A and S56D clones were digested with EcoRI and BamHI to release the eIF2α mutant genes from the plasmids. The digested fragments were separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The shuttle vector, pART7, was also digested with EcoRI and BamHI to allow the ligation of the released eIF2α fragments into the linearised vector. Ligated products were then transformed into E. coli and plated on LB plates supplemented with 100 mg/L ampicillin. Putative pART7-eIF2A S56A and S56D colonies were selected and screened via restriction enzyme digests with XbaI or HindIII, which released either a ~1.0 kb band or a ~0.6 kb band, respectively. A digestion with SacI was also performed and as pART7 contains a SacI restriction enzyme site, the positive pART7-eIF2α mutant clones were linearised in the presence of this enzyme.

The pART7-eIF2A S56A and S56D clones with the correct restriction enzyme digestion profiles were selected and digested with NotI to release the 35S-eIF2A S56A-ocs3’ and 35S-eIF2A S56D-ocs3’ fragment for ligation into the binary vector pART27. The digested fragments were separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The pART27 vector was also linearised with NotI and treated with antarctic alkaline phosphatase to prevent self-ligation. The resulting fragments and vector were mixed and ligated and the products were transformed into E. coli and plated on LB plates supplemented with 100 mg/L spectinomycin and 1 mg X-gal. Putative pART27-eIF2A S56A and S56D white colonies were selected and screened via restriction enzyme digests with two different enzymes to confirm the insertion of the 35S-eIF2A S56A or S56D-ocs3’ fragment into pART27. The digestion with HindIII released a ~0.6 kb band; while SacI released a ~2.2 kb band.

Once verified, the pART27-eIF2A S56A and S56D clones were transformed into A. tumefaciens and plated on LB plates supplemented with 100 mg/L spectinomycin and 100 mg/L rifampicin. The resultant A. tumefaciens-transformed colonies were grown and prepared for transformation into Arabidopsis for the generation of stably transformed eIFα mutant overexpression transgenic lines.
Appendix E

Generation of Hairpin Constructs for *Arabidopsis thaliana* Transformation

The generation of the hairpin constructs listed in Table 6.6 was attempted using the following cloning strategy. Firstly, the sense (hp1) and antisense (hp2) sequences of a specific portion of the gene of interest are amplified, ligated and integrated between the 35S promoter and ocs3’ of the shuttle vector pART7 (Fig. 2.2) via their specific restriction enzyme sites. The 35S-gene-ocs3’ sequence is then removed as a NotI fragment and introduced into the binary vector, pART27 (Fig. 2.3) between the left and right border sequence of the *A. tumefaciens*-derived T-DNA. The resultant expression vector containing the gene of interest is transformed into *A. tumefaciens* and used for the transformation of *Arabidopsis* plants.

A number of approaches were trialled in generating the hairpins of *Arabidopsis* p58IPK and GCN2, via the amplification of their hp1 and hp2 sequences, and these strategies are described below. The hp1 and hp2 of *Arabidopsis* p58IPK was amplified by PCR using pART27-IPK as template with the primer pairs At IPK hp1 5’ XhoI & At IPK hp1 3’ BglII and At IPK hp2 5’ BamHI & At IPK hp2 3’ XbaI. The expected size of the products generated was ~0.4 and ~0.32 kb for IPK hp1 and IPK hp2, respectively. Similarly, the hp1 and hp2 of *Arabidopsis* GCN2 was amplified by PCR using pART27-GCN2 as template with the primer pairs At GCN2 hp1 5’ XhoI & At GCN2 hp1 3’ BglIII and At GCN2 hp2 5’ BamHI & At GCN2 hp2 3’ XbaI. The expected size of the products generated was ~0.65 and ~0.52 kb for GCN2 hp1 and GCN2 hp2, respectively. These hp1 and hp2 products were then either used as template for a subsequent PCR reaction or ligated at their respective 3’ and 5’ ends to form the hairpin sequence. A schematic diagram of the generation of the hairpin sequence via the ligation of the hp1 and hp2 PCR amplifications is shown in Figure E1.

**E1 First Hairpin Cloning Strategy**

Initially, it was proposed to first amplify the hp1 and hp2 sequences of the two genes of interest, then use these products as template for a subsequent PCR reaction to amplify the full-length hairpin with the primers At IPK hp1 5’ XhoI & At IPK hp2 3’ XbaI for the IPK hairpin and the primers At GCN2 hp1 5’ XhoI & At GCN2 hp2 3’ XbaI for the GCN2 hairpin.
Figure E1 Schematic diagram of the generation of hairpins via PCR amplification and ligation. The sense (hp1) and antisense (hp2) sequences from the gene of interest (p58IPK and GCN2) are amplified by PCR with the primer pairs hp1 5’ & 3’ and hp2 5’ & 3’. The amplified products are then digested by BglII and BamHI and ligated at these compatible cohesive ends to generate the hairpin sequence. Italics and line represent name and location of restriction enzyme sites. Red arrow, open reading frame of p58IPK or GCN2; blue arrow, amplified hp1; orange arrow, amplified hp2; dotted lines, pairing between the bases.
However, no hairpin products were able to be amplified with this strategy. As hp1 and hp2 contain the sense and antisense sequences of the same gene, it was possible that the presence of these matching sequences may have affected the annealing of primers to the desired ends.

**E2 Second Hairpin Cloning Strategy**

As the initial approach was unsuccessful in generating the hairpin constructs, a more complex strategy was proposed. Firstly, the hp1 and hp2 sequences of the two genes of interest were to be amplified by PCR, then digested at their ends with the appropriate restriction enzymes, and ligated together to form the hairpin construct.

The IPK and GCN2 hp1 and hp2 both have the same generated restriction enzyme ends. Hp1 comprises of a 5’ XhoI and 3’ BglII end, while hp2 comprises of a 5’ BamHI and 3’ XbaI end. The amplified products were first digested respectively with the restriction enzymes BglII or BamHI, separated from the cleaved ends by agarose gel electrophoresis, and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The resulting digested hp1 and hp2 were mixed and allowed to ligate. As the cohesive ends generated from cutting with BglII and BamHI are compatible with each other, these overhangs would readily join together in the presence of ligase. The ligation reaction was then separated again by agarose gel electrophoresis and the ligated product of the desired size was extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). For the IPK hairpin, the size for the ligation of hp1 and hp2 was ~0.72 kb and not ~0.8 kb (hp1 + hp1) or ~0.64 kb (hp2 + hp2). For the GCN2 hairpin, the expected size for the ligation of hp1 and hp2 was ~1.17 kb and not ~1.3 kb (hp1 + hp1) or ~1.04 kb (hp2 + hp2). These gel purified products were then digested with XhoI and XbaI and mixed with pART7 plasmid that had also been digested with XhoI and XbaI for the ligation of the hairpin into the shuttle vector. Ligated products were then transformed into *E. coli* and plated on LB plates supplemented with 100 mg/L ampicillin.

Unfortunately, no colonies grew and numerous attempts failed to generate the pART7-IPK hairpin or pART7-GCN2 hairpin clone.
**E3 Third Hairpin Cloning Strategy**

As the more complex second hairpin construct strategy was unsuccessful in obtaining the desired hairpin constructs, it was reassessed and an additional step was included into the cloning strategy. The hp1 and hp2 sequences of the two genes of interest were still amplified, digested respectively with *Bgl*II or *Bam*HI, separated by agarose gel electrophoresis, and extracted from the gel. However, instead of following on with the *Xho*I and *Xba*I digests, the gel purified product was A-tailed, and digested again with *Bgl*II or *Bam*HI to remove ligated products that were joined with itself rather than the opposing hp1 or hp2 molecule. The resulting digested products were then mixed with the pCR®2.1-TOPO® plasmid and any fragment that contained the adenosine overhangs on both ends, i.e., the products that were not cleaved with the *Bgl*II or *Bam*HI digestion, were ligated into the plasmid via TA Cloning®. Ligated products were then transformed into *E. coli* and plated on LB plates supplemented with 100 mg/L ampicillin and 1 mg X-gal. Putative pCR®2.1-TOPO®-IPK hairpin and pCR®2.1-TOPO®-GCN2 hairpin white colonies were selected and screened via restriction enzyme digests with *Bgl*II and *Bam*HI or *Eco*RI. The construct should be linearised with the former restriction enzyme digests as a *Bgl*II site is present in the pCR®2.1-TOPO® plasmid but no *Bgl*II and *Bam*HI sites should be present in the hairpin construct. For the digestion of a pCR®2.1-TOPO®-IPK hairpin clone with *Eco*RI, three bands of ~0.2, ~0.22, and ~0.56 kb would be released. On the other hand, the *Eco*RI digestion of a pCR®2.1-TOPO®-GCN2 hairpin clone would release a ~1.2 kb band. The clones displaying the correct restriction enzyme digest profiles were then analysed by DNA sequencing to further verify that the correct hairpin sequences had been amplified and cloned.

The selected pCR®2.1-TOPO®-IPK hairpin and pCR®2.1-TOPO®-GCN2 hairpin clones were digested with *Xho*I and *Xba*I to release the hairpin construct from the plasmid. The digested fragment was separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The shuttle vector, pART7, was also digested with *Xho*I and *Xba*I to allow the ligation of the released hairpin sequence into the linearised vector. Ligated products were then transformed into *E. coli* and plated on LB plates supplemented with 100 mg/L ampicillin.

However, numerous attempts still failed to achieve putative pART7-IPK hairpin or pART7-GCN2 hairpin clones.
E4 Fourth Hairpin Cloning Strategy

It was concluded that it was not possible to generate the hairpin constructs via amplification of the sense and antisense gene sequences. Thus, the hairpins containing the hp1 and hp2 sequences were synthesised for each gene and obtained from GenScript (USA). Table E1 shows the hairpin sequences that were synthesised for the Arabidopsis p58IPK and GCN2 genes. A XhoI and XbaI site was added to the respective 5’ and 3’ ends of the hairpin sequence and a BglII was located in the region where the sense and antisense sequences were joined together.

Table E1: Sequence of hairpin constructs synthesised from GenScript

<table>
<thead>
<tr>
<th>Gene to be synthesised</th>
<th>Construct name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hairpin of Arabidopsis p58IPK (730 bp)</td>
<td>IPK hairpin</td>
<td>AATTCTCGAGTCAATCGGGTGAATGCTGAGTTATTCCGAGAGAGCTTACAAAGTATCAAGTTAAGCGTTATAGTGATGCCCTCGATGATCTGAATGCAGCCATTGAAAGCAGACCCTGCGCTTTCAGAAGCTTATTTTAAGCGTGCCTCTGTGCTTCGACATTATTTGCTGATACGAAGACTCTGAGAATAGTTACCAGAAATATCTGGAATTCAAATAGGAGATTCGAATGCTGAGAAAGAACTCTCTCAGCTTCATCAAGCTAAGAGTGCTTTGGAGACTGCCTCGACTCTGTATGAATCAAAGGATATTGCGAAAGCTCTTGAAATTTGTTGATAAAGTTGTTCTTGTCTTCTCTCCAGCTTGCTCTAAGGCAAAGCTTCTAAAGTGAAGCTCCTTATCTCTTCTTTTGACTCAAGTAAATCTACTGACGTTGTGGAATCTGGATTATTCCAAAATGAGAAGAAGGAATCAAATCTTCAAGATGATACTAGCTGAAGATGACAGCACTAACTCCGAAAGTGAGTCGCTGGGGTCATGGTCCTTGATTCCTTAGCTCAAGATCAAGTGCCTCAGATTAGCAAGAAAGATCTTTGAAGTGTCTAGGGTGGATATTGTACTGAAGATACGATTCCCCTATTTTCATCTGGAGTCAGACTCCAATTTCTGCATCTTCCAAGCCACTAAATAGGTCTATAAACCATACACAAAAGGTCCACCAGAACAGGATTTTGCTATATTTGAAAGCATAGGTTGCTCAATGAACTGAGTGCTTCGATGTGCAGTCAAGATGGAAACAGATTCCTCATCATGACTTTCCGGAATGATTTCTGATAAAACTCTTGAGCAGCCTCCACCAGGTTGAATATCATAACCCGACCTTCACGAGCATTGGAATTTGCCTGGTC TAGAGTAGTGAAGTGAAGAAGAAGGATTTGCCTATCGATGCTGAGGTGCTATCTGCTGAGGTTGTCTGTTTCTTCTGATGCTGAGATGCTGAGAAAAGGAGAGAGTTGCACTGCCTGCTCCCATTGCCAAACTGTTAAGAGGAGAATGTTGATGATACAAGCATCTCTTCTTTTGACTCAAGTAAATCTACTGACGTTGTGGAATCTGGATTATTCCAAAATGAGAAGAAGGAATCAAATCTTCAAGATGATACTAGCTGAAGATGACAGCACTAACTCCGAAAGTGAGTCGCTGGGGTCATGGTCCTTGATTCCTTAGCTCAAGATCAAGTGCCTCAGATTAGCAAGAAAGATCTTTGAAGTGTCTAGGGTGGATATTGTACTGAAGATACGATTCCCCTATTTTCATCTGGAGTCAGACTCCAATTTCTGCATCTTCCAAGCCACTAAATAGGTCTATAAACCATACACAAAAGGTCCACCAGAACAGGATTTTGCTATATTTGAAAGCATAGGTTGCTCAATGAACTGAGTGCTTCGATGTGCAGTCAAGATGGAAACAGATTCCTCATCATGACTTTCCGGAATGATTTCTGATAAAACTCTTGAGCAGCCTCCACCAGGTTGAATATCATAACCCGACCTTCACGAGCATTGGAATTTGCCTGGTCAGTAGTT</td>
</tr>
</tbody>
</table>

Underlined black nucleotides denote the location of the 5’ XhoI and 3’ XbaI restriction enzyme sites; underlined red nucleotides denote the location of the BglII restriction enzyme site; bolded text, sense coding sequence; italics, antisense coding sequence.
The synthesised IPK and GCN2 hairpins were obtained as clones inserted in a pUC57 plasmid and were received as a DNA pellet. The DNA was resuspended in 100 µL distilled water and the hairpin inserts were released from the vector via restriction enzyme digests with XhoI and XbaI. The digested fragments were separated from the vector via agarose gel electrophoresis and extracted from the gel using the Perfectprep® Gel Cleanup kit (Eppendorf). The fragments were then ligated into pART7 plasmid that had also been digested with XhoI and XbaI. Ligated products were transformed into E. coli and plated on LB plates supplemented with 100 mg/L ampicillin. Putative pART7-IPK hairpin or pART7-GCN2 hairpin colonies were selected and screened via restriction enzyme digests with XhoI and XbaI or BglII. A XhoI and XbaI digest of a pART7-IPK hairpin clone would release a ~0.73 kb band, whereas a XhoI and XbaI digest of the pART7-GCN2 hairpin would release a ~1.19 kb band. Conversely, for the BglII digests, the digest of the pART7-IPK hairpin would release two bands of ~0.6 and ~0.75 kb, and the digest of the pART7-GCN2 hairpin would release two bands of ~0.75 and ~0.85 kb. The clones displaying the correct restriction enzyme digest profiles were then analysed by DNA sequencing to further verify that the correct hairpin sequences had been amplified and cloned.

Due to time restraints, the cloning of the hairpin constructs was achieved only up to this stage. The next steps will be to digest the confirmed pART7 clones with NotI and release the 35S-IPK hairpin-ocs3’ and 35S-GCN2 hairpin-ocs3’ fragments for ligation into the binary vector pART27. Once ligated into this expression vector, the pART27-IPK hairpin and pART27-GCN2 hairpin clones will be transformed into A. tumefaciens and be prepared for transformation into Arabidopsis for the generation of stably transformed p58\textsuperscript{IPK} and GCN2 knockdown lines.
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