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THE MOLECULAR RESPONSE OF WHEAT ROOTS TO ALUMINIUM STRESS

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

November 1994
The most important words in science aren't "Eureka! I have found it!", but "that's funny..."
I. Asimov
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ACKNOWLEDGMENTS

There are a large number of people I would like to thank, without whom this work would not have been possible:

First and foremost, my supervisor Associate-Professor Richard Gardner, who provided encouragement and advice throughout this work and spent many hours editing and improving this thesis and my manuscripts.

Thanks also go to many of the other faculty members in the School of Biological Sciences, in particular Professor Dick Matthews, my co-supervisor Professor Dick Bellamy, and to all the others who provided information and advice.

To all the PMB members past and present, in particular to Jeannette, Susan, Keith and Colin, and to my fellow aluminium workers and wine drinkers - you have all helped to make the last few years enjoyable.

Beryl Davy, for her expertise and patience at showing me how to section my plant tissue.

To the people in the ARB group who helped me with the protein sections of this work, in particular Judy and John for advice about the protein fusions, and Jan for advice on westerns.

To all the computer experts out there who helped solve all my crises at short notice, in particular Dave, Terry and Moreland.

Professor Paul Williams for the use of the densitometer (and Susan for showing me how to use it).

To Bart, Susan and Ross for all the time and energy they spent in proof-reading.

To all the organisations who provided financial support, both for my stipend and for the trip to Cold Spring Harbor.

To my family and friends who provided welcome distractions when I needed a break.

And finally, to Bart, who helped immeasurably with this thesis, and was there for me throughout all the toughest times of the last few years.
ABSTRACT

Aluminium (Al) toxicity to plants is a significant problem, limiting agricultural production in up to 40% of the world’s arable soils. In spite of a large amount of research, there is still no consensus on the physiological mechanisms of Al toxicity in plants. In addition, very little is known about the molecular response of plants to Al stress. This body of research was aimed at identifying the changes in gene expression that occurred in the root tips of plants that had been stressed with Al.

A cDNA library made from the root tips of Al-treated wheat (*Triticum aestivum* L., cultivar Warigal) plants was differentially screened to identify clones whose expression was induced by Al stress. Seven cDNA clones, representing five different genes were identified as being induced in the presence of Al. Initial sequencing and northern analysis revealed that none of the clones isolated were full-length, and that some contained multiple cloning adaptors at their 5' ends. A new cDNA library was then constructed from the root tips of Al-treated Warigal plants, and homologues to each of the original five genes were isolated. These five clones were named wali1 to wali5 (for wheat aluminium induced).

Northern analysis showed that wali1, -3 and -5 were induced 24 to 96 h after Al treatment, and their expression declined when the Al was removed. wali4 had a similar pattern of expression with a transient increase in expression also observed after 0.5 h of Al stress. Each of these four genes was induced by inhibitory concentrations of Al in two wheat cultivars - Warigal, an Al-sensitive cultivar, and Waalt, an Al-tolerant cultivar, - and also in two inbred lines of wheat, RR (Al-tolerant) and SS (Al-sensitive). The fifth gene (wali2) had a bimodal pattern of induction, and was induced by Al only in the Al-sensitive Warigal and the Al-tolerant RR.

The nucleotide sequence of each of the wali clones was determined, and the databases were searched for homologous sequences. wali1 was found to be homologous to a group of metallothionein-like proteins (MLPs) from plants, and wali4 was homologous to phenylalanine ammonia-lyase (PAL). wali3 and wali5 encode related, cysteine-rich proteins with homology to Bowman-Birk proteinase inhibitors, and wali2 encodes a novel protein with a repeating motif of cysteine amino acids.

The induction of the wali genes was investigated in response to a number of other stresses through northern analysis. The expression of wali1, -3, -4 and -5 was induced in root tips of wheat after 2 d treatments with toxic levels of all other metals tested (Cd, Fe, Zn, Cu, Ga, In and La). The expression levels of wali1, -3, -4, and -5 also increased in the root tips of plants grown in the presence of low levels of Ca (10 μM). The transcript levels of wali1, -3 and -5 increased in wounded leaf and root tissue, whereas the transcript levels of wali4 increased only in wounded leaves. The expression of
wali2 was greatly reduced by low concentrations of Ca, and showed no induction, or a variable response with most of the other treatments.

The site of expression of wali1, -2, -3 and -5 in root tips (and wali1 also in leaf tissue) was identified using in situ hybridisation. wali1 was expressed predominantly in the meristematic tissue of the root tip, while wali3 and wali5 were expressed predominantly in the cortical tissue of the root. wali2 expression was detected primarily in the epidermis and root cap. Some changes in the site of expression of these genes were evident in the roots of Al-treated plants. In leaf tissue, wali1 expression was found in the mesophyll layer of cells.

The coding sequences for wali1, -2, -3 and -5 were each cloned into the bacterial expression vector pGEX-2T. The resultant fusion proteins between glutathione S-transferase (GST) and the walis were then successfully purified from E. coli. Antibodies were made to the wali1-GST fusion protein and purified by immunoaffinity chromatography. However, when used in western analysis, no specific bands corresponding to the native wali1 protein were identified. The wali2-GST protein was used in a south-western procedure to determine if the protein was capable of binding DNA, but no DNA binding to this protein was detected under the conditions tested. The wali3 and wali5 fusion proteins were tested in proteinase inhibitor assays, where no inhibition of either trypsin or chymotrypsin was detected. It is possible that the native wali3 and wali5 proteins may not function as proteinase inhibitors, or that the lack of activity detected for the fusion proteins may be due to incorrect folding or processing in the bacterial system.

This research constitutes the first identification of plant genes whose expression is increased by Al stress. The genes identified are also induced in response to other environmental and nutrient stresses, indicating that they form part of the plant's general response to stress.
Standard SI units, chemical abbreviations and the one and three letter codes for amino acids and nucleotides have been used throughout this thesis, as well as the abbreviations that follow:

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>cv</td>
<td>cultivar</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxy-terminus (of a polypeptide)</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine-5'-triphosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DMDC</td>
<td>dimethyl-dicarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNaSe</td>
<td>deoxyribonuclease I</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>disodium salt of ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>GCG</td>
<td>Genetics Computer Group</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase(pair)s</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>MLP</td>
<td>metallothionein-like protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>message RNA</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>N-terminus</td>
<td>amino-terminus (of a polypeptide)</td>
</tr>
<tr>
<td>ODx</td>
<td>optical density - the absorbance measured at a wavelength of x nm</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAL</td>
<td>phenylalanine ammonia-lyase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N-N'-bis (2-ethane sulfonic acid)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RH</td>
<td>relative humidity</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet (light)</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume (with respect to the percentage of a solution)</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>X-phos</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
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1.1. INTRODUCTION

Plants encounter a wide range of environmental conditions that can limit their growth or result in death. These include disorders such as a mineral imbalance; or an excess or lack of light, heat or water. Other stresses plants encounter are physical assaults (for example, wounding by feeding insects and other pests), or diseases such as those caused by fungi and viruses. The environmental stress that this thesis is primarily concerned with is Al toxicity.

Mineral stress can occur either because of an excess which leads to toxicity, or because of a scarcity which leads to a nutritional deficiency. Either mineral deficiency or toxicity can cause retardation of growth, impairment of metabolism and damage to plant tissues (Hecht-Buchholz, 1983). For example, an excess of Fe can be highly toxic to plants whereas not enough Fe (most plants require between $10^{-9}$ to $10^{-4}$ M) causes deficiency symptoms such as chlorosis and yellowing of leaves and may also lead to reduced crop yields (Guerinot and Yi, 1994). A key factor in mineral stress is the availability of the mineral. For example, although Fe is the fourth most abundant element present in the Earth's crust, after O, Si and Al, (Moeller et al., 1984), it is often not readily available to plants, especially those grown on calcareous soils (Guerinot and Yi, 1994). Almost all minerals essential to plant growth and development can be toxic when present at higher than optimal levels (for example, Mn, Mo, and various salts, Hecht-Buchholz, 1983). In contrast, Al is a mineral that is not required for plant growth (Bohn et al., 1985). However, when soil conditions become acidic, Al becomes soluble and is toxic to most plant species.

Some mineral conditions that do not result in any deleterious symptoms to the plant may nevertheless be of concern because of their effect on human nutrition. In parts of the world where a single crop such as rice, wheat, corn, potato or cassava makes up a large proportion of the diet, human deficiencies in Fe, I, amino acids or vitamins may become a problem. In addition, the presence of metal ions such as Cd may not be overly toxic to plants, but these can become a health risk in consumers of plants grown in soils which contain the metal(s) in question. For example, Cd poisoning killed hundreds of people in Japan when the metal was incorporated into rice plants via soil polluted with industrial waste (Moeller et al., 1984). Al is toxic to both plants and animals, although the relative contributions of various dietary (and other) sources of Al to human medical conditions, for example, Alzheimer's disease and other neurological disorders, remain controversial (Macdonald and Martin, 1988).
Not only are there problems associated with naturally varying soils, but human influences such as industrial pollution, use of fertilisers, and repeated growth of crops on soils are also introducing new problems to existing soils. For example, the salinity and acidity of cultivated soils are both increasing because of human cultural practices. This reduction in soil quality, combined with an increasing demand on the world food supply means that major crops are now being grown on increasingly more marginal soils. Understanding how plants respond to stresses such as Al toxicity on acid soils may lead to increased yields under these conditions.

The remainder of this introduction will cover various aspects of Al toxicity to plants (Ch. 1.2 - 1.7), with a section included on how research of the type utilised in this work has been of help in understanding other environmental stresses (Ch. 1.8). The last section of this introduction (Ch. 1.9) covers the aims of this work.

1.2. AL TOXICITY
Al is the most abundant metal in the earth's crust, comprising approximately 8% by weight (Moeller et al., 1984). Most Al in soils is insoluble, associated with complex aluminosilicates and oxides. However under acidic conditions (below pH 5) there is a release of ionic Al\(^{3+}\) and a reduction in the availability of exchangeable cations such as Ca\(^{2+}\), Mg\(^{2+}\) and K\(^+\). Although the solubility of Al is largely influenced by pH (Fig. 1.1), it is complicated by concentrations of complexing ligands, ionic strength and temperature (Driscoll and Schecher, 1990). Soil acidity limits agricultural production in approximately 40% of the world's arable soils and this is largely attributable to Al toxicity (Haug, 1984). In addition, the acidity of many of the world's soils is increasing. Factors which can increase soil acidity include the use of nitrogenous fertilisers, industrial pollution and acid rain (van Breeman, 1985). Normal rainfall can also cause acidification of soils by promoting the leaching of basic cations such as Ca\(^{2+}\), Mg\(^{2+}\), K\(^+\) and Na\(^+\) (Foy, 1984).

Several species of Al exist in soils, of which only two have been confirmed to be toxic to plants. The Al\(^{3+}\) ion is the most abundant of these two in acidic conditions. In addition a polynuclear species of Al (Al\(_{2}\)O\(_{4}\)Al\(_{12}\)(OH)\(_{24}\)(H\(_{2}\)O)\(_{12}\))\(^{7+}\), also referred to as Al\(_{13}\) has also been shown to be highly toxic to plants (reviewed in Kinraide and Ryan, 1991). Under alkaline conditions the Al(OH)\(_{4}\)\(^-\) ion was thought to be toxic but this toxicity may be attributable to Al\(_{13}\) (Kinraide, 1990). Most research performed to date on Al toxicity has focused on the stress caused to plants under acidic conditions. In the experiments performed in this thesis, Al\(^{3+}\) was expected to be the major ion present, and toxicity was assumed to be primarily due to the presence of Al\(^{3+}\), although the actual species present was not confirmed experimentally. Similarly, for much of the experimental work reviewed here, the Al species present in solution was not determined.
Although Al toxicity is a major problem in acidic soils it is also compounded by other problems related to the acidity of the soil. For example, as mentioned above there is a reduction in the availability of basic cations such as Ca$^{2+}$, Mg$^{2+}$ and K$^+$. In addition, P and Mo are less soluble under acidic conditions, and there is an increase in the concentrations of Mn as well as H$^+$, both of which can be toxic to plants (Marschner, 1991). The result can often be that although a plant may suffer from Al stress, this may also be exacerbated by deficiencies or toxicities to other minerals.

![Figure 1.1 The solubility of Al](image)

This graph is reproduced from Driscoll and Schecher, 1990. It shows the concentrations of the various species of Al present in equilibrium with the insoluble salt, Al(OH)$_3$. pH = -log[H$^+$], pAl = -log[Al species].

There are a number of substances known to be able to ameliorate the toxic effects of Al. For example, chelating substances such as citric acid can reverse root growth inhibition caused by Al (Ownby and Popham, 1989). Similarly, fluorides can also alleviate the symptoms of Al toxicity, presumably by forming Al-F complexes (such as AlF$_3$) that are non-toxic to plant growth (MacLean et al., 1992). In addition, a number of different cations can ameliorate Al toxicity in acidic conditions, with Ca$^{2+}$ and Mg$^{2+}$ being the most effective (Kinraide and Parker, 1987). This amelioration of Al toxicity by cations is not simply due to general increases in ionic strength of the culture medium (resulting in complexes or precipitates of Al) or to other effects that might reduce the effective concentration of phytotoxic Al. It is also unlikely that the positive effects of cations result solely from alleviation of the deficiency in the
cation in question. Rather, the cations may instead compete directly with Al for binding sites in the root (Kinraide and Parker, 1987; Edmeades et al., 1991).

1.3. PLANT SYMPTOMS OF AL STRESS
When susceptible plants are subjected to toxic levels of Al, a number of symptoms are observed. Root elongation is inhibited which subsequently results in a reduction of plant growth (Clarkson, 1965). The root tips of Al-stressed plants become thickened and may turn brown. The root system becomes stunted, with stubby lateral roots and a lack of fine branching. This damage and lack of growth of the root system causes the plant to become inefficient in absorbing nutrients and water and become more susceptible to drought (Foy, 1984). In the long term, plants can show symptoms of P, Ca, Fe, or Mg deficiencies (Foy, 1984; Edmeades et al., 1991).

Root growth inhibition by Al involves an inhibition of both mitosis and root elongation. In maize, a small decrease in root elongation has been observed after only 30 min of Al treatment, with larger effects observed after 3.5 h (Gunsé et al., 1992). Other researchers have also observed that Al has an effect on root elongation within a few hours (for example, onion, Clarkson, 1965; soybean, Horst et al., 1992). Similarly, inhibition of mitosis has been observed (Clarkson, 1965; Matsumoto, 1991). It is not clear whether mitosis or root elongation is inhibited first, although a report by Wallace and Anderson (1984) suggests that the rate of root elongation decreased before an effect was observed on DNA synthesis.

Several groups of researchers have followed the progression of the effects of Al toxicity on plants using light and electron microscopy. Exposure to toxic levels of aluminium results in an increase in the vacuolation of epidermal and cortical cells as well as cells in the root cap and, after prolonged exposure, in the meristematic region. There is a reduction in cell turgor, an increase in cytoplasmic disorganisation, and a reduction in the production of mucilage. Many of the root tip cells become swollen and enlarged, particularly the outer cortical and epidermal cells. Cells in the quiescent centre of the root tip may also become enlarged. The root tissue becomes disorganised, particularly in the epidermis and outer cortical cells. Many of the cells of the root, including those in the root cap, the epidermis and some cortical cells are disrupted. The earliest effects of Al stress are observed in the epidermal and outer root cap cells, with changes to meristematic cells usually observed only after prolonged exposure (several days) to Al (Bennet et al., 1985a and b; de Lima and Copeland, 1994; Hecht-Buchholz, 1983; Wagatsuma et al., 1987; Wheeler et al., 1992b). In the experiments of Bennet et al. (1985b) increased vacuolation of the outer cap cells was observed after 2 h of Al exposure, followed by vacuolation of the cap columella cells after 6.5 h, and the cap initials after 20 h.

Ultrastructural characterisation of the effects of Al toxicity have shown a number of effects. Golgi apparatus activity was disrupted in peripheral root cap cells in maize within 2 h of Al exposure (Bennet et al., 1987). Electron-dense globular deposits were observed between the cell membrane and wall of
Al-treated wheat roots after 10 h of Al treatment, and the cell walls were thickened after 2 d (Wheeler et al., 1992b). In the root cap cells of Al-treated onion plants, 'Al-structures' were observed after 2 d of Al treatment (Fiskesjö, 1990). A reduction in the size and number of amyloplasts was observed in the root cap cells of maize and wheat plants after 24 h of Al treatment (Bennet et al., 1987; de Lima and Copeland, 1994). Loss of the starch within the amyloplasts was observed, and this was correlated with an increase in activity of several enzymes involved in starch mobilisation (de Lima and Copeland, 1994).

1.4. UPTAKE OF AL

Measuring the uptake of Al into plants is complicated by the fact that there are few direct ways of measuring incorporation of the ion into plant tissues. The use of a radioactive tracer would simplify studies of this sort, but the only suitable tracer is $^{26}$Al, which is very expensive (Taylor, 1991). Instead indirect methods of following Al uptake and distribution have been employed by a number of investigators.

Several studies with wheat have shown that the initial uptake of Al into whole roots is biphasic, with an initial rapid non-linear uptake followed by a slower linear uptake (Zhang and Taylor, 1989; Pettersson and Strid, 1989). This has been interpreted as indicating that the first phase of uptake correlates with binding of Al to sites in the apoplasm, as the Al was readily extracted by chelation with citrate (Zhang and Taylor, 1989). The Al binding signified by the linear phase of uptake has been interpreted as representing either uptake into the symplasm (Pettersson and Strid, 1989) or continued binding in the apoplasm which is metabolism dependent (Zhang and Taylor, 1989). Further experiments by Zhang and Taylor (1990) support the idea that at least a portion of the linear phase of Al uptake involves metabolism-dependent binding to the apoplasm. In this work, cell walls were isolated from the roots of Al-treated plants. Some of the Al bound to these cell wall fractions could not be extracted with citrate.

From the perspective of the whole plant, the presence of Al in roots has been measured using a number of different techniques. Atomic absorption spectroscopy can measure relative amounts of Al present in plant tissue, but does not have the resolution to determine where in the roots the Al is localised. This technique was used in the work described above (Zhang and Taylor, 1989; Pettersson and Strid, 1989). Atomic absorption spectroscopy was also used to determine that the root apices of Al-sensitive wheat cultivars accumulated more Al than Al-tolerant cultivars (Delhaize et al., 1993a). The precise localisation of Al within root tips of Al treated plants has been estimated using staining with Al-reactive substances (for example, haematoxylin, Ownby, 1993; Rincón and Gonzales, 1992; Ryan et al., 1993; morin, Tice et al., 1992; aluminon, Morimura et al., 1978) and X-ray microanalysis (e.g. Delhaize et al., 1993a; Ownby, 1993). These techniques suggest that Al accumulates preferentially in the outer layers of the root tip, such as the root cap, epidermis and outer cortical cells, after short periods of Al treatment (less than 2 d).
There is disagreement concerning the intracellular site at which Al accumulates. Some work suggests that Al accumulates within the cytoplasm and nucleus, with little seen in cell walls or vacuoles (Tice et al., 1992). Other research indicates that there may be extensive binding of Al to cell walls, intercellular spaces and nuclear material (Rincón and Gonzales, 1992). Ownby (1993) detected Al accumulation predominantly in cell wall material, with little observed in nuclei, cytoplasm or vacuoles. Many of the differences observed probably result from differences in the techniques utilised and it is not known which technique is the more reliable. This uncertainty derives from the possibility that there is insufficient penetration of some staining substances into all regions of the root tip (i.e. meristematic versus epidermal regions, or intracellular versus extracellular areas). In addition, Al that is tightly complexed to substances within the root may not be detected as readily as Al which is less stringently bound. These factors are less of a problem with X-ray techniques, but sample preparation may introduce errors, or make it difficult to determine exactly which area of the cell is the site of Al accumulation. Lack of sensitivity may mean that not all the Al in cells is detected and contamination of samples with Al may also affect analyses (Tice et al., 1992).

In spite of these difficulties, it is widely accepted that some Al does enter the symplasm. The fact that many plants can accumulate Al in their leaves (e.g. Haridasan et al., 1986; Cuenca et al., 1991) argues that symplastic transport of Al can occur. However, the mode of entry of Al into the symplasm is still obscure. It is possible that Al may enter passively through damaged cells, or in areas made accessible by emerging lateral roots (Rasmussen, 1968). Some species of Al have been suggested to be capable of diffusing directly through the plasma membrane, probably electroneutral compounds such as Al(OH)₃ (Haug, 1984). Other possibilities include the uptake of Al after chelation to substances such as citrate. It is also possible that Al can enter cells through ion channels within the plasma membrane, or be taken up through endocytosis (reviewed in Haug, 1984; Roy et al., 1988; Akeson and Munns, 1990).

1.5. POSSIBLE SITES OF ACTION FOR Al IN THE PLANT

A large amount of physiological research has centred on the possible mechanisms involved in Al toxicity in plants (see reviews by Foy et al., 1978; Foy, 1984; Haug, 1984; Andersson, 1988; Bennet and Breen, 1991a; Kochian et al., 1991; Marschner, 1991; Lüttge and Clarkson, 1992; Rengel, 1992). In spite of all this research, identification of the primary target(s) of Al toxicity in the plant is still a matter of controversy. The situation is also complicated by the possibility that Al may be affecting different processes, or that some processes are affected to varying degrees in different plant species. In this section I will briefly review the evidence for the possible targets of Al toxicity.

(a) The root cap and meristem

From the perspective of the whole plant, the root system is more obviously affected by Al toxicity than the leaves of the plant. In some cases the above ground portions of plants affected by Al may show no symptoms at all. Within the root system, not all areas show the same susceptibility to Al toxicity. It
was previously postulated by Bennet and coworkers (1985b), that the root cap was the target organ of Al toxicity due to their observations that very early effects of Al stress were observed in the root cap (see Ch. 1.3). They suggest that this observation is of primary importance due to the role of the root cap in controlling root growth under other circumstances (discussed in Bennet and Breen, 1991a). Ryan et al. (1993) had observed that only the terminal portion of wheat roots were sensitive to Al, and that when Al was applied only to more mature regions of the root it had no effect on root growth. Al applied to the elongation zone of the root tip resulted in damage to the tissue, but no reduction in root growth. However, when Al was applied at the meristematic region of the root, root growth was inhibited (Ryan et al., 1993). These researchers argued against the idea that it is the root cap itself that is the target in the terminal portion of the root, because plants that have had their root caps removed were still inhibited by Al. Therefore it appears more likely that the effects of Al on the meristematic region of the root tip (the terminal 2 to 3 mm) are responsible for the inhibition of root growth and that this region is implicated in the toxic effects of Al on the whole plant.

Within the meristematic region of the root tip, there are a multitude of possible targets for Al toxicity. Extracellular targets could be the cell wall, the plasma membrane or specific proteins associated with the plasma membrane. Within the cell, other proteins may be affected by Al, alternatively Al may associate with DNA to directly inhibit cell division. Any one or combination of these possible targets may be the primary site of Al toxicity, whereas others may not contribute to Al toxicity at all, or may be a secondary effect. The rest of this section will cover some of the known effects of Al on each of these cellular components.

(b) The cell wall
Al has been shown to interact with the cell walls of plant roots. For example, Al can bind and crosslink pectins, thereby increasing the rigidity of the cell wall (Foy et al., 1978). Cell elongation occurs when load-bearing bonds in the cell wall are broken down by acidification and displacement of Ca (reviewed in Rengel, 1992). It has been suggested that if Al replaces Ca in the cell wall, H+ can no longer displace the Al to induce the wall loosening which is necessary for elongation. However, it is not known whether or not Al binding to the cell wall can result in direct inhibition of root elongation. The observation that Al applied at the elongation zone of the root did not inhibit root growth (Ryan et al., 1993) argues against a direct inhibition of cell wall expansion by Al.

(c) The plasma membrane and associated proteins
Al could have a number of effects on the plasma membrane or proteins embedded within the membrane. These effects may be difficult to separate, as changes to membrane properties may indirectly affect protein functions or activities, and vice versa. A number of effects on membranes and plasma membrane proteins have been observed, some of which are contradictory, possibly due to different experimental conditions, techniques employed, or the plant species used. For example, Zhao et al. (1987) found that Al affected membrane permeability of Quercus rubra (red oak), with decreased
permeability to water, and increased permeability to substances such as urea. They suggest that these effects were due to Al affecting the lipid packing density either by directly binding to the phospholipids or by reacting with membrane proteins. ATPase activity was found to decrease in microsomes prepared from Al-treated roots of barley relative to untreated controls (Matsumoto et al., 1992). Many studies on Al effects assess the capability of plants to transport various ions as a measure of damage to membranes. Kinraide (1988) found that Al treatment of wheat roots did not diminish the capacity of the roots to extrude protons. This was interpreted to mean that the membrane was not leaky, and the proton pump was intact. In contrast, Matsumoto (1988b) found that proton transport was inhibited by Al. K⁺ net efflux in Al-treated plants has been reported to be increased (in barley, Kasai et al., 1992), decreased (in soybean, Cakmak and Horst, 1991b; Horst et al., 1992; and wheat, Sasaki et al., 1992) or not affected (in Agrostis capillaris, McCain and Davies, 1984). Dissimilar effects on ion transport may be reported due to differences in the portion of the root studied. For example, differences in K⁺ and H⁺ effluxes in response to Al were observed in different areas of wheat roots (Miyasaka et al., 1989). Nichol et al. (1993) found that Al inhibited the influx of Ca, ammonium, and to a lesser extent K⁺, and enhanced the influx of nitrate and phosphate in barley roots. It was suggested that these effects may be caused by Al binding directly to, and affecting the activity of membrane transport proteins. However, it is also possible that Al inhibits cation uptake, and stimulates anion uptake by reducing the negative charge of the plasma membrane by binding to lipid molecules (Nichol et al., 1993). Ryan et al. (1992) measured the changes in ionic currents and ion fluxes in wheat roots and compared the changes that occurred with decreases in root elongation. They found that in some cases inhibition of root elongation occurred before any detectable changes in the current or ion flux, suggesting that the flow of current was not affecting root growth. Examples of effects on both transport proteins (in particular, Ca channels) and more general effects on the plasma membrane (lipid peroxidation) will be considered below.

It has been suggested that Al treatment can cause peroxidation of lipids. This would have several deleterious effects on the plasma membrane, as well as causing the production of free radicals which can damage other cellular components. Al can not directly participate in redox reactions that lead to radical formation, because Al has a fixed oxidation number (III, Moeller et al., 1984). However, in an in vitro study using phospholipids from bovine brain tissue, while Al was found not to be able to stimulate peroxidation by itself, it did stimulate lipid peroxidation induced by Fe at pH 5.5 (Gutteridge et al., 1985). Similar results have been obtained in other in vitro systems, and it has been suggested that Al may stimulate peroxidation by binding to membrane lipids, and altering the packing of the lipids or causing aggregation of liposomes (Ohyashiki et al., 1993; Oteiza, 1994). In a study on soybean roots, Al (as well as high Fe and low Ca) was found to increase lipid peroxidation, and an increase in the enzyme activities of superoxide dismutase and peroxidase were observed (Cakmak and Horst, 1991a; Horst et al., 1992). These enzymes are involved in the degradation of damaging free radicals, and protect cells from oxidative damage. These authors suggest that lipid peroxidation is a consequence of Al stress rather than the primary cause, as root elongation was more sensitive to Al than to lipid peroxidation. However, the initial effects of Al are likely to be restricted to the outer layer of cells. Any
changes in lipid peroxidation in a small subset of cells would be difficult to detect, as the method utilised by Cakmak and Horst (1991a) measured lipid peroxidation in the whole root tip.

Al is known to affect Ca transport. Huang et al., (1992a) found a strong correlation between Al toxicity and inhibition of Ca absorption by Al. Their experiments indicated that Al competitively inhibited Ca uptake, which is consistent with Al acting on Ca channels in the plasma membrane. Removal of Al resulted in a reversal of the Al-induced inhibition of Ca uptake (Huang et al., 1992b). Other researchers have also shown that Al inhibits the uptake of Ca (for example, Bengtsson et al., 1988; Godbold et al., 1988). In a study comparing the effects of various Ca channel blockers to the effects of Al, Rengel and Elliott (1992) found that Al acted in a similar way to verapamil in blocking Ca channels. In contrast, another Ca channel blocker, bepridil (which has a different mechanism of action to that of verapamil) acted in an additive way with Al. In experiments comparing the effects of Al and other cations on uptake of Ca, it has been found that although La by itself had a greater effect on Ca uptake than Al, the two metals together showed an effect similar to Al by itself (Rengel, 1994). This suggests that Al and La have similar effects on Ca uptake, but that Al has greater affinity for the site of action than La, which is a known Ca channel blocker, though its specific mode of action is not known (Lansman, 1990; Tester, 1990; Rengel, 1994). Recently, Piñeros and Tester (1993) reconstituted wheat root Ca channels into artificial lipid bilayers. They found that Al could block these channels completely. At this stage however, a direct correlation between inhibition of Ca channel transport and inhibition of elongation of roots in response to Al has not been made. In fact, it has been reported recently that under some growth conditions wheat root growth can be affected by Al when no reduction of Ca uptake was observed (Ryan et al., 1994).

(d) DNA

It has also been postulated that the primary target of Al may be an intracellular one. For example, as described in section 1.4, Al has been found to be associated with plant nuclei using some types of staining procedures. Nuclei have been isolated from Al-treated roots, and chemical analysis has confirmed the presence of Al (Matsumoto et al., 1977). Al can decrease the rate of DNA synthesis and DNA template activity (Minocha et al., 1992; Morimura and Matsumoto, 1978). In addition, chromatin from Al-treated roots appeared to become condensed and/or aggregated after Al treatment (Matsumoto, 1988a). Fractionation of nuclei showed that the majority of Al present was co-purified with the DNA (Matsumoto, 1991). It has been suggested that Al can bind to the phosphate groups on the DNA, possibly on both strands, thereby stabilising the double helix which would result in a blockage of cell division (Matsumoto, 1991). However, the observation by Wallace and Anderson (1984) that Al inhibited root elongation before cell division ceased tends to suggest that direct inhibition of cell division by Al binding to DNA is not the primary effect of Al toxicity.
(e) Intracellular proteins

There are many other possible intracellular targets for Al toxicity in plants. Many proteins are capable of binding Al, including proteins that may normally bind metal ions with similarities to Al, such as Fe or Mg. For example, Al can bind to and cause conformational changes to transferrin (Grossmann et al., 1993). Al can also inhibit a number of Mg-dependent enzymes, such as hexokinase or G proteins (Macdonald et al., 1987; Macdonald and Martin, 1988; Rengel and Elliott, 1992). Al is likely to bind to proteins through oxygen-donating ligands, rather than through sulfhydryl linkages, as occurs with metals such as Zn, Cu and Cd in metallothioneins (reviewed in Kägi, 1991). Acidic polypeptides also have a good ability to bind Al (Putterill and Gardner, 1988).

One protein which has been a focus of interest as a target for Al toxicity is calmodulin, which is an important component of Ca-regulated processes. Al can bind to calmodulin and cause conformational changes in vitro (Siegel and Haug, 1983). The binding of Al to calmodulin occurs with greater affinity than Ca binding to calmodulin, though Al appears to bind to different sites than Ca. However, binding of Al to calmodulin in vivo has not yet been demonstrated.

It is uncertain whether or not Al within the cytoplasm is likely to be toxic. As the pH of plant cell cytoplasm is in the range of 6.5 to 7.5 (Guern et al., 1991), very little of the Al present is likely to be soluble (Taylor, 1991). Although the concentration of toxic or reactive species of Al in the cytoplasm may be very low, it is unknown what concentrations may be required to bind to intracellular targets. It remains possible that some targets may have very high affinities for Al, such that the protein, or other target is inactivated even by very low levels of intracellular Al. However, there is probably a delay between Al uptake into the apoplasma, and uptake into the symplasm (Zhang and Taylor, 1989 and 1990). This delay suggests that Al effects in the symplasm may not occur fast enough to be the primary cause of Al toxicity. In addition, roots can recover from Al treatment when transferred to Al-free solutions, which suggests that the toxic Al may be acting outside the symplasm where it may be more easily removed (Ownby and Popham, 1989; Bennet and Breen, 1991b).

In summary, there are many potential targets of Al toxicity in plant cells. From information about the uptake of Al, it appears most likely that the primary target of Al is extracellular. However, as both the physiological concentrations and the form of Al present within Al treated cells is unknown, it is difficult to estimate the potential of Al for intracellular damage. However, due to the large capacity of the apoplasma for Al binding, and the potential for far reaching damage to the plasma membrane (e.g. lipid peroxidation), and to proteins within the plasma membrane, it is reasonable to assume that these areas of the cell are likely to be affected by Al before intracellular targets.
1.6. AL TOLERANCE IN PLANTS

As Al toxicity is a significant problem in many crop plants grown on acid soils, improving the tolerance of these plants to Al is a central goal for many breeding programmes. Although liming can alleviate some of the problems of acidic soils, the lime generally does not penetrate to the subsoil regions, and root growth is still affected when the plant grows beyond the topsoil. In addition, liming soils can be an expensive enterprise. Plants that are naturally tolerant to Al therefore represent an important resource, and breeding programmes to improve the tolerance to Al using resistant genotypes have been initiated (e.g. Devine et al., 1976). However, breeding programmes may be of limited use in plants that have no natural resistance to Al. In these cases, it is desirable to understand how other species of plants protect themselves from Al in the hope that it may prove possible to transfer that protection to susceptible species via gene transfer.

There are many possible mechanisms that may confer tolerance to Al in plants (reviewed by Taylor, 1991). Plants may prevent the toxic Al from reaching the target(s) of Al toxicity by chelating the Al externally with secreted substances. The plant may also change the acidity of the root’s immediate environment so that Al is no longer soluble or is not in a toxic form. Some plants may be Al-tolerant due to a change in the binding characteristics of the cell wall or putative Al binding proteins within the plasma membrane, or as a result of changes in the permeability of the plasma membrane. If Al does enter the cell, it may be detoxified by active efflux, or compartmentalised away from susceptible targets. Tolerance may also occur as a result of evolution of Al-tolerant proteins, or expression of Al-binding ligands within the cell (Taylor, 1991).

Much research has been dedicated to investigating the mechanisms by which Al tolerance is conferred on plants (see Taylor, 1991). Unfortunately, much of this research is of limited value as it has focused on comparing plants with differing tolerance to Al, but which are not closely related. It is likely that different species (and even varieties or cultivars within a species) may utilise different mechanisms of Al tolerance, so the most convincing research is that which compares isogenic plants that differ only in their tolerance to Al. This only represents a feasible objective when only one or a few genes are responsible for the Al tolerance. However, in many species (for example, in soybean, Hanson, 1991; alfalfa, Devine et al., 1976; and rice, T. Richardson, personal communication) resistance to Al stress is conferred by many loci. In the case of some wheat cultivars however, only one or a few genes are involved in the increased tolerance to Al of some cultivars over others. For example, the wheat cultivars used in the work described in this thesis, Waalt (Al-tolerant) and Warigal (Al-sensitive), differ in their Al tolerance by a single gene (Larkin, 1987; Wheeler et al., 1992a). These two cultivars have been further interbred to obtain two more isogenic lines, RR and SS (see Ch. 2.7.1), which may be of use in isolating a gene for Al tolerance (Putterill et al., 1991).

To date, the best evidence for any particular plant trait being associated with a plant’s tolerance to Al has been supplied by Delhaize and coworkers (1993b). They used two near-isogenic lines of wheat derived
from the Al-tolerant cultivar Carazinho and the Al-sensitive cultivar Egret. These two lines differ in their Al tolerance by a single gene (Delhaize et al., 1993a). It was found that these two lines differed in their ability to secrete malic acid, a substance known to be able to chelate Al. The Al-tolerant plants secreted five- to ten-fold more malic acid than the Al-sensitive plants. This capability was then examined in a segregating population of seedlings, and there was found to be complete agreement between the malic acid secretion of the seedlings, and their Al tolerance (Delhaize et al., 1993b). Furthermore, these authors precluded the possibility that the malic acid was being secreted in response to phosphate deficiency rather than Al toxicity. Other researchers have also found that some Al-tolerant plants secrete more organic acids (such as malic and citric acids) than Al-sensitive plants. However in most of these studies, either the plants studied were not closely related and the exudation may not have been linked to Al tolerance per se (e.g. Christiansen-Weniger et al., 1992), or the secretion may have been in response to phosphate deficiency or other factors (e.g. Miyasaka et al., 1991; Ojima et al., 1984 and 1989, Koyama et al., 1988).

As mentioned previously, it is quite likely that different Al-tolerant species of plants may detoxify Al using different mechanisms. In the future, as more is known both about the primary target(s) of Al and about exactly how plants avoid Al stress, it may be possible to combine several different approaches to create plants that are more tolerant to Al than those that are available naturally.

1.7. MOLECULAR RESPONSE OF PLANTS TO AL STRESS

Although a large amount of research has been carried out on the physiology of Al stressed plants, very little is known about the molecular response(s) of these plants to Al. When the research described in this thesis was initiated, the molecular research that had been published in this area consisted primarily of characterisation of differences in protein profiles of untreated versus Al treated plants using two-dimensional gel electrophoresis (Delhaize et al., 1991; Ownby and Hruschka, 1991; Picton et al., 1991; Rincón and Gonzales, 1991). Although this work did identify proteins that were induced or repressed by Al treatment, or that appeared to be specific to either the Al-tolerant or -sensitive cultivar, none of these proteins had at that time been further characterised or isolated.

In addition to the protein work, Czamecka et al. (1984) reported that the expression of the soybean heat shock clone pCE54 increased in hypocotyls in response to Al treatment, as well as to Cd and Ni. However in these experiments, Al was added to the culture medium at pH 6.0. At this pH the level of phytotoxic Al would have been low and the induction may have been due to factors other than Al toxicity.

Subsequent to the research presented in this thesis, other work on the molecular basis of Al toxicity in plants has been published. The two-dimensional gel electrophoresis work described above has identified a protein that increases in response to toxic levels of Al, Cd and Cu (Cruz-Ortega and Ownby,
This protein also increases in response to Ca deficiency and low pH, but is not induced by heat shock. Peptide sequencing of this protein (named TAl-18) has indicated that it is homologous to a family of pathogenesis-related proteins. Other protein-based work has identified a 51 kDa protein from a microsomal fraction that accumulates in response to Al in an Al-tolerant cultivar of wheat (Basu et al., 1994) but the identity of this protein remains unknown.

Other research performed in this laboratory has increased our knowledge of the changes in gene expression in response to Al stress in wheat roots. Richards and Gardner (1994) have found that the transcripts of the histone H3 and H4 genes were reduced in response to Al stress, although the amounts of transcripts of two heat shock genes were not affected by Al. Expression of an S-adenosyl-L-homocysteine hydrolase (SHH) gene was also reduced by Al treatment. Th SHH enzyme is involved in the activated methyl cycle in plants, and Richards and Gardner (1994) suggest that the decrease in expression observed may be due to a reduced requirement for methylation in the Al-stressed plant. These results suggest that the plants response to Al stress is different to that induced by heat shock, a conclusion also supported by the fact that the two-dimensional gel electrophoresis work showed that, unlike heat shock, there were very few changes to overall protein synthesis in Al treated plants (Pieton et al., 1991). The expression of histone genes is related to cell division (Osley, 1991), and the fact that the histone gene expression decreased only gradually suggests that cell division was not immediately stopped in Al treated plant roots.

Taken together, there is still a paucity of information on the changes in gene expression that are taking place in the roots of plants stressed with toxic levels of Al. However, many significant changes may not be detectable using the methods discussed here. Many of the effects of Al may be manifested not at the level of gene transcription or translation but by modifications to the proteins themselves. Thus much remains to be learnt about the changes in gene expression that occur in response to Al treatments.

**1.8. MOLECULAR RESEARCH INTO OTHER PLANT STRESSES**

In this section the study of some other plant stresses will be discussed. One of the first observations that environmental conditions could have an effect on gene expression was noted when the polytene chromosomes of *Drosophila* were observed to have a change in puffing pattern induced by heat shock (Ritossa, 1962). Since then a large research effort has been expended in examining the changes in gene expression that occur in response to stresses such as heat shock in both animal cells and more recently in plant systems. In the case of the heat shock response, the induced proteins were isolated, followed at a later date by cloning of heat shock induced genes (reviewed by Scharf et al., 1994). Comparison and analysis of promoters has identified heat shock elements which are necessary for the induction of gene expression in response to heat shock (reviewed in Sorger, 1991). More recently, the first steps in characterising changes in plant gene expression in response to stress have occurred with the use of techniques such as differential screening of cDNA libraries or enrichment of cDNA libraries for stress-
induced clones using subtractive hybridisation techniques (for example, cold-induced genes, Cattivelli and Bartels, 1990; van Berkel et al., 1994; salt-induced clones, Gulick and Dvorák, 1992).

The stress-induced gene expression changes that have been identified have revealed more information concerning what is occurring in the plant in response to stress. In the following section I will briefly discuss the molecular investigations into plant stresses other than Al toxicity. However, it is not possible to cover everything that is known about stress responses in plants here, since many other stresses in plants are being studied at the molecular level. For example, knowledge is being accumulated about plant responses to cold (Guy, 1990; Cattivelli and Bartels, 1992), anaerobic conditions (Dennis et al., 1992), salt stress and dehydration (Ho and Sachs, 1989; Bartels and Nelson, 1994). This work, combined with what is known about the physiology of these stresses, has suggested a number of ways in which tolerance to these stresses can be achieved (Bartels and Nelson, 1994). For example, expression of proteins that have an osmoprotective function may help counter some of the deleterious effects of cold, water and salt stresses.

(a) Heat shock
Heat shock is probably the best characterised stress at the molecular level. In response to heat stress a number of different proteins termed heat shock proteins (HSP) are induced. Some of these proteins are also induced by other stresses such as ethanol, arsenite, heavy metals, amino acid analogues, glucose starvation and calcium ionophores, indicating that there are similarities in the way plants react to some stresses. There are a number of different high molecular weight heat shock proteins which are thought to act as molecular chaperonins. These proteins bind other cellular proteins and are thought to mediate protein folding, transport across membranes and assembly of oligomeric proteins but they may also modulate receptor activities and clathrin uncoating. Ubiquitin, a protein involved in targeting of proteins for degradation, is also induced during heat stress. A number of low molecular weight HSPs are also induced; however, little is currently known of the function of these proteins (reviewed in Vierling, 1991; Schöffl et al., 1993).

Not all types of stress result in the induction of HSPs. As mentioned above they do not appear to be induced by Al stress in root tips, and they are not synthesised in response to anaerobic conditions, cold or salt stress (Czarnecka et al., 1984; Russell and Sachs, 1989; Guy, 1990). Although induced by heat shock, many of the characterised HSPs are also expressed in the absence of stress (for example, Duck et al., 1989), and may perform essential cellular functions; for example, deletion of HSP70 genes in yeast results in cell death (Craig et al., 1987).

Some species of plants can be acclimatised to normally lethal high temperatures if they are first exposed to non-lethal high temperatures (Lin et al., 1984). This thermotolerance is often correlated with an increase in expression of some HSPs, but HSPs may not be sufficient for thermotolerance (Schöffl et al., 1992). It is possible that an increase in the steady state levels of a combination of the HSPs may be
used to overcome the effects of heat stress, allowing plants to grow in environments subject to high temperatures.

(b) Wounding
In cultivated plants, wounding occurs when plants are pruned or harvested. Insect pests also cause wounding, which can allow entry to viral and fungal pathogens. These invasive agents can cause substantial damage to plant tissue. Synthesis of a number of different host proteins is induced by this type of damage. For example, proteinase inhibitors are induced in response to physical wounding. Ingestion of these proteinase inhibitors by feeding insects can interfere with the digestive processes of the insect, and may dissuade the insect from feeding, or even result in the insect's death (Ryan, 1990).

Many of the enzymes within the phenylpropanoid pathway are also induced by wounding, as well as by other stresses such as UV light and heavy metals (Ohl et al., 1990; Schuch, 1992). The enzymatic products of these genes include flavonoids (for example, anthocyanins and tannins), isoflavonoids, soluble esters, coumarins, lignin, suberin and other cell wall bound phenolics (Jones, 1984; Hahlbrock and Scheel, 1989). Some of these secondary metabolites, such as the isoflavonoid phytoalexins, are toxic to invading fungi and their production may act as a defence mechanism (Dixon et al., 1983). Other products such as the flavonoids can act as UV protectants. Several of the downstream products from the phenylpropanoid pathway are components of cell walls and may help to repair damage already inflicted, or to seal off damaged cells from the rest of the plant tissue.

Mechanical wounding can also induce the synthesis of cell wall proteins such as hydroxyproline-rich glycoproteins and proline-rich proteins (Bradley et al., 1992). The induction of these proteins may also help in the repair of damaged cell walls. Invading fungi or other pathogens can induce the synthesis of proteins which are termed 'pathogenesis-related'. The functions of all the pathogenesis-related proteins are not known, but they include chitinases and glucanases which are enzymes capable of degrading fungal cell walls (Somssich, 1994).

Overexpression of these types of wound- and pathogen-induced genes may improve the resistance of plants to invading pathogens. For example, the expression of foreign phytoalexins in transgenic tobacco plants enabled the plants to become more resistant to infection by Botrytis cinerea (Hain et al., 1993). Expression of chitinase genes in transgenic plants has also increased the resistance of the plants to infection by the fungal pathogen Rhizoctonia solani (Broglie et al., 1991; Benhamou et al., 1993). Characterisation of the promoter elements activated by pathogen attack or the wounding response might enable these promoter regions to be used to direct the expression of other protective proteins at wound sites in plants (Moffat, 1994).
(c) Heavy metal stress
The molecular response of plants to most nutritional or mineral disorders is largely unknown. However work in bacterial or yeast systems can complement our limited knowledge of mineral deficiencies and toxicities in plants. For example, little is currently known of the molecular response of plants to phosphate starvation, but phosphate mobilisation, uptake and metabolism have been characterised in yeast and bacteria (Goldstein, 1992). Similarly, relatively little is known about heavy metal toxicities in plants. In animals and yeast, metallothioneins are induced in response to toxic levels of heavy metals. Metallothioneins can bind metal ions to detoxify them, and are also thought to play a role in Zn and Cu metabolism (Hamer, 1986). Until recently, there was no evidence that plants utilised metallothioneins for metal detoxification; instead phytochelatins appear to fulfil this role (Tomsett and Thurman, 1988). Phytochelatin synthesis is induced by metals such as Cd, Cu and Zn, but not by Al (Grill et al., 1987). Recently, plant homologues to animal and fungal metallothioneins have been isolated; but the induction of these genes in response to toxic levels of metals has not been well characterised (Robinson et al., 1993). As indicated above, other stress-induced genes are known to be induced by some toxic metal conditions, such as HSPs (Czarnecka et al., 1984; Delhaize et al., 1989) and phenylalanine ammonia-lyase (PAL, Ohl et al., 1990). Perhaps overexpression of these types of genes, or metallothioneins of animal origin may alleviate metal toxicity. For example, expression of a mouse metallothionein gene in transgenic tobacco plants has been shown to confer cadmium tolerance on the plants (Pan et al., 1994).

(d) Oxidative stress
Oxidative stress occurs when plants are subjected to elevated levels of free radicals or to other active derivatives of oxygen such as the superoxide radical anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen. This occurs during senescence, wounding, ozone toxicity and possibly in response to many other stresses such as high light, chilling, drought, nutrient deficiency, pathogens and sulphur dioxide (Polle and Rennenberg, 1993; Foyer et al., 1994). In senescence, it is known that the increase in radicals in plant tissues is correlated with a concomitant decline in proteins with antioxidant properties such as superoxide dismutases (Thompson et al., 1987). Free radicals in plant tissues are potentially very damaging, as they can have a plethora of deleterious effects on many different essential cellular components such as lipids, proteins and DNA. For example, lipid peroxidation can lead to increased membrane permeability, decreased membrane fluidity and production of non-bilayer lipids (Thompson et al., 1987).

There are a number of different substances with antioxidant properties that have the potential to protect plant tissues in conditions which result in oxidative stress. These include vitamins E and C, glutathione and also the enzymes catalase, superoxide dismutases and peroxidases (Thompson et al., 1987). Approaches to increase the levels of these substances in plants may help to decrease their susceptibility to oxidative stress, and perhaps also to delay senescence in some tissues. However, a coordinated approach will probably be required in most plants. For example, superoxide dismutase catalyses the conversion of superoxide radical anion (together with protons) into hydrogen peroxide and oxygen
(Rabinowitch and Fridovich, 1983). As hydrogen peroxide is itself potentially damaging, particularly because it gives rise to the more toxic hydroxyl radical in the presence of metals such as Fe (via the Haber Weiss reaction), then the overproduction of superoxide dismutase by itself may not have a protective function. However, in some systems overexpression of superoxide dismutase has improved the stress tolerance of plants (e.g. Gupta et al., 1993) although there are other examples where this has not proven beneficial (reviewed in Foyer et al., 1994). As oxidative stress may occur under many different stress conditions, it will be interesting to test the protection of plants that have been engineered to withstand oxidative stress against a range of environmental stresses.

(e) Summary
The molecular changes occurring in stressed plants can indicate the type of damage that occurs during a given stress. As mentioned in the examples given above, heat stress, which might be expected to denature some cellular proteins, results in the induction of proteins that may help retain the correct conformations of other proteins, and also in the induction of proteins that are used to identify inactivated proteins for degradation. In the case of wounding or pathogen attack, substances which are potentially toxic to feeding insects or invading fungi are produced. When plants are subjected to high levels of light they synthesise UV protectant compounds such as flavonoids. Collectively, these observations suggest that developing a better understanding of what molecular events take place in response to any given stress, for example Al toxicity, may enable predictions to be made about the types of damage which cells experience.

Much of the molecular research directed towards characterising stresses suffered by plants is still at an early stage. Stress-induced cDNAs or proteins have been identified for some stresses, but the function of many of these genes is still unknown. In addition, the signal transduction pathways by which plants respond to external stress stimuli are only now being examined (Leigh, 1993). Very little of the information gained from studies into stress has been applied to engineering stress-resistant plants. The increase of knowledge over the next few years should prove interesting as many hypotheses for improving plant protection are tested.

1.9. AIMS OF THIS THESIS
Although there has been a large amount of research into the physiology of Al stress, two central unanswered issues remain. The first is determining exactly how Al tolerant plants resist Al stress, in the hope that in the future we may be able to transfer these abilities with the use of transgenic technologies, to plants with little natural protection from Al stress. The second is characterising processes which occur in plants stressed with Al so that the target of Al toxicity might be identified. It is towards this second issue that the work described in this thesis has been directed.
The initial aim of this work was to characterise the changes in gene expression that occur in response to Al stress. The approach taken has been to differentially screen a cDNA library constructed from the root tips of Al-treated plants. Wheat was chosen as the plant system for these studies because its responses to Al are well characterised and because of the availability of sensitive and resistant isogenic lines. In addition, a cDNA library constructed from the root tips of Al-stressed wheat was available. Other researchers in this laboratory currently working with wheat had established methods for RNA isolation and hydroponic growth of the plant. The work describing the genes isolated and their response to Al stress is presented in Chapter 3.

The second aim of this thesis was then to discover whether or not the Al-induced genes were specific to Al stress, or whether other environmental stresses were also effective at inducing the expression of these genes. In addition, it was of interest to discover whether the Al-induced genes were expressed in similar cell types, and whether the cellular pattern of expression of these genes changed in response to Al stress. The work directed at answering these questions, through northern analyses and in situ detection of mRNA, is presented in Chapter 4.

Ultimately, it is desirable to know what the function of the Al-induced proteins is. Some information towards this can be obtained from the work presented in Chapters 3 and 4. In addition, the work presented in Chapter 5 has initiated research directed at furthering our knowledge of the properties of the proteins encoded by the Al-induced genes. Specifically, Chapter 5 describes the cloning of the coding regions from four of the Al-induced genes into a bacterial expression vector. The recombinant proteins were isolated, and used in some preliminary experiments aimed at understanding the biochemical functions of the proteins encoded by the Al-induced genes.
2.1. ENZYMES, CHEMICALS AND RADIOCHEMICALS

Enzymes and chemicals used in this work were purchased from the following suppliers: Amersham, BDH Chemicals New Zealand Ltd, Bethesda Research Laboratories (BRL), Boehringer Mannheim, Pharmacia LKB, Promega Biotec, Sigma Chemical Company, US Biochemical (USB).

Radiolabelled dCTP [α-32P] was purchased from New England Nuclear and also from ICN Biomedicals, Inc. The dCTP from both suppliers had a specific activity of 3000 Cimmol⁻¹ and was supplied at a concentration of 10 CiL⁻¹.

2.2. BUFFERS AND SOLUTIONS

Unless otherwise stated, all percentages are weight / volume.

coupling buffer 0.5 M NaCl, 0.1 M NaHCO₃ (pH 8.3)
Denhardt's solution 0.02% Ficoll, 0.02% PVP, 0.02% BSA
DNA hybridisation buffer 1% SDS, 1 M NaCl, 10% dextran sulphate
5X ligase buffer 25% PEG 8000, 5 μM rATP, 5 μM DTT, 50 mM MgCl₂, 250 mM Tris-Cl (pH 7.6)
MTPBS 150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄
PBS 0.1 M NaCl, 0.1 M sodium phosphate (pH 7.5)
phenol water saturated phenol
phenol/chloroform phenol:chloroform:isoamyl alcohol (25:24:1)
phenol (TLE buffered) liquefied Ultra-pure™ phenol extracted three times with TLE
SDS gel loading buffer 0.05 M Tris-Cl (pH 6.8), 0.1 M DTT, 2% SDS, 0.1% bromophenol blue, 10% (v/v) glycerol
SM 100 mM NaCl, 8 mM MgSO₄, 0.01% gelatin, 50 mM Tris-Cl (pH 7.5)
solution I 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl (pH 8.0)
solution II 0.2 M NaOH, 1% SDS
solution III 3 M potassium / 5 M acetate
SSC 150 mM NaCl, 15 mM tri-sodium citrate (pH 7.0)
TBE 90 mM H₃BO₃, 2 mM EDTA, 90 mM Tris-Cl (pH 8.0)
TBS-T 0.1% Tween-20, 137 mM NaCl, 20 mM Tris-Cl (pH 7.6)
TC 150 mM NaCl, 2.5 mM CaCl₂, 50 mM Tris-Cl (pH 8.0)
TE 1 mM EDTA, 10 mM Tris-Cl (pH 8.0)
TLE  0.2 M Tris-Cl, 0.1 M LiCl, 5 mM EDTA (pH 8.2)
TS   0.4 M NaOH, 0.6 M NaCl

2.3. VECTORS

2.3.1. BACTERIOPHAGE
A cDNA library constructed by K. Richards and A. Königstorfer of this laboratory was used in this work. mRNA isolated from the root tips of Warigal plants treated with 10 μM Al for 2 d was ligated into the bacteriophage Lambda ZAP® II. The helper phage R408 was used for the in vivo excision of pBluescript plasmids from Lambda ZAP® II clones. Both Lambda ZAP® II and R408 were purchased from Stratagene.

2.3.2. PLASMID
Plasmids used during the course of this work are listed below:

<table>
<thead>
<tr>
<th>plasmid</th>
<th>supplier / reference</th>
<th>use / description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSPORT 1</td>
<td>BRL</td>
<td>cDNA library construction, subcloning</td>
</tr>
<tr>
<td>pBluescript</td>
<td>Stratagene</td>
<td>subcloning</td>
</tr>
<tr>
<td>pBC</td>
<td>Stratagene</td>
<td>subcloning</td>
</tr>
<tr>
<td>pGEX-2T</td>
<td>Pharmacia LKB</td>
<td>production of <em>Escherichia coli</em> fusion proteins</td>
</tr>
<tr>
<td>pTA250.2</td>
<td>Appels and Dvorák, 1982</td>
<td>contains a 1.2 kb Sal I to BamH I fragment of the wheat 26S rRNA gene cloned into the plasmid pBR322</td>
</tr>
<tr>
<td>wali1</td>
<td>this work, Table 3.3</td>
<td>cDNA encoding a plant MLP in pSPORT 1</td>
</tr>
<tr>
<td>wali2</td>
<td>this work, Table 3.3</td>
<td>cDNA encoding a cysteine-rich protein of unknown function in pSPORT 1</td>
</tr>
<tr>
<td>wali3</td>
<td>this work, Table 3.3</td>
<td>cDNA encoding a putative Bowman-Birk proteinase inhibitor in pSPORT 1</td>
</tr>
<tr>
<td>wali4</td>
<td>this work, Table 3.3</td>
<td>partial cDNA encoding PAL in pSPORT 1</td>
</tr>
<tr>
<td>wali5</td>
<td>this work, Table 3.3</td>
<td>cDNA encoding a putative Bowman-Birk proteinase inhibitor in pSPORT 1</td>
</tr>
<tr>
<td>1.4</td>
<td>this work, Table 5.2</td>
<td>fusion of wali1 and GST in pGEX-2T</td>
</tr>
<tr>
<td>2.13</td>
<td>this work, Table 5.2</td>
<td>fusion of wali2 and GST in pGEX-2T</td>
</tr>
<tr>
<td>3.7</td>
<td>this work, Table 5.2</td>
<td>fusion of wali3 and GST in pGEX-2T</td>
</tr>
<tr>
<td>5.5</td>
<td>this work, Table 5.2</td>
<td>fusion of wali5 and GST in pGEX-2T</td>
</tr>
</tbody>
</table>

2.4. OLIGONUCLEOTIDES
The oligonucleotides used to clone wali2, -3 and -5 into pGEX-2T with the use of PCR are listed below (oligonucleotides supplied as part of cDNA construction kits, or used for sequencing purposes only are not included):
27

<table>
<thead>
<tr>
<th>name</th>
<th>sequence (5'-3')</th>
<th>use (see Ch. 5.2.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCS1</td>
<td>CCT GGT CAT GGG ATC CCT CTC ACA</td>
<td>used to clone wali3 and wali5 into pGEX-2T</td>
</tr>
<tr>
<td>KCS2</td>
<td>CAC CAA CCA GGG ATC CAT GGG TAT</td>
<td>used to clone wali2 into pGEX-2T</td>
</tr>
<tr>
<td>forward</td>
<td>GTT TTC CCA GTC ACG AC</td>
<td>used to clone wali2, -3 and -5 into pGEX-2T</td>
</tr>
</tbody>
</table>

2.5. BACTERIA

2.5.1. BACTERIAL STRAINS

The following Escherichia coli strains were used during the course of this work. Details on the genotypes of these strains can be found in Sambrook et al. (1989).

<table>
<thead>
<tr>
<th>strain</th>
<th>use</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>host for propagation of plasmids, and production of fusion proteins</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>host for Lambda ZAP® II clones, allows blue / white screening for inserts</td>
</tr>
<tr>
<td>PLK-F'</td>
<td>host for Lambda ZAP® II clones, strain used for amplification</td>
</tr>
</tbody>
</table>

2.5.2. MEDIA

Descriptions below are for liquid media. When a solid support was required for the growth of bacteria, 1.5% Davis agar was added as a solidifying agent.

L

<table>
<thead>
<tr>
<th></th>
<th>1% bacto-tryptone, 0.5% yeast extract, 1% NaCl (pH 7.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2XL</td>
<td>2% bacto-tryptone, 1% yeast extract, 0.1% NaCl, 10 mM glucose (pH 7.2)</td>
</tr>
<tr>
<td>NZCYM</td>
<td>1% bacto-casitone, 0.1% casamino acids, 0.5% yeast extract, 0.5% NaCl, 0.2% MgSO₄ (pH 7.3)</td>
</tr>
<tr>
<td>Terrific broth</td>
<td>1.2% bacto-tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄</td>
</tr>
</tbody>
</table>

2.5.3. ANTIBIOTICS

Antibiotics were added to liquid and solid media as a selection for the maintenance of plasmids. Ampicillin was used at a concentration of 100 mgL⁻¹, tetracycline at 12.5 mgL⁻¹, and chloramphenicol was used at 30 mgL⁻¹. Chloramphenicol was also used (at a concentration of 170 mgL⁻¹) for the amplification of plasmids (not containing a gene for chloramphenicol resistance), while restricting bacterial growth.

2.5.4. BACTERIAL GROWTH AND STORAGE

Bacteria were grown in liquid media containing any required antibiotics at the concentrations designated above. Bacteria were inoculated into the sterile media, contained within a sterile tube or flask with a capacity of at least five volumes that of the media. Growth of the bacteria, except where
otherwise stated, was for 16 to 18 h at 37°C with constant shaking to ensure adequate aeration of the culture.

Bacteria were streaked to single colonies on solid media containing any antibiotics required for maintenance of plasmids. The plates were incubated for 16 to 18 h at 37°C in an inverted position. Bacteria were grown on solid media for short term storage (the plates were kept at 4°C for up to six months).

For longer term storage of bacteria, 70 μL of DMSO was added to 930 μL of an overnight culture and gently mixed. The bacteria / DMSO mix was then stored at -80°C.

2.5.5. PREPARATION OF COMPETENT CELLS
Competent DH5α cells were prepared using a method supplied by D. Alexander (Calgene Inc., Davis, USA, personal communication). DH5α bacteria were grown overnight at 30°C in 2XL broth. An aliquot of this culture was used to inoculate 100 mL of prewarmed (30°C) 2XL broth, which was then grown at 30°C with shaking (150 - 200 rpm) until the culture reached an OD₆₀₀ of 0.45 - 0.55. The cells were chilled in an ice/water mix for 2 h then harvested by centrifugation at 2 500X g for 15 min at 4°C. The pellets were resuspended gently in 50 mL of a freshly made, chilled salt solution containing 100 mM CaCl₂, 70 mM MnCl₂, 40 mM sodium acetate at pH 5.5. The resuspended cells were incubated in the ice/water for a further 45 min before centrifugation at 1 800X g for 10 min at 4°C. The pellets were again resuspended in a total of 5 mL of the chilled salt solution. Glycerol (80%) was added dropwise with gentle swirling to give a final concentration of 15%. Aliquots of the cells (200 μL) were dispensed and stored at -80°C until required.

2.5.6. TRANSFORMATION OF E. COLI
Competent cells prepared by the above method were transformed as follows: DNA (up to 10 ng) in a volume of 100 μL was added to a recently thawed 200 μL aliquot of competent cells. The bacteria/DNA mix was incubated on ice for 30 min, followed by heatshock at 37°C for 5 min. A prewarmed aliquot (3.7 mL) of 2XL broth was added, and the cells were incubated at 37°C for 100 min at 40 rpm. Portions of the transformation were then plated on L plates containing the appropriate antibiotic for selection of transformants. To screen for the presence of inserts in the plasmids pSPORT 1, pBluescript, or pBC, 20 μL of IPTG (20 gL⁻¹ aqueous solution) and 40 μL of X-Gal (20 gL⁻¹ in dimethylformamide) were spread on the L plates. Inserts in the multiple cloning sites of these plasmids disrupted the β-galactosidase gene causing the colonies to be white in colour, compared to the blue colour of colonies containing plasmids with no insert.

MAX Efficiency DH5α™ Competent Cells (from BRL) were also used when cells of especially high competency were required. These cells were transformed according to the instructions supplied by the manufacturer.
2.6. BACTERIOPHAGE

2.6.1. PREPARATION OF PLATING BACTERIA
PLK-F' or XL1-Blue cells from a freshly streaked (less than a week old) L plate with tetracycline, were used to inoculate 20 mL of L broth containing 200 µL of 20% maltose and 200 µL of 1 M MgSO₄. The culture was grown overnight at 30° C with shaking. The cells from the overnight culture were harvested by centrifugation at 1000× g for 10 min, then resuspended in 10 mM MgSO₄ to give an OD₆₀₀ of 1.0. The plating bacteria were stored at 4° C and used for up to two weeks.

2.6.2. PLATING BACTERIOPHAGE
Bacteriophage (stored in SM buffer) were plated on both large (150 mm diameter) and small plates (80 mm diameter). The following instructions give amounts used for the large plates, with bracketed values giving the amounts used for the small plates. Up to 10 000 pfu (1 000 pfu) were incubated with 300 µL (200 µL) of plating bacteria and 300 µL (200 µL) of 10 mM MgSO₄ at 37° C for 30 min. The bacteriophage/bacteria mix were then added to 6.5 mL (3 mL) of 0.7% agarose in NZCYM (at 42° C), and poured onto prewarmed (37° C) NZCYM plates. Plates were incubated overnight in a humid container.

2.6.3. PICKING BACTERIOPHAGE PLAQUES
A sterile glass Pasteur pipette was inserted into the agar so that it surrounded the plaque of interest. The plaque was separated from the surrounding agar using gentle suction from the pipette bulb and ejected into 200 µL of SM buffer. Chloroform was added (50 µL) to lyse any remaining bacterial cells, and the plaques were left at room temperature for 2 h to allow the bacteriophage particles to diffuse into the buffer. The bacteriophage were stored at 4° C.

2.6.4. MAXIPLAQUES
For rapid rescreening of large numbers of bacteriophage, a large plaque assay was used (Meeks-Wagner et al., 1989). XL1-Blue plating bacteria (400 µL) were added to 6.5 mL 0.7% agarose in NZCYM, and poured onto a prewarmed large NZCYM plate. Once the top agarose had set, 0.5 µL aliquots of each bacteriophage eluate were spotted onto the plate at 2 to 3 cm intervals. The plate was then incubated overnight at 37° C in a humid container.

2.6.5. EXCISION OF PHAGEMID
Excision of the pBluescript phagemid from a bacteriophage of interest was performed essentially as described in the ZAP-cDNA™ Synthesis Kit instruction manual supplied by Stratagene, using the helper phage R408. The f1 bacteriophage origin of replication has been cloned into the Lambda ZAP® II bacteriophage in two parts (a site of initiation and a site of termination). When E. coli is infected with both Lambda ZAP® II and R408 bacteriophage, the helper phage synthesises the DNA
between the initiator and terminator sites, and a single copy of the DNA is released and circularised. The DNA that is released from the Lambda ZAP® II bacteriophage corresponds to the pBluescript phagemid and contains the sequences cloned into Lambda ZAP® II.

2.7. PLANTS

2.7.1. PLANTS USED IN THIS WORK

The wheat (*Triticum aestivum* L.) cultivars Warigal (Al-sensitive) and Waalt (Al-tolerant) were used in this work. Waalt is an Al-tolerant segregant derived from Warigal (Larkin, 1987). Waalt and Warigal differ in their Al-tolerance by a single semi-dominant gene (Wheeler et al., 1992a). Two other, more isogenic lines, SS and RR were also used. These lines were derived as follows: An heterozygous plant from a F2 population of seeds (from a cross between Waalt and Warigal) was backcrossed twice to Warigal. A second backcross was then allowed to self, and homozygous sensitive (SS) and resistant (RR) plants were identified by progeny analysis. SS and RR should be 15/16 identical genetically (Putterill et al., 1991).

2.7.2. MEDIA

For most experiments, wheat plants were grown in Ruakura medium, a low ionic strength growth medium containing 0.1 mM MgSO4, 0.3 mM KNO3, 0.2 mM NaCl, 0.15 mM NH4NO3, 5 μM FeCl3, 5 μM (NH4)H2PO4, 5 μM H3BO3, 1 μM MnSO4, 1 μM ZnSO4, 0.2 μM CuSO4, 0.2 μM CoCl2, 0.5 mM CaSO4, adjusted to pH 4.3 with HCl. This medium approximates soil solution composition (Edmeades et al., 1985). For experimental treatments with Al or other toxic metals, the metal of interest was added to the medium from stock solutions to give the desired concentration. Details of the stock solutions used and working concentrations are given below:

<table>
<thead>
<tr>
<th>metal</th>
<th>stock solution</th>
<th>working strength(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>20 mM Al2(SO4)3</td>
<td>10 or 100 μM1</td>
</tr>
<tr>
<td>Cd</td>
<td>250 mM CdCl2</td>
<td>2.5 - 250 μM</td>
</tr>
<tr>
<td>Fe</td>
<td>20 mM FeCl3</td>
<td>50 μM</td>
</tr>
<tr>
<td>Zn</td>
<td>0.3 M ZnSO4</td>
<td>50 - 200 μM</td>
</tr>
<tr>
<td>Cu</td>
<td>50 mM CuSO4</td>
<td>1 - 2.5 μM</td>
</tr>
<tr>
<td>In</td>
<td>0.5 M InCl3</td>
<td>2.5 μM</td>
</tr>
<tr>
<td>Ga</td>
<td>278 mM GaCl3</td>
<td>2.5 μM</td>
</tr>
<tr>
<td>La</td>
<td>5 mM LaCl3</td>
<td>2.5 - 5 μM</td>
</tr>
</tbody>
</table>

For experiments where plants were grown in media lacking one or more essential nutrients, or with low levels of one nutrient, the following media were used (most are based on Ruakura medium):

---

1 10 or 100 μM Al solutions contain 5 or 50 μM Al2(SO4)3 respectively
<table>
<thead>
<tr>
<th>treatment</th>
<th>composition²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca only</td>
<td>0.4 mM CaCl₂</td>
</tr>
<tr>
<td>low Ca</td>
<td>Ruakura medium with only 10 μM CaSO₄ but with 0.49 mM Na₂SO₄</td>
</tr>
<tr>
<td>no P</td>
<td>Ruakura medium with no (NH₄)H₂PO₄</td>
</tr>
<tr>
<td>no Mg</td>
<td>Ruakura medium with no MgSO₄ but with 1 mM Na₂SO₄</td>
</tr>
<tr>
<td>no Fe</td>
<td>Ruakura medium with no FeCl₃</td>
</tr>
<tr>
<td>no K</td>
<td>Ruakura medium with no KNO₃ but with 0.3 mM NaNO₃</td>
</tr>
</tbody>
</table>

2.7.3. **HYDROPONIC GROWTH AND TREATMENTS OF PLANTS**

Wheat seeds were surface sterilised in 1.5% hypochlorite for 10 min, then rinsed three times with sterile distilled water. The seeds were spread over 10 cm by 10 cm stainless steel grids (80 - 100 seeds per grid), placed on damp filter paper in a sealed container, and left to germinate in the dark in a controlled environment chamber on a cycle of 16 h at 22°C, 8 h at 18°C for 2 d. After this time, the germinated seeds on their grids were placed over plastic pots containing 300 mL of Ruakura medium. The seedlings were then grown in the controlled environment chamber on a cycle of 16 h days (65% RH, 22°C, 190 μmolm⁻²s⁻¹ light) and 8 h nights (85% RH, 18°C, in the dark). After 2 d the seedlings were transferred to larger pots containing 1 L of Ruakura medium, which was changed daily thereafter. The plants were grown for an additional 3 d in the larger pots before the commencement of any treatments. Throughout the growth and treatments (except for the low O₂ treatment) of the plants the medium was aerated continuously using an aquarium pump connected via plastic tubing to 22 gauge needles inserted into the sides of the pots.

Most treatments used throughout this work had a duration of 2 d. The exceptions to this were the time courses of Al and Cd treatments, the heatshock treatment, and one of the wounding timepoints. To commence a treatment, the plants were transferred to a 1 L pot containing the appropriate media for a treatment (see Ch. 2.7.2 above). When plants were shifted to a medium lacking a component of the medium they were previously grown in (for example in nutrient deficiency treatments, or in recoveries from Al or Cd treatments in the time courses), their roots were rinsed gently in sterile MilliQ water (or in unsupplemented Ruakura medium in the case of the Al time course).

In the time course experiments, the seedlings remained in the Cd (50 μM) or Al (10 μM) supplemented media for 0.5 to 96 h. To simulate recovery from Al or Cd, some plants were grown in the presence of the toxic metal for 24 h, then transferred to unsupplemented Ruakura medium and grown for a further 4 to 96 h.

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² all media were adjusted to pH 4.3 with HCl
For wounding of root tissue, the terminal 3 - 5 mm of the root tips were excised and incubated for 6 h or 2 d in aerated Ruakura medium. Leaf tissue was wounded by cutting the leaf blade into 0.5 - 1.0 cm segments and incubated for 6 h or 2 d in petri dishes lined with 3MM Whatman filter paper dampened with Ruakura medium. Some crushing of both the root and leaf tissue also occurred due to handling with forceps.

In the heat shock treatments, grids of seedlings were transferred to pots containing Ruakura medium preheated to 42° C. These plants were then grown for an additional 4 h while the medium was maintained at 42° C (in a waterbath operated within the controlled environment chamber).

Two other treatments were used where the plants were grown in Ruakura medium. In low O₂ treatments, the medium was degassed under a vacuum for 2 h, and this medium was not aerated during plant growth. The oxygen content of the low O₂ and the control treatments were monitored before the plants were placed in the medium, and after the plants had grown in the medium for 24 h (i.e. before the medium was replaced). These oxygen measurements were taken using a YSI (model 54 ARC) dissolved oxygen meter, according to the instructions supplied by the manufacturer. In dark treatments, the plants were transferred (in their pot of Ruakura medium) to a wire cage surrounded by metal foil within the controlled environment chamber. The foil was loosely layered to allow some circulation of the air, while blocking out all light. The temperature within this cage was monitored to ensure that it remained similar to that of the rest of the chamber.

When root growth was monitored throughout a treatment, 10 to 15 randomly chosen roots (only the primary roots of the plants were selected) from plants growing within each pot, were measured with a ruler before and after the treatment. The means and standard errors of the root growth were calculated using standard statistical methods (Sokal and Rohlf, 1981).

To harvest samples, the seedlings were removed from the medium and gently blotted dry on paper towels. Root tips (3 - 5 mm from the apex) and leaf tissue (the whole leaf blade) were excised from the plants and immediately frozen in liquid nitrogen. To reduce the effect of pot-to-pot variation in the time course experiments, approximately 20 to 30 seedlings were collected from each of three different pots for each time point. In the Cd time course, the seedlings used in the control (0 h) were harvested from each of the experimental pots before the addition of Cd to the media. The seedlings in the control time point in the Al time course, and also the seedlings used in all other treatments were collected from one pot only. Tissue was stored at -80° C until required for RNA or protein isolation.

2.7.4. FIXATION, EMBEDDING AND SECTIONING
When tissue was required for in situ hybridisations, it was harvested using fresh, sharp scalpel blades to minimise any tearing of the tissue that might otherwise occur (O'Brien and McCully, 1981). Tissue was cut into sections no longer than 5 mm in length (root apices and leaf blades). Once the plant
tissue was excised it was immediately immersed in freshly prepared paraformaldehyde fixative (4% paraformaldehyde, 50 mM PIPES pH 6.8). Fixation proceeded for 18 h at 4°C with stirring, after which the tissue was transferred to 50% ethanol and incubated at 4°C for 4 h. The 50% ethanol was then replaced with 70% ethanol, and the tissue incubated for at least 1 h at 4°C. The plant tissue was wrapped in paper tissues and enclosed in plastic Tissue-Tek® cassettes. The samples were then dehydrated through 70%, 95% and 100% ethanol, transferred into xylene and infiltrated with paraffin (Paraplast) using a Tissue-Tek® Vacuum Infiltration Processor (operated by B. Davy, Histology Unit, School of Biological Sciences, University of Auckland). Tissue was then arranged in cassettes, and embedded in paraffin. Sections were taken using a R. Jung (Heidelberg) Rotary Microtome at 8 μm thickness. Sections were floated on warm (42°C) distilled deionised water, then taken up on Superfrost® Plus slides (supplied by Salmond Smith Biolabs). The sections were baked onto the slides for 18 to 48 h at 42°C, after which they were stored in a vacuum dessicator at room temperature until required. Unsectioned, embedded tissue was also stored at room temperature in the vacuum dessicator.

2.7.5. **HAEMATOXYLIN STAINING**
Haemotoxylin staining of the roots of untreated and Al-treated plants was occasionally undertaken to check the effect of the treatments on the plants (Larkin, 1987; Ownby, 1993). The haematoxylin stain was made up fresh before each use by dissolving 0.2 g haematoxylin and 0.021 g NaIO₃ in 100 mL distilled, deionised water for 1 h in the dark. Plants were rinsed three times in distilled deionised water for 15 min, stained for 15 min, then rinsed three times in distilled deionised water for 15 min again. The roots were then examined by eye, and by using a dissecting microscope. A stained tip indicated sensitivity to the Al treatment.

2.7.6. **TOLUIDINE BLUE STAINING**
Sections of wheat root tips and leaf tissue were stained with toluidine blue to check the integrity of the sections. Dewaxed, rehydrated sections were flooded with toluidine blue stain (0.05% toluidine blue in sodium acetate buffer, pH 4.4), and incubated for 30 s to 2 min. The sections were then rinsed under a running tap, mounted with aqueous mounting medium and examined under a microscope.

2.8. **ISOLATION OF PLASMID DNA**
Plasmid DNA was isolated using a range of different methods depending on requirements. DNA suitable for sequencing was isolated using the Magic™ Minipreps DNA purification system from Promega, according to the manufacturer’s instructions. Quick screening of colonies during subcloning was performed using a one step miniprep method developed by Chowdhury (1991). Plasmid DNA required for other purposes was isolated using the alkaline lysis extraction procedures (both small and large scale) detailed in Sambrook *et al.* (1989).
2.9. ISOLATION OF TOTAL PLANT RNA
To minimise contaminations with RNAses during extractions of RNA the following precautions were used: clean gloves were worn at all times; solutions (except those containing Tris-Cl) were treated with 0.1% DMDC and autoclaved; Tris-Cl solutions were made up using DMDC-treated water and chemicals kept exclusively for RNA work; corex tubes were soaked in SDS; and all glassware and mortars and pestles were baked at 200° C for 4 h prior to use.

Total plant RNA was isolated using a scaled down version of a phenol/SDS method (Ausubel et al., 1987). Root tips or leaf tissue were ground under liquid nitrogen using mortars and pestles. The tissue was then added to a centrifuge tube containing 2.25 mL TLE, 0.25 mL 10% SDS and 1.25 mL phenol (TLE buffered) on ice. The mixture was vortexed, 1.25 mL chloroform was added, and the mixture was vortexed again. The plant cell debris and the phenol was separated from the aqueous phase by centrifugation at 12 700X g, for 15 min at 4° C. The aqueous phase was extracted with 1.25 mL phenol (TLE buffered) and 1.25 mL chloroform, centrifuged as described above; then extracted with 2.5 mL chloroform and centrifuged again. The volume of the aqueous phase was estimated, and 0.25 volumes of 10 M LiCl was added. The RNA was allowed to precipitate overnight at 4° C. The RNA was collected by centrifugation at 15 900X g for 30 min at 4° C, washed with 2 mL 2 M LiCl, then centrifuged at 15 900X g for 20 min at 4° C. The pellet of RNA was resuspended in 1 to 2 mL of water, and reprecipitated by the addition of 0.25 volumes of 10 M LiCl, followed by incubation at 4° C for at least 4 h. The RNA was recovered by centrifugation at 15 900X g for 30 min at 4° C, and washed with 2 M LiCl as described above. The pellet was resuspended in 1 to 2 mL of water and precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol at -20° C overnight. The RNA was again collected by centrifugation at 15 900X g for 30 min at 4° C, washed with 70% ethanol, dried and resuspended in water. The RNA was stored at -80° C.

2.10. ISOLATION OF PROTEIN
2.10.1. BACTERIAL PROTEINS
Bacterial proteins were isolated from bacteria grown in liquid culture. Bacterial cells were harvested by centrifugation (for 1 min in a microfuge) of a 1 mL aliquot of the culture. The cells were then resuspended in 50 µL of SDS gel-loading buffer and incubated at 100° C for 5 min. The mixture was cooled on ice, and debris removed by centrifugation in a microfuge for 1 min. A portion of the supernatant (usually 10 µL) was then analysed by electrophoresis through an SDS-polyacrylamide gel (Ch. 2.12.1).

2.10.2. GST FUSION PROTEINS
An overnight culture (20 mL) of DH5α containing the plasmid of interest (pGEX-2T, or an engineered derivative) was used to inoculate 400 mL of L broth containing ampicillin. The culture was then grown at 37° C with shaking until the OD₆₀₀ reached a value between 0.9 and 1.2. A 1 mL sample of
the culture was taken for later analysis, and 400 μL of 0.1 M IPTG was added to the remainder of the culture, which was then grown for a further 2 to 4 h. Again, a 1 mL sample was collected for analysis, and the rest of the culture was centrifuged at 6 400X g for 15 min at 4°C. The harvested cells were resuspended in 30 mL of ice-cold MtPBS, and stored at -20°C overnight. The next day the cells were thawed, and then lysed by two passages through a French Pressure Cell (at 500 - 1 500 psi), followed by the addition of 3 mL of 10% Triton X-100 in MtPBS. The lysate was incubated on ice for 30 min, and debris was removed by centrifugation at 22 900X g for 10 min at 4°C. A 50 μL aliquot of the supernatant was collected for later analysis, and the rest was passaged over a glutathione sepharose column. The glutathione column was constructed by packing a 300 μL aliquot of glutathione sepharose beads (from Pharmacia) in a column, allowing the storage fluid to drain, then washing the column with two 3 mL aliquots of ice-cold MtPBS. After the lysate had been passed over the column, a 50 μL sample was taken to assess binding of the fusion protein to the sepharose beads. The column was washed four times with 3 mL aliquots of ice-cold MtPBS then equilibrated with 3 mL TC buffer. Fresh elution buffer was then prepared (10 mM reduced glutathione in TC), and three 0.3 mL aliquots were passed over the column, and the eluates (containing the fusion protein) were pooled.

2.10.3. PLANT PROTEINS
Proteins were extracted from root tips and leaf tissue. The plant tissue was ground under liquid nitrogen, then added to an (approximately) equal volume of SDS gel-loading buffer. The mixture was vortexed, then incubated at 100°C for 5 min. Cellular debris was removed from the extract by centrifugation in a microfuge for 5 min, and a portion of the supernatant was analysed by electrophoresis on SDS-polyacrylamide gels.

2.11. DNA AND RNA MANIPULATIONS
2.11.1. ELECTROPHORESIS
DNA and RNA samples were electrophoresed through horizontal submerged agarose (usually 1%) gels as described in Sambrook et al. (1989). Loading dye (0.2 volumes of 40% sucrose, 0.04% bromophenol blue, 0.04% xylene cyanol FF) was added to the DNA or RNA and the samples were loaded into wells in the gels (RNA samples were heat denatured at 65°C for 5 min prior to loading). The gels were electrophoresed in TBE buffer in a field of 2 to 6 Vcm⁻¹. After electrophoresis, the gels were stained in 100 μgL⁻¹ ethidium bromide in TBE for approximately 30 min. The nucleic acids were visualised by placing the gel on a 302 nm UV transilluminator.

To determine the size of the DNA or RNA in the gels, molecular weight standards were co-

electrophoresed. For DNA size determination the BRL 1 kb and 123 bp ladders were used, and for RNA size determination the BRL 0.24 - 9.5 kb or 0.16 - 1.77 kb RNA ladders were used.
When RNA was electrophoresed, standard procedures were used to avoid RNAse contamination of the samples (see Ch. 2.9). In addition, the electrophoresis tanks were soaked in 3% \( \text{H}_2\text{O}_2 \) for 10 min, then rinsed in sterile distilled deionised water prior to electrophoresis.

Sodium phosphate agarose gels were used to electrophorese RNA samples for northern analysis. The RNA was denatured with glyoxal and DMSO and electrophoresed as described in Sambrook et al. (1989), except that sodium iodoacetate was omitted from the gel. The gels used generally contained 1.2% agarose.

2.11.2. **Restriction Enzyme digestions**

Restriction enzyme digests were carried out in small volumes (15 - 100 \( \mu \text{L} \)) containing the substrate DNA, 1 to 10 units of the desired enzyme(s) per \( \mu \text{g} \) DNA, and 0.1 volume of the 10X buffer supplied by the manufacturer. Digests were allowed to proceed at the recommended temperature for 1 to 16 h. Digests containing more than one enzyme used the buffer that optimised the activity of all the enzymes used.

2.11.3. **Fragment isolation**

DNA fragments of interest were purified away from other contaminating nucleic acids by electrophoresis through a TBE agarose (or low gelling temperature agarose from SeaPlaque®) gel. After electrophoresis, the fragment of interest was excised from the gel using a clean scalpel blade. The DNA was then isolated from the agarose using either a Geneclean® II Kit (from Bio 101) or the Magic™ PCR Preps System (from Promega) according to the instructions supplied by the manufacturers.

2.11.4. **Dephosphorylation**

To reduce the self-ligation of vector DNA without inclusion of insert DNA, the cut vector DNA was sometimes dephosphorylated. After restriction enzyme digestion, calf intestinal alkaline phosphatase (CIP, used at 1 unit per \( \mu \text{g} \) DNA) was added to the reaction, and incubated for 30 min at 37° C. The CIP was inactivated by heating to 65° C for 15 min, then removed from the DNA by extraction with phenol/chloroform. The DNA was purified by precipitation with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol and recovered by centrifugation.

2.11.5. **Generation of Blunt-ended termini**

When required, DNA ends were blunted using the Klenow fragment of *E. coli* DNA polymerase I. Reactions (in 20 - 50 \( \mu \text{L} \)) contained the substrate DNA, 25 \( \mu \text{M} \) of each dNTP, 0.1 volume of 10X Klenow buffer (0.1 M Tris-Cl [pH 7.4], 60 mM MgCl₂, 20 mM 2-mercaptoethanol) and 6 to 12 units (1-2 \( \mu \text{L} \)) of Klenow. The reactions were incubated at room temperature for 1 h.
2.11.6. **LIGATION**

DNA was purified prior to ligations by either extraction with phenol/chloroform followed by ethanol precipitation, by purification with the Magic™ DNA Clean-Up System (Promega), or by isolating a fragment from an agarose gel (Ch. 2.11.3). Ligations were usually performed in small volumes (15 - 30 µL), and contained the DNA(s) of interest, 0.2 volumes of 5X ligase buffer, and 1 to 5 units of T4 DNA ligase. Ligation reactions were incubated at either room temperature for 1 h, or at 14° C overnight.

2.11.7. **DNASE I DELETION SUBCLONING**

Some sequencing subclones were generated using a DNAse I deletion strategy developed by Lin *et al.* (1985). The procedure of Lin *et al.* was followed except that all phenol/chloroform extractions and DNA precipitations were replaced with purifications using the Magic™ DNA Clean-Up System from Promega according to the manufacturer's instructions. This change resulted in a higher yield of DNA (and therefore recombinant colonies).

2.11.8. **RANDOM PRIME LABELLING**

DNA fragments isolated from agarose gels (Ch. 2.11.3), or total cDNA (2.11.12), were labelled with dCTP \( [\alpha-32P] \) using a random prime labelling kit from GIBCO BRL. The instructions supplied with the kit were followed except that half-volume reactions were often performed. Unincorporated nucleotides were removed from the reactions by centrifugation through a sepharose G50 column.

2.11.9. **GENERATION OF DIG-LABELLED RNA TRANSCRIPTS**

Plasmid DNA templates (the wali cDNA clones in pSPORT 1) were linearised by restriction enzyme digestion prior to RNA transcript generation. To remove any contaminating RNAses, the digests were extracted twice with phenol/chloroform, three times with chloroform, then precipitated with ethanol. RNA transcripts were generated using a DIG RNA labelling kit from Boehringer Mannheim, according to the manufacturer's instructions.

2.11.10. **POLYMERASE CHAIN REACTION**

Polymerase chain reactions (PCRs) were set up in 25 µL with 5 ng of the template DNA, 1 µM of each primer, 0.2 mM of each dNTP, 3 µL of 25 mM MgCl₂, 1.25 µL of 20X Tfl buffer (supplied with the enzyme), and 2 units of Tfl thermostable DNA polymerase (Epicentre Technologies). Reactions were overlaid with paraffin oil and subjected to 30 cycles of 94° C for 1 min, 60° C for 1.5 min, 72° C for 2 min using a Techne PHC-3 thermal cycler.

2.11.11. **MRNA PURIFICATION**

Polyadenylated RNA was isolated from total plant RNA (Ch. 2.9) by affinity chromatography on oligo(dT)-cellulose. A mRNA Purification kit from Pharmacia was used according to the manufacturer's instructions.
2.11.12. CDNA SYNTHESIS

CDNA was synthesised from purified mRNA (Ch. 2.11.11) using some of the components from the SuperScript Plasmid System pSPORT 1 cDNA library construction kit (supplied by GIBCO BRL) for use as probes. The mRNA (approximately 1 μg) was heat denatured at 70° C for 10 min with 1 μg of oligo(dT) primer (15-mer) followed by quick cooling on ice. To this mixture was added 4 μL of 5X first strand buffer (375 M KCl, 15 mM MgCl2, 250 mM Tris-Cl, pH 8.3), 2 μL of 0.1 M DTT, 1 μL of 10 mM dNTP mix, and 0.5 μL RNasin. These components were mixed, prewarmed to 37° C, and 2 μL of SuperScript reverse transcriptase (200 unitsμL⁻¹) was added. To monitor the success of the CDNA synthesis, a 2 μL aliquot of the reaction was removed and added to 0.5 μL dCTP [α-32P]. This side reaction and the main reaction were incubated at 37° C for 1 h, then cooled on ice. The side reaction was stopped by the addition of 1 μL 0.25 M EDTA and 7 μL water.

To synthesise the second strand of the cDNA the remaining 18 μL of the first strand synthesis was mixed with 30 μL of 5X second strand buffer (450 mM KCl, 23 mM MgCl2, 0.75 mM β-nicotinamide adenine dinucleotide, 50 mM (NH4)2SO4, 100 mM Tris-Cl, pH 6.9), 3 μL of 10 mM dNTP mix, 10 units of E. coli DNA ligase, 40 units of E. coli DNA polymerase, 2 units of E. coli RNAse H, and the volume was made up to 150 μL with water (NB the enzymes were added to the reaction last). A 10 μL aliquot of the reaction was removed and added to 0.5 μL dCTP [α-32P]. The side and main reactions were incubated at 16° C for 2 h, then the side reaction was stopped with the addition of 1 μL 0.25 M EDTA. The main reaction was also stopped with 6 μL 0.25 M EDTA, then extracted with phenol/chloroform, chloroform, and precipitated with ethanol.

A portion (5 μL) of each of the side reactions was electrophoresed through an agarose gel, which was then dried on a vacuum gel drier at 60° C for 1.5 h. The gel was then autoradiographed to estimate the size range of the cDNA. The 1 kb ladder from GIBCO BRL was radioactively labelled by nick translation (using a kit from GIBCO BRL according to the manufacturer's instructions) and coelectrophoresed for size comparison.

2.11.13. CDNA LIBRARY CONSTRUCTION

A CDNA library was constructed using the SuperScript Plasmid System pSPORT 1 cDNA library construction kit supplied by GIBCO BRL. The protocols supplied by the manufacturer were followed with some modifications. The first and second strand synthesis reactions were performed as described in section 2.11.12 above, except that no side reactions were taken, and no radioactive nucleotides were incorporated into the cDNA. In addition, the Not I primer adaptor was used in place of the oligo(dT) primer used in the first strand synthesis. After the second strand reaction incubation was complete, the reaction was not stopped by the addition of EDTA; instead 10 units of T4 DNA polymerase were added to the 150 μL reaction and it was incubated at 16° C for 5 min, then cooled on ice. The T4 DNA polymerase ensured that the termini of the cDNA were blunt-ended. The reaction was stopped
with the addition of 10 µL of 0.25 M EDTA. The reaction was then extracted with phenol/chloroform and precipitated with ethanol as described in the manual.

The cDNA was resuspended in a total volume of 30 µL and a 5 µL aliquot of this was electrophoresed through an agarose gel to determine the yield and size range of the cDNA. The Sal I adaptors were then ligated to the cDNA as described in the protocol, using a 16° C incubation for 2.5 d. The cDNA was again extracted with phenol/chloroform and ethanol precipitated, then digested with Not I, and purified again as described in the manual. The cDNA was then size fractionated using a Sephacryl S-500 HR Chromatography Column supplied with the kit. The protocols for the size fractionation were followed, except that as no radioactive nucleotides were incorporated in the cDNA synthesis, no Cerenkov counting of the fractions was done. The cDNA was instead assumed to be in the seventh to twelfth fractions based on the volumes of the fractions. These fractions were pooled, and 15 µL each of fractions 1, 2, 4, 7-12, 14, 17 and 20 were electrophoresed through an agarose gel to determine the yield and size of the isolated cDNA (shown in Fig. 3.4).

An aliquot of the size-fractionated cDNA (approximately 10 ng) was ligated to 50 ng of the cut vector, pSPORT 1 (supplied in the kit as Not I and Sal I digested, purified away from the linker piece of DNA), in a 20 µL reaction containing 1 µL of 10 mM rATP, 4 µL of 5X DNA ligase buffer (with the same composition as the buffer listed in Section 2.2, but supplied with the cDNA construction kit) and 1 unit of T4 DNA ligase. The DNA was ligated at room temperature for 4 h, then at 4° C for a further 24 h. After this time, the ligation was stored at -20° C. Portions of the ligation were transformed into MAX Efficiency DH5α™ Competent Cells (from BRL, see Ch. 2.5.6).

2.11.14. QUANTIFICATION OF NUCLEIC ACIDS
The concentrations of DNA, RNA and oligonucleotides were quantified in a number of ways. The most accurate method determined the concentration of the nucleic acids by the absorbance of a sample at 260 nm using a scanning spectrophotometer. The concentrations of each nucleic acid were calculated using the conversions listed in Sambrook et al. (1989). DNA concentrations were also estimated by electrophoresing a sample through an agarose gel and comparing to DNA samples of known concentration, or by using an ethidium bromide plate assay described in the SuperScript Plasmid System manual supplied by GIBCO BRL as part of the pSPORT 1 cDNA library construction kit.

2.12. PROTEIN MANIPULATIONS
2.12.1. ELECTROPHORESIS
Proteins were electrophoresed through SDS-polyacrylamide gels using the protocols detailed in Sambrook et al. (1989). To aid the resolution of small proteins, glycerol was also included in the resolving gel at a concentration of 13.3% (v/v) as recommended in Schägger and von Jagow (1987).
The gels were electrophoresed at a voltage of 8 Vcm⁻¹ until the sample had passed through the stacking gel, and up to 15 Vcm⁻¹ while the sample was passing through the resolving gel.

After electrophoresis, the gels were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue R250, 45% methanol [bulk quality], 45% acetic acid [bulk quality]) at 65°C for 30 to 60 min, and destained in 20% methanol, 7% acetic acid (both bulk quality) at room temperature overnight. Destaining was enhanced by replacing the destaining solution two to three times during the procedure, by including some absorbent material (filter paper or a piece of sponge) in the solution, and also by heating the gel and the destaining solution to 65°C.

The stained gel was dried on a vacuum gel drier at 80°C for approximately one hour. The gels were either dried onto 3MM filter paper, or dried between two pieces of clear cellophane.

2.12.2. QUANTIFICATION

Protein concentrations were estimated using a Bio-Rad Protein Assay based on the method of Bradford (1976), according to the protocol supplied by the manufacturer. Known concentrations of Immunoglobulin G were used as protein standards.

2.12.3. SERINE PROTEINASE ACTIVITY ASSAY

A casein degradation method (Kakade et al., 1969) was adapted to assay for serine proteinase inhibitor activity. Trypsin or chymotrypsin (0 to 10 μg) was diluted in 180 μL of 0.1 M sodium phosphate (pH 7.6) to give a range of enzyme concentrations. Soybean trypsin inhibitor (positive control), or the protein of interest, or no protein was added in 0.1 M sodium phosphate (pH 7.6) buffer to the enzyme solution to give a total volume of 200 μL. The mixture was warmed to 37°C, then 200 μL of a 2% casein in 0.1 M sodium phosphate (pH 7.6, also warmed to 37°C) solution was added. The mixture was incubated at 37°C for 20 min, then stopped by the addition of 600 μL 5% trichloroacetic acid. The reactions were vortexed, and left to precipitate at room temperature for 1 h. The reactions were centrifuged in a microfuge for 5 min, and the supernatant collected. The OD₂₈₀ of the supernatant was determined.

2.12.4. IMMUNOAFFINITY PURIFICATION OF ANTIBODIES

Purification of antibodies from rabbit serum was performed using a procedure based on information supplied by Pharmacia and a protocol by Harlow and Lane (1988). Approximately 2 mg each of the recombinant proteins GST and wali1-GST were dialysed overnight at 4°C against coupling buffer, to dilute out the Tris-Cl in the protein buffers. CNBr-activated Sepharose 4B from Pharmacia (1 g) was washed and reswelled on a sintered glass filter with 200 mL of 1 mM HCl. The dialysed protein solutions were removed from the dialysis bags and warmed to room temperature. An aliquot of each protein was removed, and the protein concentration determined (Ch. 2.12.2). Unfortunately a large proportion (up to 85%) of the protein was lost during the dialysis (presumably bound to the dialysis
The protein solutions were made up to 2.5 mL with coupling buffer, and each was added to half of the reswelled sepharose. The sepharose and protein were incubated together at room temperature for 2 h, after which the slurries were poured into chromatography columns. The protein content of the eluates from the columns (containing any unbound protein) was determined. Approximately 250 µg of wali1-GST protein and 550 µg of GST protein were bound to each of the two sepharose columns.

The two columns (each had a bed volume of approximately 1 mL) were washed with 10 mL coupling buffer. Any remaining active groups on the sepharose beads were blocked by incubating the columns with 0.1 M Tris-Cl (pH 8.0) for 2 h at room temperature. The columns were washed alternately with 5 mL of 0.5 M NaCl, 0.1 M sodium acetate (pH 4.0), and with 5 mL of 0.5 M NaCl, 0.1 M Tris-Cl (pH 8.0) a total of three times. The columns were allowed to drain completely before each new addition of a wash or elution buffer. The columns were then washed sequentially with 10 mL of 0.01 M Tris-Cl (pH 7.5), 10 mL of 0.1 M glycine (pH 2.5), and 10 mL of 0.01 M Tris-Cl (pH 8.8). The last wash was continued until the pH of the eluate reached 8.8. The columns were washed with 10 mL of 0.1 M triethylamine (pH 11.5, freshly prepared), and then with 0.01 M Tris-Cl (pH 7.5) until the pH of the eluate reached 7.5.

The rabbit serum (a 4 mL aliquot) was thawed and diluted to 40 mL with 0.01 M Tris-Cl (pH 7.5). The diluted serum was passed through the GST-sepharose column three times to bind out the anti-GST antibodies present in the serum. The eluate was passed over the wali1-GST-sepharose column three times to bind any anti-wali1 antibodies present. Each of the two columns was washed with 20 mL of 0.01 M Tris-Cl (pH 7.5), and then with 20 mL of 0.5 M NaCl, 0.01 M Tris-Cl (pH 7.5). Four collection tubes were prepared (two for each column) containing 1 mL of 1 M Tris-Cl (pH 8.0). The antibodies bound by acid-sensitive interactions were released by passing 10 mL of 0.1 M glycine (pH 2.5) over the columns. The eluates were each collected in one of the collection tubes. The columns were washed with 0.01 M Tris-Cl (pH 8.8) until the eluates reached a pH of 8.8, and the antibodies bound by base-sensitive interactions were released by passing 10 mL of 0.1 M triethylamine (pH 11.5) over the columns. Again, the eluates were each collected in one of the tubes containing 1 M Tris-Cl (pH 7.5), which neutralised the eluates. The two eluates collected from each column were then combined. The columns were washed with 0.01 M Tris-Cl (pH 7.5) until the pH of the eluates reached pH 7.5 and stored at 4°C. The antibody solutions were then dialysed against PBS containing 0.02% sodium azide.

To remove more of the anti-GST antibodies from the anti-wali1 antibody solution, the GST-sepharose column was drained and washed sequentially with 10 mL of 0.01 M Tris-Cl (pH 7.5), 10 mL of 0.1 M glycine (pH 2.5), and with 0.01 M Tris-Cl (pH 8.8) until the eluate reached pH 8.8. The column was washed with 10 mL of 0.1 M triethylamine (pH 11.5), and then with 0.01 M Tris-Cl (pH 7.5) until the eluate reached pH 7.5. The anti-wali1 antibody solution was passed over the GST-sepharose column to bind out any anti-GST antibodies, and the flow through (containing the anti-wali1 antibodies) was
collected. The column was rinsed with 0.01 M Tris-Cl (pH 7.5) until the eluate reached pH 7.5. This last procedure to remove the anti-GST antibodies from the anti-wal1 antibody solution was repeated.

2.13. TRANSFER OF NUCLEIC ACIDS AND PROTEINS TO MEMBRANES

2.13.1. DOT BLOTTING

DNA samples were dot blotted onto Hybond-N+ membranes using a protocol supplied by the manufacturer (Amersham). Protein samples were dot blotted onto Hybond-C Super membranes, also following protocols supplied by Amersham.

2.13.2. SOUTHERN TRANSFER

This method is a modification of that of Southern (1975). DNA was electrophoresed through agarose gels, after which the gels were soaked in TS for 20 min. The DNA in the gels was then transferred to Hybond-N+ membranes by capillary transfer. The gel and the nylon membrane were arranged in a stack as shown in Figure 2.1. The transfer of DNA from the agarose gel to the membrane was mediated by TS buffer, and the transfer was allowed to proceed for 4 to 16 h. The stack was dismantled and the gel and membrane were neutralised in NS for 30 min. The gel was restained with ethidium bromide to check the efficiency of transfer and the membrane was air-dried prior to hybridisation.

2.13.3. NORTHERN TRANSFER

RNA molecules electrophoresed through sodium phosphate gels were transferred directly to Hybond-N+ membranes by capillary transfer (see Fig. 2.1) with no pre-treatment of the gels. The RNA was transferred for 3 to 4 h using 50 mM NaOH as the transfer buffer. After transfer the stack was dismantled and the membrane rinsed in 2X SSC and air-dried. The gel was stained in ethidium bromide to check the efficiency of transfer.

Figure 2.1 Capillary transfer of nucleic acids

Transfer of DNA or RNA to nylon membranes was effected through capillary transfer as shown here. The gladwrap contained a 'window' cut out around the gel, so that the passage of the transfer solution had to occur through the gel. In Southern transfers the transfer solution was TS, in northern transfers it was 50 mM NaOH. The gel in the stack was placed upside down, relative to how it was electrophoresed.
2.13.4. WESTERN TRANSFER

Proteins separated within SDS-polyacrylamide gels (Ch. 2.12.1) were transferred to Hybond-C Super or PVDF membranes by electroblotting, according to a procedure recommended by Amersham. A 'sandwich' of the gel, membrane, 3MM filter paper, and Scotch Brite scouring pads was assembled as shown in Figure 2.2. The gel was blotted for 18 h at 4° C at 20 to 50 mA in 0.15 M glycine, 20% methanol, 0.025 M Tris-Cl (pH 8.3). Shorter transfers were sometimes used, for example 2 h at 4° C at up to 400 mA. After transfer, the apparatus was dismantled, and the membrane was rinsed in PBS. The gel was restained to check the efficiency of transfer.

![Figure 2.2 Western transfer of proteins](image)

Transfer of protein to membranes occurred through electroblotting as shown here. The plastic holders slot into the blotting apparatus so that the gel and membrane 'sandwich' are held tightly together.

2.13.5. PLAQUE LIFTS

Plaque lifts were carried out essentially as recommended by DuPont onto Colony/PlaqueScreen (DuPont) or Hybond-N+ (Amersham) nylon membranes. The plates containing bacteriophage plaques were chilled at 4° C for a minimum of 1 h prior to performing the plaque lifts to harden the top agarose. The plaque lifts were taken by placing the membranes onto the surface of the plates for 2 min. The membranes were floated DNA side up on a solution of 0.5 M NaOH for 2 min, and blotted dry on filter paper for 2 min. The membranes were neutralised by transferring them to a solution of 1 M Tris-Cl (pH 7.5) for 2 min, blotted on filter paper and allowed to air dry for 1 h. If replicate filters were required the entire procedure was repeated.

2.13.6. COLONY LIFTS

Colony lifts were performed using a variation of the procedure described in Sambrook *et al.* (1989). Colony/PlaqueScreen membranes (DuPont) were placed on large L plates containing ampicillin. Between 2 600 and 2 800 colonies were spread on top of each membrane, and the plates incubated at 37° C overnight. These plates (the master plates) were chilled at 4° C for 1 h. New membranes were placed on L plates containing ampicillin (one for each replica required). A replica of each master plate was taken on a piece of sterile velvet stretched over a replicating block. The colonies on the velvet
were transferred to the replica plates. The master plate was re-incubated at 37°C for 2 h to regenerate the colonies, and stored at 4°C. The replica plates were also incubated at 37°C for approximately 4 h until the transferred colonies became visible. The membranes on the replica plates were transferred to L plates containing ampicillin and chloramphenicol and incubated at 37°C overnight to amplify the plasmid DNA.

To lyse the bacterial cells, the filters were removed from the bacterial plates and placed (colony side up) on two layers of 3MM filter paper soaked in 10% SDS for 5 min. The DNA on the filters was denatured and fixed to the membranes by placing the membranes sequentially on filter paper soaked in 0.5 M NaOH, 1.5 M NaCl; then 1.5 M NaCl, 0.5 M Tris-Cl (pH 7.5); and 2X SSC, each for 5 min.

The filters were incubated separately in 100 mL of 1 M NaCl, 1 mM EDTA, 0.1% SDS, 50 mM Tris-Cl (pH 8.0) at 42°C for 4 h. The bacterial debris was removed from the filters by gently wiping them with clean tissues, then the membranes were air dried. The filters were incubated in a sufficient quantity of DNA hybridisation buffer to ensure that the membranes stayed wet, at 65°C for 30 min. The membranes were rinsed in 2X SSC, and incubated in 2X SSC, 0.1% SDS, 100 mgL⁻¹ proteinase K for 1 h at 65°C. The filters were washed twice in 2X SSC, 0.1% SDS for 15 min at 65°C prior to prehybridisation and hybridisation (Ch. 2.14.1).

2.14. HYBRIDISATION TECHNIQUES

2.14.1. HYBRIDISATION TO DNA TARGETS

Membranes containing DNA targets (dot blots, Southern's, plaque and colony lifts) were prehybridised in DNA hybridisation solution at 65°C for at least 2 h. Only the minimum amount of prehybridisation solution was used to ensure that all the filters remained wet throughout the hybridisation. After prehybridisation the probe (labelled by random priming, Ch. 2.11.8) and salmon sperm or herring sperm DNA at 100 mgL⁻¹ (final concentration in the hybridisation solution) were heat denatured by boiling for 2 to 5 min, and added to the hybridisation solution. The membranes were incubated overnight at 65°C with constant movement to ensure good coverage of the hybridisation solution.

After hybridisation, the filters were washed twice in 2X SSC at room temperature for 5 min. They were then washed twice in 2X SSC, 0.1% SDS at 65°C (though sometimes at temperatures up to 85°C, depending on the stringency required) for 20 to 30 min. The wet filters were sealed in plastic bags and autoradiographed by exposure to DuPont Cronex X-ray film for periods ranging from 1 h to 3 weeks at -80°C (some shorter exposures were performed at room temperature).

2.14.2. NORTHERN ANALYSIS

Hybridisations to northern blots essentially followed the procedure of Virca et al. (1990). Membranes were prehybridised in 50 mM PIPES (pH 6.8), 100 mM NaCl, 50 mM sodium phosphate (pH 7.0),
1 mM EDTA, 5% SDS, 100 mgL⁻¹ denatured salmon sperm or herring sperm DNA, for at least 15 min at 65° C. The prehybridisation buffer was usually discarded, and replaced with fresh 50 mM PIPES (pH 6.8), 100 mM NaCl, 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, 5% SDS containing the heat-denatured probe. Towards the end of this work it was found that discarding the prehybridisation buffer was not necessary, and the heat-denatured probe was added directly to the prehybridisation solution. The probe was hybridised to the membranes overnight at 65° C with constant movement. After hybridisation, the filters were washed and autoradiographed as described in section 2.14.1. Autoradiographs were scanned using an Ultrascan XL Densitometer according to the manufacturer's recommendations.

2.14.3. WESTERN ANALYSIS
Western analysis of proteins was carried out using an ECL Western Blotting Analysis System from Amersham following the protocol supplied by the manufacturer. TBS-T was used to dilute the antibody solutions and to wash the membranes.

2.14.4. SOUTH-WESTERN ANALYSIS
A procedure by Sukegawa and Blobel (1993) was modified to detect DNA-binding proteins. Two identical membranes containing electroblotted protein (Ch. 2.13.4) were simultaneously analysed during this procedure. The proteins on one membrane were renatured for 18 h at 4° C in 100 mM KCl, 1% Triton X-100, 10% glycerol, 1 mM ZnCl₂, 50 mM Tris-Cl (pH 7.5). This membrane was then rinsed for 30 min in reaction buffer (100 mM KCl, 1% Triton X-100, 10% glycerol, 0.1 mM ZnCl₂, 50 mM Tris-Cl [pH 7.5]), blocked in reaction buffer containing 5% Anchor non-fat milk powder for 2 h at 4° C, and rinsed in reaction buffer to remove the milk.

The second membrane was treated in a similar way to the first membrane except that the renaturation buffer was 100 mM KCl, 1% Triton X-100, 10% glycerol, 50 mM EDTA, 10 mM DTT, 50 mM Tris-Cl (pH 7.5) and the reaction buffer was 100 mM KCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA, 2 mM DTT, 50 mM Tris-Cl (pH 7.5).

Total Warigal genomic DNA (kindly supplied by T. Richardson) was sonicated until its average size was approximately 800 bp, and labelled by random priming (Ch. 2.11.8). The membranes were incubated in the appropriate reaction buffer containing half of the heat denatured probe DNA for 3 h at 4° C. The membranes were then washed five times in reaction buffer for 10 min at 4° C.

The two membranes were sealed in plastic bags and autoradiographed as described in section 2.14.1.

2.14.5. IN SITU HYBRIDISATION
The in situ hybridisation protocol used was based on the methods of Cox and Goldberg (1988) and Bochenek and Hirsch (1990), with some modifications. Before hybridisation, the paraffin was
removed from the sections by soaking for 5 min each in two changes of xylene, and the tissue was rehydrated through an ethanol series (100%, 95%, 70%, 50%, then H2O). The slides were incubated in 0.2 N HCl for 20 min, rinsed twice in 2X SSC for 5 min, and incubated for 30 min at 37° C in 10 mgL⁻¹ proteinase K, 0.05 M EDTA, 0.1 M Tris (pH 7.5). The slides were rinsed twice in water, incubated in 0.1 M triethanolamine (pH 8.0, prepared fresh) for 5 min, and transferred to a fresh container of 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride and incubated for 10 min. The sections were washed twice in 2X SSC for 5 min, dehydrated through an ethanol series (50%, 70%, 95%, 100%) and dried under vacuum for 1 h.

The hybridisation solution (50% formamide, 0.3 M NaCl, 0.01 M Tris (pH 7.5), 1 mM EDTA, 1X Denhardt's solution, 0.07 M DTT, 150 mgL⁻¹ E. coli tRNA, 0.5 gL⁻¹ salmon sperm DNA, 1 mgL⁻¹ DIG-labelled RNA probe) was heat denatured, and approximately 100 µL was added to each slide, and overlaid with a parafilm coverslip. Slides were incubated for 16 to 18 h in a sealed humid container at 45° C. The coverslips were removed by soaking in 4X SSC, then the slides were washed twice in 2X SSC for 20 min at room temperature and three times in 0.2X SSC for 20 min at 65° C. Detection of hybridised transcripts was as described in Bochenek and Hirsch (1990) except that the MgCl₂ was omitted from buffer III. When required, the slides were covered with parafilm coverslips. All sections in a particular experiment were left for the same time (typically 18 h) for colour development. Slides were mounted with an aqueous mounting medium.

The aqueous mounting medium was prepared by dissolving 10 g gelatin in 60 mL water with gentle heating. A few phenol crystals and 70 mL glycerol were added, and the mixture was filtered through miracloth and stored at 4° C. When required for use, the medium was liquefied by heating to 65° C for a few minutes.

2.14.6 REMOVAL OF BOUND PROBES
Membranes were often sequentially hybridised with more than one probe. After each hybridisation, bound probe was removed by immersing the membrane in boiling 0.1% SDS. The SDS solution containing the membrane was allowed to cool to room temperature, after which the membrane was hybridised with the next probe, or sealed in a plastic bag and stored at 4° C.

2.15. COMPUTING
2.15.1. SEQUENCE ANALYSIS
The computer programs of Devereux et al. (1984), comprising the Genetics Computer Group (GCG) package version 7.1.1-Unix installed on a Silicon Graphics 4D-30 workstation, were used for the alignment and analysis of sequence data and the searching of sequence databases. Unless otherwise stated, the default parameters for the GCG programs were utilised. Database searches were also conducted at the NCBI (National Center for Biotechnology Information, USA) using the BLAST
network service (Altschul et al., 1990). Databases accessible from this service for which sequences are available are: GenBank, the EMBL Data Library, the Database of Expressed Sequence Tags, the Brookhaven Protein Data Bank, SWISS-PROT, PIR, Kabat Sequences of immunological interest, and TFD (transcription factor database).

2.15.2. GENERAL
Word processing, compilation and formatting of references, graph and figure drawing were performed using the programs Microsoft Word (version 5.0), EndNote Plus (version 1.3.1), Cricket Graph (version 1.3.2), and MacDraw® Pro 1.0v1 respectively on a Macintosh LC II and LC III. Integration of densitometry data was performed using GelScan XL software (version 2.1, Pharmacia) on an IBM compatible 486 computer.

2.16. PHOTOGRAPHY
Photographs of agarose gels were taken through A001, A002 and A003 Kokin filters with Polaroid 665 or 667 film. Autoradiographs were developed using an Agfa Curix 60 film processor. Photomicrographs were taken with an Olympus 35 mm camera body attached to the OM light path of an Olympus IMT-2 inverted research microscope, using 60 ASA Tungsten Professional Colour Reversal film from Kodak. All other photography was done by the School of Biological Sciences Graphics unit (University of Auckland).
3.1. INTRODUCTION
At the time this work was initiated very little was known about the molecular effect of Al on plants. Although there was a large body of published research dealing with the growth and physiology of plants suffering from Al toxicity, there was no consensus on the physiological mechanism(s) of Al toxicity in plants. Work on the molecular effect of Al toxicity was limited to the use of two-dimensional gel electrophoresis to identify proteins induced by Al treatment. Several protein 'spots' had been identified that were induced by Al (Delhaize et al., 1991; Ownby and Hruschka, 1991; Picton et al., 1991), but none had been further characterised. This chapter therefore describes work aimed at identifying genes induced by Al treatment in plants by the use of differential screening of a cDNA library.

A cDNA library in the bacteriophage vector λ ZAP II had been constructed by Dr Andreas Königstorfer and Keith Richards of this laboratory. This library was constructed from mRNA isolated from root tips of the Al-sensitive wheat cultivar Warigal that had been grown in the presence of 10 μM Al for two days. This library will be referred to as the Wg+Al(ZAP) cDNA library. The first part of this chapter describes the differential screening of the Wg+Al(ZAP) cDNA library to identify genes whose expression was increased with Al treatment in the root tips of plants, relative to untreated plants (Ch. 3.2). A second library, in the pSPORT vector, was then constructed and used to identify full-length (except in the case of the clone wali4) copies of each of the cDNA clones identified as being induced by Al-treatment (Ch. 3.3). Northern analysis was used to identify the timing of the Al induced expression of these clones (Ch. 3.4). Northern analysis was also used to investigate whether or not Al induction was specific to the sensitive cultivar Warigal, or if it also occurred in the closely related cultivar Waalt (Al-tolerant), as well as two lines of wheat, SS and RR which were derived from a cross between Waalt and Warigal (Ch. 3.5). The last experimental section of this chapter (Ch. 3.6) describes the information derived from the nucleotide sequence of each clone.

3.2. IDENTIFICATION OF AL INDUCED CDNA CLONES
3.2.1. DIFFERENTIAL SCREENING OF THE Wg+Al(ZAP) CDNA LIBRARY
The amplified Wg+Al(ZAP) cDNA library constructed by Dr Andreas Königstorfer and Keith Richards was used in the preliminary identification of Al-induced clones. Between 2 000 and 10 000 pfu were plated on nine large (15 cm diameter) petri dishes and duplicate plaque lifts were made. Total RNA was isolated from the root tips of Warigal plants that were either untreated, or grown in the presence of
10 μM Al for 2 d. cDNA was synthesised from mRNA that had been purified from these total RNA preparations. Approximately 50 - 100 ng of each type of cDNA was labelled and used to probe the duplicate plaque lifts. The two probes will be referred to as the 'Wg+Al' probe (derived from Warigal plants treated with Al) and the 'Wg' probe (derived from untreated Warigal plants). An example of the results obtained by this differential screening is shown in Figure 3.1. Approximately 55 000 clones from the Wg+Al(ZAP) cDNA library were screened in this way.

![Figure 3.1 Differential screening of the Wg+Al(ZAP) cDNA library](image-url)

Plaques which exhibited enhanced hybridisation to the Wg+Al probe relative to the Wg probe were picked from the plates and used in further rounds of screening. A second differential screen of 150 plaques chosen from the primary screen was performed by plating the bacteriophage directly onto a lawn of bacteria to yield large plaques (Ch. 2.6.4). Duplicate filters were again taken from the plates and hybridised to either the Wg+Al or the Wg probe. The bacteriophage that showed greater hybridisation to the Wg+Al probe were chosen for further purification (43 of 150). If the plaques were picked from high density areas of the original library platings (for example, when the picked bacteriophage came from plates with up to 10 000 plaques), they were then purified so that only one bacteriophage was present in each eluate. This was achieved by replating the mixed plaques at low densities, taking duplicate plaque lifts, hybridising to the Wg+Al or the Wg probe, and picking well-spaced single plaques which hybridised preferentially to the Wg+Al probe. If the plaques were picked from low density areas (for example, from plates with only 2 000 plaques) then this step was not performed. Plasmids were rescued from the candidate bacteriophage by superinfection with the R408 helper phage. Plasmid DNA was isolated from the clones and dot blotted onto duplicate nylon
membranes and rescreened. Eleven clones were selected whose expression appeared to be increased or induced by the Al treatment.

3.2.2. Preliminary Characterisation of Putative Differential cDNA Clones

To confirm the differential expression of the genes represented by the eleven cDNA clones, preliminary northern hybridisations were performed. Northern blots containing RNA isolated from the root tips of Warigal plants that had been grown either with or without 10 μM Al for a two day period were probed with the labelled inserts of the clones. Some examples of the results obtained are shown in Figure 3.2 and the information derived from the northern hybridisations is summarised in Table 3.1. Most of the clones were not full-length, as determined by comparing the size of the insert to the size of the hybridising band on the northern blot (Table 3.1). Only clones which hybridised to a mRNA whose expression increased with the Al treatment (relative to the untreated plants) were selected for further characterisation. Three of the eleven clones were not pursued further (D, F and O), as the expression of the hybridising mRNA did not change substantially after treatment with Al. In addition, the insert size of clone H was larger than the hybridising mRNA (see Table 3.1), so this clone was deemed to be 'complex', and possibly made up of more than one cDNA.

![Figure 3.2 Preliminary northern analysis of the putative differential cDNA clones](image)

Examples are shown of autoradiographs of northern hybridisations using labelled inserts of the putative differential cDNA clones as probes (Chs. 2.11.1, 2.13.3, 2.14.2, 2.11.8). Lanes contain approximately 10 μg of total RNA from the root tips of Warigal seedlings that were either untreated (-) or treated with 10 μM Al for 2 d (+), see Chs. 2.7.2, 2.7.3 and 2.9. After hybridisation, the membranes were washed in 2X SSC, 0.1% SDS at 65°C. Labels underneath each autoradiograph indicate which of the cDNA clones were used as probes. The two treatments shown for each probe, came from the same northern gel and autoradiograph (one intervening lane was removed in each case). Of the northern hybridisations shown here, E and R were selected for further study.

The remaining seven clones all showed induction by Al and were further characterised by determining the nucleotide sequence of their 5' and 3' ends. Some sequence information was also obtained from the complex clone H. Comparisons of the nucleotide sequences with each other and with the databases revealed several similarities. For example, the clones E, G and P were related to each other, with E and G more homologous to each other than to P (Table 3.1). Similarly, clones 2 and R were highly homologous to each other. It was also found that two of the isolated clones (1 and N) had homology to previously isolated genes from other plant species. This homology will be discussed in detail in Chapter 3.6. One representative of each gene was characterised further. These five clones (1, 2, G, N and P) were renumbered 1.0 to 5.0 for future work.
Table 3.1 Characteristics of the putative differential clones.

<table>
<thead>
<tr>
<th>clone</th>
<th>insert size</th>
<th>mRNA size</th>
<th>Al induction</th>
<th>sequence information</th>
<th>clone number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
<td>700</td>
<td>5X</td>
<td>homology to ids-1 (67%)</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>1300</td>
<td>3-5X</td>
<td>homologous to R (97%)a</td>
<td>2.0</td>
</tr>
<tr>
<td>D</td>
<td>1000</td>
<td>1200</td>
<td>-2X</td>
<td>not characterised</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>700</td>
<td>700</td>
<td>5-10X</td>
<td>homologous to G (98%) and P (64%)a</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>900</td>
<td>1800</td>
<td>NI</td>
<td>not characterised</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>500</td>
<td>700</td>
<td>5-10X</td>
<td>homologous to E and P (59%)</td>
<td>3.0</td>
</tr>
<tr>
<td>H</td>
<td>1400</td>
<td>700</td>
<td>5-10X</td>
<td>no homology to known sequencesa</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>650</td>
<td>2700</td>
<td>2-3X</td>
<td>homology to PAL (71%)a</td>
<td>4.0</td>
</tr>
<tr>
<td>O</td>
<td>200</td>
<td>1000</td>
<td>NI</td>
<td>not characterised</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>550</td>
<td>700</td>
<td>5-10X</td>
<td>homologous to E and G</td>
<td>5.0</td>
</tr>
<tr>
<td>R</td>
<td>400</td>
<td>1300</td>
<td>3-5X</td>
<td>homologous to 2</td>
<td></td>
</tr>
</tbody>
</table>

Sizes of inserts (in bp) are estimates from gel electrophoresis, and sizes of the mRNAs are estimates from northern hybridisations. The level of Al induction was estimated by eye from the northern hybridisations. The clone number refers to the renumbering of the clones used in further work. Homologies (nucleotide identities) reported are approximate only, due to the small amount of sequence information obtained from each clone (approximately 300 - 500 nucleotides). This sequence information was single stranded only and contained a number of nucleotide ambiguities. Clone 1 (1.0) was compared to the barley ids-1 sequence (accession number X58540) and clone N (4.0) was compared to the rice PAL sequence (accession number X16099).

a'NI' = not induced; 'a' = clone contained multiple adaptors, or a stretch of poly(A) nucleotides at the 5' end of the clone (see text).

The sequence information also indicated that several of the clones were unusual, containing either a stretch of poly(A) nucleotides at both ends of the clone (clones 2, E and H), or having multiple adaptors from the library construction ligated into the 5' end of the clones (clone N). In the case of clone N, database searches with the nucleotide sequences indicated that N was homologous to the rice phenylalanine ammonia-lyase (PAL) gene (Fig. 3.3). This homology diverged at the 5' end of clone N, where it was subsequently found that the sequence was homologous to the adaptor sequences (Fig. 3.3).

3.3. ISOLATION OF FULL-LENGTH CDNA CLONES

The initial northern and sequence analyses revealed that most of the clones isolated were not full-length and that many of them contained, at their 5' end, multiple copies of the cloning adaptors, or a poly(A) stretch at both ends of the clone. For these reasons a new library was constructed so that full-length cDNA clones could be isolated.

3.3.1. CONSTRUCTION OF A PSPORT 1 CDNA LIBRARY

Total RNA was isolated from the root tips of Warigal plants grown in the presence of 10 µM Al for 2 d. Poly(A) mRNA (17 µg) was isolated from approximately 1 mg of this RNA. A total of 5 µg of this mRNA was then used in the construction of a cDNA library in the plasmid pSPORT 1. A summary of
the method employed for library construction is shown in Figure 3.4A. The synthesised cDNA ranged in size from 50 bp to 6 kb, with an average size of approximately 1 kb (Fig. 3.4B). After addition of adaptors and size fractionation, approximately 700 ng of cDNA was obtained that was ready for ligation into the pSPORT 1 plasmid (lane 4, Fig. 3.4C). A portion of this cDNA (10 ng) was ligated to 50 ng of Not I - Sal I digested pSPORT 1 DNA. Five percent of this ligation was transformed into MAX Efficiency DH5α cells (Ch. 2.5.6). A total of 26 000 colonies were obtained from this transformation. If the whole ligation was transformed into cells of equivalent competency then it would be possible to produce a library containing 520 000 clones. This library will be referred to as the Wg+Al(SPORT) cDNA library. If larger numbers of clones were to be required in future, more of the size fractionated cDNA could be ligated into pSPORT 1 or any other suitable plasmid or bacteriophage vector.

<table>
<thead>
<tr>
<th>Figure 3.3</th>
<th>Comparison of the 5' sequence of clone N (4.0) with the rice PAL nucleotide sequence.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>GAATTGGCCAGCGGCGGCAGGAGGCCGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGGCCTTGACCAATGTGGA</td>
</tr>
<tr>
<td>rice</td>
<td>GCAAGCCGCAACTACCGGCTCATGCCAGAAGCTCCGGCCGCTGTCGAGCAGGAGCCCTCACGAGGCCGC...</td>
</tr>
<tr>
<td>N</td>
<td>TTGACCAAGAGGTGCCGAGGGCCGCCGCCGCGTGCGCGGCCGAGGAGGACTGCCAACAAGCAGAAAACGTATCG</td>
</tr>
<tr>
<td>rice</td>
<td>CTGCCGGCAGAGCTGGCCGGCCCGCCGCTGCGCGCTCCGCACGCCGCGCGCGATCG</td>
</tr>
<tr>
<td>N</td>
<td>CCGATTGTGGATCCGGCTTACCGGGCTCTACCGGTTCGTGGCTGGCAAGAGCTCCAGGATCTTTAGGCCCAGGA</td>
</tr>
<tr>
<td>rice</td>
<td>TGGAGGCGCCGCTTCCGCTCGCTTCGCTGCGGAGAGCTGGCTGATATTCTACGCGGCGCA</td>
</tr>
<tr>
<td>N</td>
<td>GAAGAGCGGCAGGTCTCCTGGCCGAAAGGTCTTCGCTGCTGCGCTGACGAGCTGCAAGGAGGAGAGAGACATCG</td>
</tr>
<tr>
<td>rice</td>
<td>GAGGCTCAAGTCCCCCGCCGGAGGAGGTCGCCAAGAAGGTTCTGCTGCGAAGCTGCAAGGCGATCGA</td>
</tr>
</tbody>
</table>

The comparison between the sequence of N (4.0) and the rice (Oryza sativa) PAL gene was made using the GCG program GAP. At the 5' end of the N sequence, where the homology diverges, sequences that are similar to portions of the EcoR I adaptors are highlighted with double underlining, and sequences similar to the Linker-Primer are highlighted with dotted underlining. Numbering of the rice PAL gene is as given in the genomic clone, accession number X16099 (Minami et al., 1989). The sequence of N starts with the EcoR I site that the clone was inserted into in the λZAP bacteriophage. The composition of the adaptor and Linker-Primer that were used in the cDNA cloning are also shown.

To determine whether or not the Wg+Al(SPORT) recombinants did in fact contain inserts, a small proportion (500 colonies) was plated on L+amp plates containing IPTG and X-Gal. The majority of these colonies were white (94%), indicating that they almost certainly did contain inserts. A total of 12
Figure 3.4 Construction of the Wg+Al(SPORT) cDNA library

(A) Overview of cDNA library construction, adapted from the SuperScript Plasmid System instruction manual (see also Ch. 2.11.13 for details). (B) cDNA (one sixth of total), lane 1; BRL 1 kb ladder, lane 2. (C) Size fractionation of cDNA. Fractions (20) were collected from the cDNA column (Ch. 2.11.13), and 15 µL of the following fractions were electrophoresed on a 1% agarose gel: fraction 1, lane 1; fraction 2, lane 2; fraction 4, lane 3; fractions 7-12 (pooled), lane 4; fraction 14, lane 5; fraction 17, lane 6; fraction 20, lane 7. The BRL 1 kb ladder is in lane 8 for size comparison. Fractions 7-12 were used in the library construction. (D) DNA from randomly picked colonies from the cDNA library, cut with Not I and Sal I to release the inserts from the vector DNA. Blue colony, lane 2; white colonies, lanes 3-7 and 9-14; BRL 1 kb ladder, lanes 1 and 15; BRL 123 bp ladder, lane 8.

colonies (eleven white and one blue) were chosen at random to check the size range of inserts. The plasmid DNA isolated from these clones was digested with Not I and Sal I (the restriction enzymes used in the construction of the library) and electrophoresed through an agarose gel (Fig. 3.4D). Each of the
plasmids (including the plasmid isolated from the blue colony) contained an insert, with sizes ranging from 350 bp to 1400 bp. The average size of the inserts was approximately 800 bp.

3.3.2. ISOLATION OF AL INDUCED CLONES

A total of 13 700 colonies from the Wg+Al(SPORT) library were plated on five large L+amp plates, with between 2 600 and 2 800 colonies on each plate. Two replica colony lifts were taken from each plate, and used to identify those colonies which contained plasmids having homology to the Al-induced clones 1.0 to 5.0 (see section 3.2 and Table 3.1). Homologues to each of the five clones were identified (see Fig. 3.5 for an example) and purified. For clones 1.0, 3.0 and 4.0, a total of 13 700 colonies were screened, and five, six and three colonies respectively were identified which hybridised to the probes. For clones 2.0 and 5.0, a total of 8 500 colonies were screened, and four colonies hybridised to each probe. For clones 1.0, 2.0, 3.0 and 5.0, the hybridising pSPORT 1 clone with the longest insert size was chosen for further study. These clones were named wali1, wali2, wali3 and wali5 (for wheat aluminium induced).

Figure 3.5 Colony screening of the Wg+Al(SPORT) cDNA library

An example of one of the colony screens used to identify clones homologous to the differential clones 1.0 - 5.0. The insert from clone 1.0 was isolated (Ch. 2.11.3), labelled (Ch. 2.11.8) and hybridised to the colony lift (Chs. 2.13.6, 2.14.1). After hybridisation, the filter was washed in 2X SSC, 0.1% SDS at 65° C. At this stringency of washing, some background hybridisation to many of the colonies on the filter is visible - this facilitated picking the correct colonies for further work (A). When the colony hybridisations were washed at a higher temperature (85° C), this background hybridisation was eliminated (B).

In the case of clone 4.0, three homologous clones were isolated from the Wg+Al(SPORT) cDNA library. Two of these clones yielded inserts which probably encoded full-length copies (or nearly full-length copies) of clone 4.0 (insert sizes of 2.4 kb and 2.2 kb), and the other had a 0.8 kb insert. However, the 0.8 kb clone showed the strongest hybridisation to the clone 4.0 probe. As it was known from the preliminary sequencing of clone 4.0 that it encoded PAL (Table 3.1) and PAL in many plants is
encoded by a gene family where the members are known to be differentially regulated (Jones, 1984; Schuch, 1992), each of the three clones isolated was sequenced from the 3' end (and the 5' end in the case of the 0.8 kb clone) to determine which of the three showed the greatest homology to clone 4.0. From this information the shorter of the three clones was found to be homologous to clone 4.0, and this clone was named wali4 and used in subsequent work. As several PAL genes already had been isolated from various plant species, finding and sequencing a full-length copy of this gene from wheat was not deemed necessary.

3.4. AL INDUCTION OF THE cDNA CLONES

From the preliminary northern analysis (see Table 3.1 and Fig. 3.2) it was known that the transcripts of each of the wali genes increased in response to Al treatment in the (Al-sensitive) cultivar Warigal. Therefore the timing of induction by Al was characterised for each of the wali genes. Figure 3.6 shows transcript levels of the wali genes in the root tips of Warigal plants treated for varying lengths of time with Al. The comparison includes root tips from plants that were treated with Al for 24 hours and then allowed to recover in medium without Al for varying lengths of time. Relative lane loadings of RNA were estimated by hybridisation to a wheat 26S rDNA probe (Appels and Dvorák, 1982). Densitometry was then performed on each of the hybridising bands, and the relative transcript abundances are shown in Figure 3.6B.

The expression of wali1 increased dramatically after 24 h of Al treatment, and continued to rise up to 96 h when treatments ceased (Fig. 3.6). During recovery from the Al treatment (for up to 96 h), the expression of wali1 decreased, although it did not return to basal levels. The expression patterns of wali3 and wali5 were similar to wali1 except that the expression of these two genes reached a maximum after 48 h of Al treatment. wali4 had a lower level of induction with a transient increase after 0.5 h, followed by a second increase in transcript abundance after 24 h. The transcripts of wali4 returned to pre-treatment levels during the 96 h recovery from the Al treatment. Similarly, wali2 had a complex pattern of induction, with a transient peak of expression after 0.5 h of Al treatment, and a second increase after 24 h. The expression of wali2 did not return to pretreatment levels even after a recovery period of 96 h.

3.5. THE AL RESPONSE OF THE WALI CLONES IN OTHER WHEAT CULTIVARS

A second series of northern hybridisations was performed to observe the effect of Al concentration on induction of the wali genes in Warigal and to determine whether higher levels of Al are required to induce the wali genes in the more tolerant wheat cultivar, Waalt (Fig. 3.7). wali1, -3, -4, and -5 behaved similarly in these experiments and are considered first. These four genes showed increased expression levels with increasing concentrations of Al in Warigal roots. In Waalt, expression of wali1,
Figure 3.6 Northern analysis of clones wali-5 over a time course of Al treatment.

(A) Northern hybridisations showing a time course of wali gene induction by Al. Lanes contain approximately 10 µg of total RNA extracted from the root tips of Warigal seedlings that were either untreated (0), treated with 10 µM Al for 0.5, 2, 4, 24, 48, or 96 h or Al treated for 24 h, followed by recovery in Al-free media for 4 (r4), 24 (r24), or 96 h (r96) (Chs. 2.7.3, 2.9 and 2.13.3). The membrane was successively hybridised with 32P-labelled insert DNA isolated from each of the wali clones and then with a 26S wheat rRNA probe (from pTA250.2, Ch. 2.11.8, 2.14.2). The rRNA probe served to estimate relative loadings of RNA in each lane. In this and all other northern hybridisations shown in this work, only the portion of each autoradiograph that has the hybridising bands is shown. (B) Densitometry (Ch. 2.14.2, 2.15.2) was performed and the expression level of each band in (A) was determined relative to that of the rRNA band. The fold-induction of each treatment was calculated relative to the untreated (0) sample.
3, 4 and 5 was induced only at the inhibitory concentration of Al (100 μM). A reduction in transcript abundance of wali1, -3, and -5 was observed in Waalt at the lower, non-inhibitory concentration of Al (10 μM), relative to untreated roots. At this level of Al, Waalt seedlings usually show no reduction of root growth.

wali2 showed a different pattern of induction from the other four genes. The level of expression of wali2 was increased in Warigal after 2 d of treatment with 10 μM Al, but reduced after 2 d in 100 μM Al. In addition, the expression of wali2 was not significantly affected in Waalt at 10 μM Al (in contrast to the reduction in expression found for wali1, -3 and -5), or at the higher Al concentration (100 μM).

The wali genes differed in their basal levels of expression between the two wheat cultivars (Fig. 3.7). wali2 had a decreased level of expression in Waalt relative to Warigal, while the other four genes had slightly higher expression levels in Waalt.

RNA samples from wheat roots that had been treated with toxic levels of Cd (250 μM), or had been heat shocked for 4 h were included in the northern analysis to determine if the wali genes were induced in response to these stresses. Both of these stress treatments created severe effects on the plants. For example, in the case of the Cd treatment, I was unable to isolate RNA from plants that were exposed to the Cd for long periods of time (i.e. 2 d). Warigal plants subjected to heat shock for up to 2 d are also generally dead before this time (K. Richards, personal communication). For each of the wali genes, expression levels were generally down-regulated by both Cd stress and heat-shock treatment (Fig. 3.7). The exception is wali4, where expression levels are relatively unchanged by Cd treatment.

Leaf tissue from plants whose roots had been stressed with Al was also examined to determine whether the wali genes are expressed in other tissues (Fig. 3.7). wali1 was the only gene of the five exhibiting detectable expression in leaves. There was no induction of wali1 in leaves by Al; in fact transcript levels appeared to decrease slightly in response to the 10 μM Al treatment.

During the course of this work, seed from the near isogenic wheat lines SS and RR (Ch. 2.7.1) became available. Given that each of the wali clones exhibited some differences in expression in cultivars Waalt and Warigal, I decided to investigate whether similar differences occurred in SS and RR. Northern analysis was performed using RNA that had been isolated from the root tips of SS and RR plants which had been either untreated, or treated with moderate (10 μM) or high (100 μM) levels of Al (Fig. 3.8). wali1, -4 and -5 showed a very similar reaction to the Al treatments in SS plants as had previously been observed for Warigal. mRNA levels generally increased with increasing amounts of Al. wali3 mRNA levels also increased dramatically with 10 μM Al treatment, however the 100 μM Al treatment did not show a greater increase than the lower Al treatment. In the case of RR plants, wali1, -3 and -5 transcripts increased with increasing concentrations of Al (although the increase at 10 μM Al was not as
large as that which occurred in SS); whereas wali4 showed only a slight increase in expression with the 100 μM Al treatment.

**Figure 3.7 Northern analysis of wali1-5 expression in Warigal and Waalt plants**

(A and B) Northern hybridisation of the wali clones to total RNA isolated (Ch. 2.9) from wheat plants after different treatments. Lanes contain approximately 10 μg of total RNA extracted from either the roots or leaves of the sensitive cultivar Warigal (Wg) and the tolerant cultivar Waalt (Wt). Plants were treated with 0, 10, or 100 μM Al for 48 h, or with 250 μM Cd for 4 h, or heatshocked (h/s) at 42°C for 4 h (Ch. 2.7.3). Two separate gels (A and B) were electrophoresed and alkali blotted, and the membranes hybridised successively with 32P-labelled insert DNA isolated from each of the wali clones and a 26S wheat rRNA probe (from pTA250.2, see Ch. 2.13.3, 2.14.2). The rRNA probe served as a control to estimate the relative lane loadings of RNA in each lane. (C) Densitometry was performed and the expression level of each band in A and B was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the untreated (0) root sample from Warigal.
In the case of wali2, the response of this gene to Al in the SS plants was similar to the response observed in Waalt plants (compare Fig. 3.8 to Fig. 3.7). wali2 expression did not increase in response to Al treatment in SS plants. Although the northern shown in Fig. 3.8 has only very faint bands (observable on the original autoradiograph, and in the densitometry results), this result was observed in other northern analyses (see Ch. 4 and Appendix 3). Similarly, wali2 transcripts showed an identical response in RR plants as that which occurred in Warigal. Transcripts homologous to wali2 increased in RR plants treated with 10 μM Al, and decreased in RR plants treated with 100 μM Al.

3.6. NUCLEOTIDE SEQUENCE ANALYSIS OF THE WALI CLONES

To further characterise the five wali clones, the nucleotide sequence of each of the clones was determined. Restriction fragments from each of the clones were isolated, and subcloned into pUC-based plasmid vectors (listed in Ch. 2.3.2). Some subclones (of wali4) were also obtained using a DNAse I deletion strategy (Ch. 2.11.7). A summary of the subclones obtained and how they were derived, along with the final sequencing strategy is presented in Appendix 1. Double-stranded DNA purified from these subclones (Ch. 2.8) was sequenced by the Centre for Gene Technology Sequencing Unit using dye-labelled primers homologous to regions in the multiple cloning sites of these plasmids.

The sequence information obtained from these subclones was corrected and aligned into a contiguous sequence for each of the wali clones, using the University of Wisconsin GCG programs 'GELENTER', 'GELOVERLAP' and 'GELASSEMBLE'. The complete sequences were submitted to Genbank and assigned the following accession numbers: wali1, L11879; wali2, L11880; wali3, L11881; wali4, L11883; wali5, L11882. The nucleotide sequences of each of the wali clones, along with their deduced amino acid sequences, are shown in Figure 3.9.

Each of the five wali clones has the general structure expected for cDNA clones, with a poly-A tract at the 3' end. The problems encountered with the clones isolated from the Wg+Al(ZAP) library, namely the presence of multiple adaptors at the 5' end of the clones, was not evident in any of the wali clones isolated from the Wg+Al(SPORT) library.

Each of the sequences was scanned for putative polyadenylation signals. In animal cells, the consensus polyadenylation signal is AATAAA (found between 10 and 40 bp from the poly(A) tail). However plant polyadenylation signals appear to be more variable, not only in the sequence of the signal, but also in its location and frequency (Heidecker and Messing, 1986; Dean et al., 1986). Potential polyadenylation signals in each of the wali clones have been underlined in Figure 3.9, and a summary of their sequences and locations is presented in Table 3.2. The potential signals found in wali3 and wali5 are the only ones that match exactly the consensus sequence, but the signals in wali5 are located further upstream than might be expected. Each of the wali clones (with the exception of wali5) has a putative polyadenylation signal within 40 bp of the poly(A) tract. As a consensus has not yet been reached on the
polyadenylation signals utilised in plants, it cannot be predicted whether these sequences are sufficient for polyadenylation in these genes, or whether other sequences are involved.

Figure 3.8 Northern analysis of wali1-5 in SS and RR plants

(A) Northern hybridisation of the wali clones to total RNA isolated (Ch. 2.9) from wheat plants after Al treatments (Ch. 2.7.3). Lanes contain approximately 4 µg of total RNA extracted from the roots of the sensitive cultivar SS and the tolerant cultivar RR. Plants were treated with 0, 10, or 100 µM Al for 48 h. For other details of the northern analysis, see the figure legend to Fig. 3.7. (B) Densitometry was performed and the expression level of each band in (A) was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the untreated (0) sample from SS.

Figure 3.9 Nucleotide and deduced amino acid sequence of the wali clones

wali1

<table>
<thead>
<tr>
<th>Nucleotide Sequence</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCCTCGACACAAAGCTCATCTTCTTCTTTTGAAATCTTTTTGGTTTCATCAGATTTC</td>
<td>MSCNCGSGCSCGSDCKCGK</td>
</tr>
<tr>
<td>GAGGATGTCTTGGACTGATCCGGGTGCAGCTGCAGCTGCAAGTGCGGGAA</td>
<td>MYPDLTEQGSAAAAVAAVV</td>
</tr>
<tr>
<td>GATGTACCTGATCTGAGGAGGAGGGAGTGCCGCGCCAGGTCGCCCGCCGTCGGTCGT</td>
<td>LGVAPENKAGQFEVAAAGQ</td>
</tr>
<tr>
<td>CCTCGGCGTGAGGAGGACAGGAGGAGGACAGGAGGCAGGTCGCCCGCCGTCGGTCGT</td>
<td>EGCSCKDNCCKCPNCN*</td>
</tr>
</tbody>
</table>
301  CACTCGTGATGGTGAGATATGCAGTCGCCCTCTGTATCTGTGATGGAG  360
361  TCGAGCAAGGGTGCTGTCGCTGCGTGCGGTGTTTTACCTGTCTCCTCCGCCATGTCT  420
421  TGCCCTTGGTGCCCTGCTGTGTGATGTCGACGTCGCCCTCGTAAATGCTTATCTATC  480
481  TCCACATGATGGAGTGATGTAATATGTAAGAATGAAATGATTACCCTAATTAACCTACTCC  540
541  TAAATCGCTACTTCCGGTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  575

wali2

1  CTTGTGAGCCCGTAGAAATTAGTTTCACAAACCACCCAGCTAGCTAGCTAGGTTATGGTGATCCCGA  60
   MGMCSDD
61  CAAGAAGCTAGCACGGCCCGGCTGAGGGTGTCTGTGGTGATGTCGCTGCGCTGGGCAATGCCCTGCT  120
   KKRSSPGVALWCVVLGIALL
121  CACTGTCAGCCGCGCCGGGCCAGTTGAGGTCTGTCGAGAGACGCCGGCAATTTGCTGAAGAGCA  180
   VVDAGGSEGRRAGQLAEEH
181  TGGGGATGCGCCCTACTGCTGCGCGCCTGCAAGGCCGGTGAGCTGCAACCTGGGACC  240
   GDAAYCSALCKGRSQSDFGP
241  GTGTTCACATAGTGCTTCTTACGAGGAGGTACAGGACAGAGAGAGAGCTGAG  300
   CYNECCLYERTQGEARGAG
301  TGGAGTTGAGGGCCACGTGTCTCTGAGAGTGGCGACGGAACAGGGGGTGGACGCGGAAGGA  360
   GVGGPGAGLLEMPTRKVGEEE
361  AGAAGAGAACAAAGGCGAGAGCACCAGCTGCTCGTTGAGCGACCCGCTGCTGGCTA  420
   EEKTKEQGDOTVRDLSDPVPWCY
421  CGACAGTTGCCTGGAACATCCAGAAATTTGGACTAAGACATGCTAATGATGCTACGC  480
   DACREHPEDYNQCNVNDCYA
481  TGAGAATATGCGCCGAGCCCGCATGGCTGGTGCCCATGGAGAGAGTCGAAGGAGAAGAAGC  540
   ENMPDAAWLVAHHTGGEEA
541  AAGAGGAGGAGTGTTGTAGTCTCCATGAGGGGTGTCGACGATGAGGGTCGACGATGGGC  600
   RGGVVDVPMGMGVQHEAAAGA
601  ACTGTACGGGGCAACTGCGATGAGTATTTGGCCCAAACACTATGATGAAAGGTCTCCCGG  660
   LSRGNCDEYCRKHYDEGSPG
661  GTACAGGCCACTGCCAGTATATGTCGCCACGCTCTAGGCTGAGCAGAAGAGAGAAGAG  720
   YRHCOYMCPRLRVRHGVAEGE
721  AAGAAGCAAGAGAAAGAGCTGTTGTAGTGGCTGACAGTGTGTTGGAAGCCACCTGGCAGACT  780
   EARGGAVDGLTVVGKPHCDW
781  GTTCGCCGACTGTAAGTGTAACACCGCTGGCTGCTGCGGATGTTGGAGAAGAGGTCGCAAGAGA  840
   FRDSCVTPCWRCGRKVRQEE
Sequences similar to the polyadenylation signal consensus (AATAAA) are underlined. Stop codons are represented with *.
In the putative protein sequence of wali2, Cys residues that are spaced in the following way - Cys-X₃-Cys-X₁₀₋₁₄-Cys-X₃-Cys (where X can be any amino acid), are highlighted with double lines above and below the Cys residues, and the entire Cys-motif is underlined.
When the sequence of the wali clones was complete, the University of Wisconsin GCG program 'MAP' was used to determine which was the most likely open reading frame (ORF) for each clone. In the case of wali1, the deduced protein sequence shown in Figure 3.9 was the longest ORF found in the clone. In addition, this reading frame utilises the first ATG triplet found in wali1. There is no upstream, in-frame stop codon in wali1 so it remains possible that the clone is not full-length (i.e. that there is a more 5' ATG not included in the wali1 sequence). However it is reasonable to infer that the initiating methionine indicated in Figure 3.9, does in fact denote the 5' limit of the coding region of this gene, because there are similarities between this clone and homologous genes from other plant species (see Fig. 3.10).

In the case of wali2, the ORF indicated in Figure 3.9 is the longest one present. In addition, this ORF utilises the first ATG present in the sequence. There is a stop codon in this reading frame upstream of the ATG, which indicates that this clone is full-length.

wali4 is a partial clone which encodes the enzyme PAL. The homology to other PAL genes indicated the reading frame which is shown in Figure 3.9. In addition, this is the longest ORF present in this clone. As wali4 is a partial clone, the initiating ATG is not present at the 5' end of the sequence obtained.

The situation with wali3 and wali5 is more complicated. These two clones are homologous to each other (62% nucleotide identity), so it is reasonable to assume that they utilise the same reading frame, and therefore encode related proteins. The reading frames shown in Figure 3.9 correspond to the longest ORF present in wali3. Two different reading frames are present in wali5 that could encode proteins of 9.5 or > 14 kDa. The smaller translational product utilises the first ATG found in the wali5 sequence. If the larger of the two open reading frames is used, it would imply that wali5 is not full-length, because this reading frame lacks an ATG. The larger of the two reading frames was investigated in wali3; in which the larger reading frame was found to possess a stop codon located close to the 5' end. On this basis, the 9.5 kDa reading frame has been identified as the gene product of both genes. In the reading
frames of wali5, the ATG triplet identified as the initialising codon has an in-frame, stop codon located 5' to the ATG. This indicates that wali5 (and also wali3 by homology) is full-length, and contains an entire coding region.

Table 3.3 lists features of each of the wali clones, based on the reading frames inferred from the analysis presented.

<table>
<thead>
<tr>
<th>clone</th>
<th>insert size</th>
<th>mRNA size</th>
<th>protein size</th>
<th>pI</th>
<th>homology</th>
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</thead>
<tbody>
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<td>wali1</td>
<td>575</td>
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<td>7.4</td>
<td>4.3</td>
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<td>wali2</td>
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<tr>
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<td>582</td>
<td>700</td>
<td>9.5</td>
<td>8.2</td>
<td>wali5, B-B proteinase inhibitors</td>
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<td>742</td>
<td>2700</td>
<td>-</td>
<td>-</td>
<td>phenylalanine ammonia-lyase</td>
</tr>
<tr>
<td>wali5</td>
<td>541</td>
<td>700</td>
<td>9.5</td>
<td>7.9</td>
<td>wali3, B-B proteinase inhibitors</td>
</tr>
</tbody>
</table>

Sizes (in bp) of cDNA inserts are taken from the nucleotide sequences. Sizes (in nucleotides) of mRNAs have been estimated from northern hybridisations. Protein size (in kDa) is based on the predicted size of the entire coding region. Similarly, the pI has been calculated from the entire coding region. B-B = Bowman-Birk.

In surveying a range of mRNAs, Kozak (1984) observed that initiation of translation occurs at the first ATG triplet in 95% of the examined mRNAs. However the context of the initiating codon is also important, as the sequence surrounding the ATG appears to be involved in the recognition and initiation of translation (Heidecker and Messing, 1986). The consensus sequence surrounding the ATG of plant mRNAs, determined by Lütcke et al. (1987) is shown in Table 3.4. Table 3.4 also shows the sequence surrounding the first ATG of wali1, -2, -3 and -5, and includes the sequence surrounding the other in-frame ATG codons within the coding regions of each of the clones (each of the ATGs are listed in the order in which they occur in the sequences). In each case the first ATG in the open reading frames of the wali clones matches the consensus at least as well as the other ATGs. In animal systems, the nucleotide at position -3 (the A in the ATG is numbered +1) is known to be particularly important in the process of recognition of the ATG. However, for plants, no clear preference has been observed for the -3 nucleotide under in vitro conditions (Lütcke et al., 1987). The consensus sequence given in Table 3.4 is based on a survey of 61 plant genes, for which positions +4 and +5 were found to be the most highly conserved. However, there is no experimental evidence indicating which nucleotides are the most important in translation initiation in plants. The fact that the first ATG of each sequence follows the consensus at least as well as the other ATGs, combined with its location, indicates that it is reasonable to infer that the first ATG is the preferred initiation codon.

The codon bias for each of the putative coding regions was also investigated. It has previously been noted that many genes from monocotyledons have a preponderance of G or C nucleotides at the third position of the codons (Brinkmann et al., 1987). This bias also occurs in wali1, -3, -4 and -5 with G/C
contents of 87%, 89%, 75% and 89% respectively in the codons' third position. (Note that the value calculated for wali4 is only from the partial nucleotide sequence of this gene, and may not be accurate for the whole gene.) In contrast, wali2 has a G/C content in the third codon position of 57%. The difference in third position G/C preference may be anticipated to affect the translatability of the mRNAs (Brinkmann et al., 1987). This might happen in a number of ways, for example certain types of tRNAs may be more abundant than others, causing those messages with a preponderance of the codons homologous to the anticodons of the abundant tRNAs to be translated more efficiently. Another possibility is that the G/C content may influence the stability or the secondary structure of the mRNAs or how efficiently the message is processed by the ribosomes. This means that the wali2 message may have a different translational efficiency from the other wali mRNAs.

| Table 3.4 Putative initiation codons of the wali clones |
|-----------------|-----------------|-----------------|
| clone     | sequence | location |
| wali1     | GAGG ATG TC  | 65             |
| wali2     | GAGG ATG TA  | 122            |
| wali3     | AGCC ATG GG  | 44             |
| wali4     | GGGT ATG TG  | 50             |
| wali5     | GGAG ATG CC  | 332            |
| wali6     | GAAT ATG CC  | 488            |
| wali7     | CCCA ATG GG  | 566            |
| consensus | AACG ATG AA  | 55             |
| wali9     | GGTC ATG GG  | 106            |
| wali10    | AACC ATG AA  | 73             |
| wali11    | GGTC ATG GG  | 124            |
| wali12    | CGAA ATG TG  | 274            |
| wali13    | GCGT ATG AA  | 292            |
| consensus | AAAA ATG GC  |                |

Nucleotides which match the consensus are underlined. The location refers to the numbering of the A in the ATG which is identified in the complete sequence provided in Fig. 3.9. The consensus sequence is from Lütke et al. (1987).

The G/C content of the wali2 open reading frame was quite different from the other clones and the possibility was investigated that another reading frame might exist in the wali2 clone which might exhibit a much higher G/C content. The G/C content of the first and second positions of the codons in the wali2 open reading frame was therefore calculated. These positions had G/C contents of 65% and 50%, which is still much lower than the third position G/C content of the other wali clones. Within wali2, the longest alternative open reading frame (to the one shown in Fig. 3.9) would encode a protein of 75 amino acids, situated in the wali2 cDNA between nucleotides 465 and 705 (as numbered in Fig. 3.9). The G/C content of the third position of the codons in this open reading frame is 70%. The significance of this open reading frame is unknown, and there are no sequences in the databases with significant homology to this region of wali2.

The nucleotide and inferred protein sequences of each of the wali clones were used in searches against all sequences currently held in the databases available using the BLAST network service to identify
homologous genes from other organisms. The homologies found are summarised in Table 3.3, and are considered in order below:

wali1

The wali1 sequence exhibits homology to a group of plant genes that have been referred to as metallothionein-like, based on similarities to animal and fungal metallothioneins. Figure 3.10 shows an alignment of the wali1 sequence and eight other metallothionein-like proteins (MLPs) isolated from castor bean (Ricinus communis; A. Weig and E. Komor, unpublished), kiwifruit (Actinidia deliciosa; Ledger and Gardner, 1994), mimulus (Mimulus guttatus; de Miranda et al., 1990), pea (Pisum sativum; Evans et al., 1990), barley (Hordeum vulgare; Okumura et al., 1991, clone ids-1 mentioned in Table 3.1), Arabidopsis thaliana (K. Takahashi, unpublished), soybean (Glycine max; Kawashima et al., 1991) and maize (Zea mays; de Framond, 1991). The proteins all contain a central hydrophobic region separating two Cys-rich domains, which each contain three Cys-X-Cys motifs. (One of the motifs in the Arabidopsis, soybean, kiwifruit and castor bean genes has the sequence Cys-Gly-Gly-Cys.) The four genes containing the Cys-Gly-Gly-Cys motifs also contain an additional two Cys amino acids close to the N-terminus of the proteins. Robinson et al. (1993) have proposed that the MLPs be divided into two subgroups based on these differences (type 1, of which wali1 is a member with only Cys-X-Cys motifs, and type 2, with the different spacing of one motif and an additional two Cys residues). They propose that the differences in the pattern of Cys residues may have implications in the metal ion binding capabilities of the two groups of proteins, though experimental evidence for this is lacking at present.

The Cys-rich regions within the MLPs have homology to the animal and fungal metallothioneins (de Miranda et al., 1990; Evans et al., 1990). The central hydrophobic domain of the MLPs is distinct with no homologous sequences found in animal and fungal metallothioneins; this central domain also contains aromatic amino acids, which are not present in other eukaryotic metallothioneins (de Miranda et al., 1990).

Other genes encoding metallothionein-like proteins have been isolated recently that have not been included in Figure 3.10. They include another Arabidopsis clone (Zhou and Goldsborough, 1994), and a clone isolated from Brassica napus (Buchanan-Wollaston, 1994). Both of the putative proteins that are encoded by these clones are type 1, although the Brassica napus sequence lacks the central hydrophobic domain. In addition, four proteins closely related to the plant MLPs shown in Figure 3.10 are also present in the BLAST-accessible databases, but these have not been included in the figure as they lack one or more of the Cys-X-Cys motifs. Three of these genes appear to form a distinct subgroup of the metallothionein-like proteins, with two isolated as 'expressed sequence tags' (from rice and Arabidopsis) and the other isolated in this laboratory by S. Ledger (Ledger and Gardner, 1994). The other related gene encodes a MLP from barley (Klemsdal et al., 1991), which lacks the last Cys-X-Cys motif found in the proteins illustrated in Figure 3.10.
The identification of the homology between wali1 and these genes implies that wali1 is a member of this family of related metallothionein-like proteins. When more is known about the functions and metal-binding characteristics of these proteins, the significance of the differences which have been found between the various subgroups may become clear.

**Figure 3.10 Comparison of plant metallothionein-like proteins**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Sequence</th>
</tr>
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<tr>
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</tr>
<tr>
<td>soybean</td>
<td>MSCCGGNCGCSCGCCKCGNGCGGCCKMYPDL...SY.TESTTTTETL</td>
</tr>
<tr>
<td>castor bean</td>
<td>MSCCGGNCGCSCGCCKCGNGCGGCCKMYPDM...SFS.ETKTTTETL</td>
</tr>
<tr>
<td>kiwifruit</td>
<td>MSCCGGKCSCSCSSCGSGSCCGGCCKMYPDL...SYS.EMTTTETL</td>
</tr>
<tr>
<td>pea</td>
<td>MS......GCSCGSSCNCGDSCCNKRRSSGL...SYS.EMETTETV</td>
</tr>
<tr>
<td>mimulus</td>
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</tr>
<tr>
<td>barley</td>
<td>MS......GSCGSGGCSCDDCKDGKMYPLTEQGSAAAQVAVV</td>
</tr>
<tr>
<td>wheat</td>
<td>MS......CSCGSGGCSCDDCKDGKMYPLTEQGSAAAQVAVV</td>
</tr>
<tr>
<td>Consensus</td>
<td>MS-------C-GCSSSCGCSCGGCCCKMYPLDYS-S-ETTTTET-</td>
</tr>
</tbody>
</table>

Alignment of the deduced amino acid sequences of the wali1 clone (wheat) and other plant MLPs. The conserved Cys residues are highlighted by black boxes. Gaps (represented by dots) have been introduced into the sequences to maximise the alignment. The consensus sequence was generated using the PRETTY program from GCG. The plant sequences were obtained from the Genbank and SwissProt databases as follows: barley, accession number X58540; Arabidopsis, accession number X62818; pea, accession number P20830; mimulus, accession number P20238; kiwifruit, accession number L27811; castor bean, accession number P30564; maize, accession number P30571. The soybean sequence is also included. References for each of the sequences are given in the text.

**wali2**

The wali2 sequence encodes a 37.5 kDa protein with a calculated pI of 5.3 and has no significant homology to any other sequences currently in the databases. It has no obvious leader sequence. An interesting feature is that the putative protein contains 24 Cys residues (of 347 aa), 16 of which are...
aligned into four Cys-X₃-Cys-X₁₀₋₁₄-Cys-X₃-Cys motifs (see Fig. 3.9). A further four Cys amino acids are arranged in a variation of this motif (i.e. Cys-X₅-Cys-X₄-Cys-X₃-Cys). A search of the databases for other proteins containing this repeated motif of Cys amino acids revealed a number of proteins; however, most of these were highly Cys-rich proteins (such as keratin or metallothioneins) containing many other Cys residues in addition to the ones located in the motif. Some other proteins identified in this search contained only one of the motifs, such as the snake neurotoxins. The remaining proteins, with more than one motif, included a diverse range, such as the 'sevenless' protein from *Drosophila melanogaster* (two motifs, accession numbers P13368 and P20806), an adhesion molecule from *Homo sapiens* (three motifs, accession number P23352), a protein-tyrosine kinase receptor precursor from *Caenorhabditis elegans* (three motifs, accession number P24348), and vicilin precursors from *Gossypium hirsutum* (two and three motifs respectively, accession numbers P09799 and P09801). Apart from these cysteine motifs, no similarities were found between any of these proteins and the putative protein encoded by wali2.

**wali3 and wali5**

wali3 and wali5 have 62% nucleotide sequence identity (74% amino acid similarity), and exhibit 51% and 54% amino acid similarity (respectively) to a maize wound induced protein (*WIPI*, Rohrmeier and Lehle, 1993). These putative proteins also share some features with Bowman-Birk serine proteinase inhibitors. An alignment of these proteins is shown in Figure 3.11.

Bowman-Birk inhibitors are small (7 to 8 kDa), cysteine-rich proteins which inhibit serine proteinases. These proteins are termed 'double-headed' inhibitors because they can simultaneously inhibit two proteinases. In many cases, each of the two reactive sites of the inhibitor (marker with asterisks in Fig. 3.11) inhibits a different serine proteinase. The amino acid which is immediately N-terminal to the Ser residue at each of the reactive sites helps to determine the specificity of the inhibitor. For example Leu, Tyr, or Phe at this position inhibits chymotrypsin; Arg or Lys inhibits trypsin; and Ala inhibits elastase (Ikenaka and Norioka, 1986). However, other amino acids are also important in the specificity of these inhibitors, and Ser is not absolutely required in the reactive site (for example the first reactive site of the peanut inhibitor is Arg-Arg, see Fig. 3.11; Norioka and Ikenaka, 1983).

Most Bowman-Birk inhibitors characterised to date have been isolated from the seeds of legumes, where they are abundant (Ikenaka and Norioka, 1986). However, the presence of these proteins is not restricted to seeds; for example, an alfalfa protein was isolated from leaves, and was also shown to be wound-inducible (Brown et al., 1985). Comparison of the predicted protein sequence of Bowman-Birk inhibitors from cDNA clones with the actual protein sequence indicates that an N-terminal leader sequence is cleaved from the mature protein. The leader peptide from the cowpea inhibitor is relatively hydrophobic (Hilder et al., 1989), as are the N-terminal portions of the wali3 and wali5 putative proteins. However, Hilder et al. (1989) propose that this leader sequence does not function as a signal
Figure 3.11 Comparison of plant Bowman-Birk serine proteinase inhibitors with the putative inhibitors wali3, wali5 and WIP1.

<table>
<thead>
<tr>
<th>wali3</th>
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<th>cowpea</th>
<th>alfalfa</th>
<th>peanut</th>
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<tr>
<td>Consensus</td>
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</table>

The consensus of the alignment was generated using the GCG program PRETTY. Residues that are double-underlined in the consensus are common to all the sequences shown. Dots indicate gaps introduced into the alignment to optimise homology between the sequences. The asterisks denote the reactive sites. The numbered Cys amino acids are disulphide bonded as follows: Cys(1)-Cys(14), Cys(2)-Cys(6), Cys(3)-Cys(13), Cys(4)-Cys(5), Cys(7)-Cys(9), Cys(8)-Cys(12), Cys(10)-Cys(11). The sequences were obtained from the Genbank database as follows: WIP1 from maize (Zea mays), accession number X71396 (Rohrmeier and Lehle, 1993); cowpea (Vigna unguiculata, Hilder et al., 1989); alfalfa (Medicago sativa L.), P16346 (Brown et al., 1985); peanut (Arachis hypogaea), P01066 (Norioka and Ikenaka, 1983); millet (Setaria italica), P19860 (Tashiro et al., 1990). The soybean (Glycine max) sequence is also included (Joudrier et al., 1987). For both soybean and cowpea, both protein and cDNA sequences have been obtained (the amino acid sequence derived from the cDNA clone is included to show the N-terminal leaders).

The consensus of the alignment was generated using the GCG program PRETTY. Residues that are double-underlined in the consensus are common to all the sequences shown. Dots indicate gaps introduced into the alignment to optimise homology between the sequences. The asterisks denote the reactive sites. The numbered Cys amino acids are disulphide bonded as follows: Cys(1)-Cys(14), Cys(2)-Cys(6), Cys(3)-Cys(13), Cys(4)-Cys(5), Cys(7)-Cys(9), Cys(8)-Cys(12), Cys(10)-Cys(11). The sequences were obtained from the Genbank database as follows: WIP1 from maize (Zea mays), accession number X71396 (Rohrmeier and Lehle, 1993); cowpea (Vigna unguiculata, Hilder et al., 1989); alfalfa (Medicago sativa L.), P16346 (Brown et al., 1985); peanut (Arachis hypogaea), P01066 (Norioka and Ikenaka, 1983); millet (Setaria italica), P19860 (Tashiro et al., 1990). The soybean (Glycine max) sequence is also included (Joudrier et al., 1987). For both soybean and cowpea, both protein and cDNA sequences have been obtained (the amino acid sequence derived from the cDNA clone is included to show the N-terminal leaders).

peptide for a number of reasons. For example, the proteins are thought to be cytoplasmic, and the N-terminal amino acid of the mature proteins from cowpea (and also soybean) is a serine. The cleavage of signal peptides from mature proteins has been investigated by von Heijne (1983), and the cleavage of the cowpea inhibitor at the Ser residue does not follow von Heijne's rules (Hilder et al., 1989). Similarly, when the hydrophobic N-terminal portion of the wali3 and wali5 proteins was scanned for signal-
sequence cleavage sites, none were found. As the hydrophobic leader does not appear to act as a signal sequence, and it is not found in the mature proteins, it may instead keep the proteins in an inactive form, or it may target the protein to a particular location in the cell. As some of the mature proteins commence with a Ser, it might be speculated that the leader may have been cleaved from the mature protein by a serine proteinase, which it subsequently could inhibit. Wali3 and Wali5 both have a Ser residue between the hydrophobic leader and the rest of the protein (the 22nd amino acid).

The crystal structure of Bowman-Birk inhibitors has been determined (Chen et al., 1992) and the seven disulphide bridges have been identified which link the 14 Cys residues. The disulphide bridges in these proteins are important to their three-dimensional structure. Wali3 and Wali5 (and WIP1) have only 10 of the 14 cysteine amino acids found in most Bowman-Birk inhibitors. It is notable that the four missing Cys residues correspond to two pairs of covalently linked cysteines (Cys4-Cys5 and Cys10-Cys11). By homology, the remaining 10 Cys residues present in these putative proteins are expected to form five disulphide bridges (see Fig. 3.11 legend for details of the disulphide bonding). Similarly, in the other example of the inhibitors where some Cys residues are not present (the millet sequence, see Fig. 3.11), two pairs of Cys involved in two disulphide bonds are absent (one is the Cys10-Cys11 pair, also missing in Wali3, Wali5 and WIP1). Only eight of the possible 14 cysteines are conserved between all of the sequences shown. In addition to the Cys residues, only three other amino acids (one serine residue from the second reactive site, and two aspartic acid residues) are conserved between all of the proteins. Another highly conserved amino acid is the other Ser residue in the first reactive site, which is present in all the sequences presented except for the peanut inhibitor; and a Pro residue situated two amino acids C-terminal to the first reactive site Ser is also conserved. This Pro is present in all of the established Bowman-Birk proteinase inhibitors, but not in Wali3, Wali5 or WIP1.

Wali4
The putative protein encoded by Wali4 is homologous to the enzyme phenylalanine ammonia-lyase (PAL). PAL catalyses the first committed step in the phenylpropanoid pathway (Jones, 1984). The wali4 clone contains sequences that correspond to the C-terminal 129 amino acids of the PAL protein. Over this region the wheat PAL protein is homologous to other plant PAL genes currently in the databases, with amino acid identity ranging from 63% (e.g. tomato [Lycopersicon esculentum], Lee et al., 1992) to 68% (e.g. sweet potato [Ipomoea batatas], Tanaka et al., 1989). An alignment of the wali4 amino acid sequence to the homologous region found in other plant PAL proteins is shown in Fig. 3.12.

3.7. DISCUSSION

3.7.1 ISOLATION OF DIFFERENTIAL cDNA CLONES
The goal of the work in this section of the thesis was to characterise the molecular response of plants to Al stress. Plants were grown hydroponically in a low ionic strength medium, where it is possible to
control Al toxicity, in contrast to soil systems which are much more complex. The wheat cultivar Warigal was chosen for this research, because its response to Al had been well studied, and a closely related cultivar was available that differed in its Al tolerance (Waalt, see Ch. 2.7.1). Arabidopsis thaliana, a plant used widely as a model system, was not selected for this study since there are difficulties in growing Arabidopsis hydroponically, and at the time this work was commenced, less was known about the response of Arabidopsis to Al. The root tips of Warigal plants treated with Al were used as the starting material for identifying clones whose expression was induced by Al. The root tip
was harvested for RNA isolations from the plants because it has been shown that the root tip is involved in sensing Al in the environment (Ryan et al., 1993). As a practical consideration, it is also much easier to extract RNA from root tips than it is from entire roots. A 2 d treatment with Al was chosen because this length of time causes a clear phenotypic difference between control plants and plants that have been treated with Al (i.e. the roots are visibly shorter and the tips thickened).

Five cDNA clones (wali1 - 5) representing four different gene families were identified on the basis of their induction by Al stress of wheat roots. Not every gene induced by Al would have been identified in this differential screening for several reasons. Firstly, clones may have been 'missed' during the screening. There is some evidence for this, related to the number of homologues of each wali clone that were isolated from the libraries. Assuming that the wali clones were represented equally in both the Wg+Al(ZAP) library and the Wg+Al(SPORT) library, then larger numbers of these clones should have been identified. For example, as four homologues of wali2 were found in 8 500 colonies of the Wg+Al(SPORT) library, approximately 26 homologues should have been detected in the 55 000 plaques screened in the Wg+Al(ZAP) library; this implies that wali2 was probably 'missed' many times during the screening of the Wg+Al(ZAP) library. This success rate means that it would have been unlikely that I would have identified differential cDNA clones which are expressed at much lower levels than the wali clones (which ranged from 0.02 - 0.05% of the Wg+Al(SPORT) library). Secondly, rare differential mRNAs would not have been identified in the differential screening because the probes used represent a population of cDNAs, and rare messages within the library will not be labelled intensely enough to be detected. Differential screening is not considered sensitive enough to reliably detect mRNAs that are present at much less than 0.01 - 0.05% of the total mRNA population (Sambrook et al., 1989).

Other differential clones may have been missed in the screening shown here, since the level of induction of particular cDNA clones was used to determine whether or not to continue with their characterisation. Many clones were discarded that only showed slight differences in hybridisation to the two probes ('Wg' and 'Wg+Al'). It is possible that differences in transfer of nucleic acids to the duplicate membranes during the differential screening masked greater changes in expression levels of some of these clones. In addition, differential clones may not have been picked from the initial library screen, due to the methodological difficulties inherent in differential screening. However, the fact that some duplication of clones occurred during the differential screening suggests that there is not an unlimited number of major changes in gene expression occurring in response to Al stress.

Previous protein analysis of the Waalt and Warigal cultivars identified a single protein (Q/q, molecular mass of 26 kD, pl of 6.5, Picton et al., 1991) that was consistently induced following Al treatment. None of the proteins encoded by the wali genes correspond in size to this protein. The three wali genes whose mRNA accumulated to the highest levels, wali1, 3 and 5 (Fig. 3.6 and 3.7), encode proteins too small to have been observed in the protein work (<20 kD, see Picton et al., 1991).
3.7.2 Expression of the wali clones

Total RNA extracted from the wheat plants was used in the northern hybridisations shown in this chapter. Hybridisations with the control 26S rRNA probe served to estimate the relative loadings in each lane of the gel, and as a check that there was equal transfer efficiency across all lanes of each northern gel. However, the use of this rRNA probe as an internal standard is based on a number of assumptions. For example, the relative amounts of rRNA to mRNA in different samples or treatments is assumed to be approximately equal. It also assumes that the RNA isolation procedure that was used did not result in changes in the ratio of rRNA:mRNA for any of the different treatments (i.e. that the presence of metal ions such as Al did not affect the efficiency of isolation of the rRNA or mRNA). At this stage it is uncertain whether or not these assumptions are valid. Other 'control probes' have been used in northern analyses, such as probes homologous to 'housekeeping' genes (for example, actin, tubulin or ubiquitin) that are assumed to be ubiquitous in all sample types. However, it is not clear that these genes will be unaffected by Al treatment, particularly since cell division and other essential cellular functions are known to be affected in root tips. The rRNA probe therefore was chosen as the best control available at present.

Densitometry was used to normalise the results obtained from the northern hybridisations. There are several possible areas where inaccuracies may be introduced into this sort of data, and these are discussed in Appendix 3. Also included in Appendix 3 are the densitometry values (after normalisation to the rRNA probe, and calculation of the level of expression relative to the untreated plants), of all treatments that were repeated in this work. This Appendix also contains information from the other northern analyses presented in Chapter 4 and Appendix 2. It can be seen, from a comparison of the results obtained from similar treatments, that many of the values obtained are similar between experiments.

Four of the five wali genes had similar patterns of induction by Al (wali1, 3, 4 and 5). All four showed induction, returned to near basal levels when the roots were removed from medium containing Al, and were induced in all cultivars tested (Waalt, Warigal, SS and RR). The response to Al treatment of the fifth gene, wali2, was notably different. wali2 had a complex bimodal induction pattern, and its expression did not decrease after removal of Al. Induction was seen in Warigal and RR but only at the lower of the two Al concentrations tested. For these reasons wali2 will be considered separately to the other four wali genes in the discussion below.

Higher levels of Al were required for induction of the four wali genes in the tolerant cultivar compared to the sensitive cultivar (Fig. 3.6). This result supports the conclusion that induction of these wali genes is a specific response by the roots to the inhibition of growth by Al. It also suggests that these four genes may be induced in response to Al in other wheat cultivars. Homologues of wali1 (Evans et al., 1990; de Miranda et al., 1990) and wali4 (Ohl et al., 1990) have been shown to be induced (or repressed) in
response to varying concentrations of metal ions and other environmental stresses in other plants; thus it seems likely that they might also be induced by Al treatment in different species.

The genes wali1, 3 and 5 did not show induction within four hours of the introduction of Al stress, but were induced by 24 h (Fig. 3.6). Thus they fit the category of 'late' induction genes, and probably are not part of the initial response of the root to Al, but are involved in the downstream effects. The bias observed towards late genes probably derives from my use of a cDNA library constructed from root tips treated for two days with Al.

Somewhat surprisingly, all four wali genes (-1, -3, -4 and -5) were induced to higher levels by 100 μM Al in the sensitive cultivar Warigal than they were at 10 μM Al. The lower concentration of Al is sufficient to halt root elongation almost completely in Warigal (see Picton et al., 1991). It therefore appears likely that the four wali genes are not responding to the cessation of root growth per se, but to some other effect of the Al treatment. For example, the more severe treatment with 100 μM Al affects the ability of the root tip to recover and continue growing if Al is removed (Picton and Richards, unpublished data), and also has a more drastic effect on protein synthesis (Picton et al., 1991).

wali2 is quite different from the other wali genes. Firstly, the expression of wali2 was only induced at low Al concentrations. Secondly, there were cultivar differences in the induction response observed for Warigal and RR, but not for Waalt or SS. The Al-response of wali2's expression is probably not linked to the Al-resistance gene of Waalt, because if this were to be the case, we would expect to see the same response in RR as is seen in Waalt. Thirdly, wali2 is also different in that the G/C content in its codons' third position is substantially lower than in the other wali clones. This may have implications in the ultimate protein levels of each of the genes.

Expression levels of all five wali genes were reduced or unaffected after 4 h exposure to cadmium stress or to heat shock (relative to untreated controls). The pattern of Al-induced changes in protein synthesis (Picton et al., 1991), combined with the fact that none of the wali clones corresponded to known heat shock genes, suggests that the plant's reaction to Al stress is different from that to heat shock. Recently it has been shown that heat shock genes corresponding to hsp70 and hsp17 are not induced by Al stress (Richards and Gardner, 1994; Ch. 1.7). The cadmium and heat shock treatments used in the experiment shown here (Fig. 3.7) were very severe. It is possible that the wali genes may be induced by less severe and longer exposures to treatments of these types, as the Al treatments used were not as severe, and a change in wali gene expression usually did not occur until after 24 h of Al stress. It is also possible that the expression levels of the wali genes may increase in response to other environmental or nutrient stresses in plants. This possibility is explored in Chapter 4.
3.7.3 Possible Functions of the Wali Clones

The only function attributable to any of the Al-induced genes at this stage is to wali4, which encodes a protein with high homology to PAL (Fig. 3.12). PAL expression in plants has been shown to be regulated developmentally and by various environmental stresses such as wounding, HgCl2, UV light and fungal elicitors (Ohl et al., 1990). Many of the plant metabolites synthesised via pathways downstream from PAL, including flavonoids and anthocyanins, have high affinity for Al (Malterud, 1982; Taylor, 1991). A reduction in the effective cellular concentration of one or more of these compounds due to sequestering by Al may lead to the induction of PAL. Another possibility is that the root thickening or the 'browning' that occurs in the presence of Al requires the biosynthesis of lignin, tannins or other downstream products of the PAL enzyme.

wali1 is homologous to a group of cysteine-rich plant proteins which are similar to animal metallothioneins, and which are termed metallothionein-like proteins (MLPs). It is highly unlikely that MLPs bind Al, since Al co-ordination by proteins involves carboxyl groups rather than sulphhydryl groups, and it has been shown that animal metallothionein does not bind Al (Putterill and Gardner, 1988). Since the barley MLP gene is induced in iron-deficient growth conditions (Okumura et al., 1992), and since the Fe3+ and Al3+ ions are very similar (e.g. Martin et al., 1987), one possibility is that Al may induce Fe deficiency in wheat roots. However, lowered Fe concentrations have not been observed consistently in wheat roots or leaves after long term treatment with Al (Wheeler et al., 1992c). Another possibility is that Al might induce wali1 by interfering with the plant's normal pathways for uptake or homeostasis of other metal ions. For example, it is known that the MLP gene from mimulus decreases in abundance in response to Cu, Cd and Zn (de Miranda et al., 1990), and in vivo experiments suggest the MLP may bind to these ions (Tommey et al., 1991; Evans et al., 1992). However, the only ion consistently altered by Al treatment of wheat roots is Mg (Wheeler et al., 1992c), and a possible role for Mg in regulation of MLPs has not, to date, been investigated.

wali2 encodes a novel, cysteine-rich protein with no significant homology to any sequences found in the database. The periodicity of the cysteines in the protein encoded by wali2 (Fig. 3.9) is reminiscent of Cys2Cys2 Zn fingers, although conserved amino acids present at other locations in the Cys2Cys2 Zn finger motifs are not present in wali2, and the precise spacing of the cysteine pairs differs (Glover, 1989). Comparisons of the Cys motifs in wali2 also showed no obvious homologies between the motifs except for the Cys residues. The large number of cysteines in the wali2 protein are suggestive of either coordination to metal ions such as Zn, or a large number of disulphide bridges in the protein. It is possible that these Cys motifs make up a repeating structural domain in the protein, though so far there is no indication of its role in wali2.

wali3 and wali5 show some similarities to Bowman-Birk serine proteinase inhibitors. Although the amino acid homology is quite low (Fig. 3.11) both the conservation of key cysteine amino acids with an essential role in the structure of the protein and the presence of reactive site serine amino acids, suggest
that wali3 and wali5 do indeed belong to this class of proteinase inhibitors. Such proteinase inhibitors may be induced by the plant in response to the Al treatment in order to reduce the chances of infection or further damage that might be caused by organisms taking advantage of the Al-induced damage to the plant's root system.

Another possibility for the induction of some of the wali genes in response to Al treatment, is that Al toxicity may be causing oxidative stress to the plant, perhaps by interactions of the Al ion with the plant cells' plasma membranes (Gutteridge et al., 1985; Ch. 1.5). Alternatively, the Al-caused damage and disruption to epidermal and cortical cells (e.g. Bennet et al., 1985a, see Ch. 1.3) may be similar to that caused by mechanical wounding because free radicals are known to have a role in wounding of plant tissues (Thompson et al., 1987). Proteinase inhibitors have been reported to have the ability to reduce the formation of oxygen radicals though the mechanism by which they have this effect is unknown at present (Frenkel et al., 1987). If the wali3 and wali5 proteins also have this ability, they may be induced in the plant to protect against the damaging effects of any oxygen radicals present. Similarly, animal metallothioneins have been reported to be efficient scavengers of hydroxyl radicals (Thornally and Vasák, 1985). If the plant metallothionein-like proteins do in fact have this ability, then wali1 may also be induced in response to Al-caused oxidative damage to the plant.
STUDIES ON THE EXPRESSION OF THE WALI GENES

4.1. INTRODUCTION
Chapter 3 described the isolation of five cDNA clones (wali1-5) whose expression increased in response to Al stress. Several of these clones were shown to possess homology with genes isolated from other species that had been subjected to different types of stress (see Ch. 3.7). However, the northern analyses presented in Chapter 3 indicated that plants heat shocked or treated with highly toxic amounts of Cd showed no increase in expression of any of the wali genes. The conflict inherent in these two sets of observations lead me to investigate whether factors other than Al stress could, in fact, induce the expression of the wali genes in wheat. This chapter of the thesis therefore presents the results of experiments carried out to investigate further the expression of the wali genes.

Section 4.2 of this chapter describes a series of northern analyses, designed to determine whether or not the wali genes are induced by other stresses. The stresses tested were toxic levels of other metals, reductions in the concentrations of some nutrient minerals, and wounding. Toxic metal stresses and wounding were investigated because it was known that homologues of wali3, -4 and -5 are induced by at least one stress of these types. The effects of low nutrient conditions were investigated since it is known that Al stress affects mineral uptake and can result in symptoms typical of mineral deficiency (Ch. 1.3). If the wali genes could be induced by a reduction in the availability of a particular nutrient it is possible that this nutrient may have a role in the induction of these genes during Al stress. Most of the experiments presented here were done in duplicate - the duplicate results are presented in Appendix 2. Also included in Appendix 2 are the results obtained from plants that were grown in medium with low levels of O2 or grown continually in the dark. These treatments were not included in this chapter due to the difficulties in reproducing the experimental conditions. A comparison of all treatments that were repeated in the northern hybridisation experiments presented here and in Appendix 2 is shown in Appendix 3.

Although northern analysis can provide information on the relative levels of expression of a gene in a particular plant organ, it does not have the resolution to determine which cells are responding to the stress. For example, it was not known whether all of the wali genes were expressed in identical areas of the root tip. In addition, although an increase in expression may be detected in response to Al stress, it was not known whether all cell types expressing the wali genes were responding to the stress, or if only certain subsets of the cells respond. In order to derive spatial information about the expression of the
wali genes, *in situ* detection of the wali mRNAs was performed in root tips of untreated and Al-stressed plants (Ch. 4.3).

### 4.2. INDUCTION OF THE WALI CLONES BY OTHER STRESSES

The northern analyses which are presented in this chapter were carried out using the wheat line SS (see Ch. 2.7.1). SS plants were used primarily because of a lack of availability of Warigal seed. All of the wali genes (with the exception of wali2) yielded a similar response to Al in both SS and Warigal, and these two lines of wheat are closely related. Differences between the responses of Warigal and SS should therefore be minimal.

The root growth of the plants during the treatments used in this section was also measured. This information allowed me to estimate what effect (if any) the treatments had on the plants. Many of the metal ion concentrations used in this portion of the work were based on recommendations by David Wheeler of AgResearch, Ruakura.

#### 4.2.1. Cd TOXICITY

This experiment was performed with the help of Keith Richards of this laboratory. He treated SS plants with a range of Cd concentrations (0 - 200 μM), and measured root growth after 2 d (Fig. 4.1A). Levels of Cd up to 25 μM did not greatly reduce root growth. High concentrations of Cd had a more severe effect on plant root growth, and it was not always possible to isolate RNA from plant root tips that had been treated with these higher concentrations of Cd. RNA that was isolated from plants treated with 0 to 100 μM Cd was resolved by electrophoresis through a sodium phosphate agarose gel and blotted to a nylon membrane by Keith Richards. I then hybridised this membrane with probes generated from each of the wali clones, to determine what changes in expression occurred. A 26S rRNA probe from the plasmid pTA250.2 was also hybridised to the membrane to determine the relative loading levels of the RNA (Fig. 4.1B). I then performed densitometry on each of the bands shown in Figure 4.1B, and calculated the relative induction of each treatment on the expression of the wali genes compared to the untreated sample (Fig. 4.1C).

In general, for each of wali1, -3 and -5, the higher the concentration of Cd used to treat the plants, the greater the increase in expression of each of the genes. wali4 showed a similar trend of increased mRNA levels in response to higher Cd, although the increases in expression were not as great, and more variability was evident. In the case of wali2, no consistent response was observed in the transcript levels observed over the range of Cd concentrations used.

The decrease in root growth with 50 μM Cd treatment (Fig. 4.1A) was of a similar magnitude to that observed for treatment with 10 μM Al (for example, see Fig. 4.3). A concentration of 50 μM was therefore chosen for further analysis of transcript level changes by northern hybridisation. Figure 4.2A
**Figure 4.1 Effect of varying Cd concentrations on root growth and expression of the wali genes**

(A) Effect of differing concentrations of Cd on root elongation in SS plants. Values were calculated as the mean increase in root length over the 2 d treatment for 10 root measurements, bars show SE. (B) Autoradiographs of northern hybridisations to total RNA extracted from the root tips of the treated plants. The membrane was probed successively with radiolabelled inserts from the wali clones and a 26S rRNA probe. All treatments were electrophoresed through the same gel, and come from the same autoradiograph (some intervening lanes have been removed). (C) Relative expression levels. Densitometry was performed and the expression level of each band in (B) was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the untreated (0) sample. No RNA was obtained from plants treated with 150 or 200 µM Cd, and only a small amount from plants treated with 100 µM Cd.

SS plants were grown in Ruakura medium for 5 d after germination, then transferred to Ruakura medium containing 0 - 200 µM Cd for 48 h. (A) Effect of differing concentrations of Cd on root elongation in SS plants. Values were calculated as the mean increase in root length over the 2 d treatment for 10 root measurements, bars show SE. (B) Autoradiographs of northern hybridisations to total RNA extracted from the root tips of the treated plants. The membrane was probed successively with radiolabelled inserts from the wali clones and a 26S rRNA probe. All treatments were electrophoresed through the same gel, and come from the same autoradiograph (some intervening lanes have been removed). (C) Relative expression levels. Densitometry was performed and the expression level of each band in (B) was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the untreated (0) sample. No RNA was obtained from plants treated with 150 or 200 µM Cd, and only a small amount from plants treated with 100 µM Cd.
shows root growth during 50 μM Cd treatment and recovery. The root growth of plants treated with 50 μM Cd was inhibited relative to the root growth of untreated plants. When plants were grown in 50 μM Cd for 24 h, then transferred to media without Cd, their root growth showed some recovery after 72 h. Figure 4.2B shows the transcript levels of the wali genes in the root tips of wheat plants treated with 50 μM Cd for varying lengths of time. Keith Richards of this laboratory then isolated RNA from these plants, and provided me with the filter used to derive the northern blots shown in Figure 4.2B. Due to variation in the amounts of RNA in different lanes (as indicated by hybridisation to the 26S rRNA probe), the results were normalised by densitometry. The relative transcript abundances are shown in Figure 4.2C.

The relative transcript abundance of each of the wali clones increased with the Cd treatment, although there were differences in timing and levels (Fig. 4.2C). In general, the expression levels of wali1, -2, -3, -4 and -5 continued to increase throughout the Cd treatment (up to 72 h). When plants were treated with Cd for 24 h, followed by recovery in Cd-free medium, the transcript levels of wali2, -4 and -5 decreased, with the expression of wali2 and -4 reducing to pretreatment levels. The expression levels of wali1 and wali3 showed no clear change during the recovery from the Cd treatment (compare the 24 h Cd treatment with the recovery treatments). The transcript increases observed with the wali2 probe contrast with the lack of induction observed with the Cd treatments shown in Figure 4.1.

4.2.2. OTHER TOXIC METALS
Since transcription of the wali genes increased in response to both Al and Cd treatments, their responses to 2 d treatments with inhibitory levels of several other metal ions in the wheat line SS was investigated (Fig. 4.3). Figure 4.3A shows the effect of the different toxic metal treatments on root growth. Each of the metal treatments slowed root growth. wali1, -3, -4 and -5 all showed increases in their transcript levels with all of the metal ions tested (Fe, Zn, Cu, Ga, In and La, Fig. 4.3C). However the wali4 transcript increase was less than two-fold higher with the La treatment than with the control treatment, and therefore may not represent a significant induction. The expression of wali2 was inhibited by several of the metal treatments (Fe, Cu, In and 100 μM Al), but was not affected by the other treatments.

This entire experiment was repeated using the cultivar Warigal (Appendix 2) with similar results for four of the wali genes. The main differences observed were in the absolute levels of induction observed for each treatment. However, there were some differences in the expression of wali2. For example, as was already established in Chapter 3.5, there was a difference in the Al response of wali2 in Warigal and SS, with induction by 10 μM Al in Warigal (Fig. A2.3C) but not in SS (Fig. 4.3C). For five of the other metals (Cd, Fe, Ga, In, Zn), the wali2 transcript changes observed in Figure 4.3 were qualitatively similar to those observed in Appendix 2; however, the responses observed to Cu and La were opposite in the two cultivars. A summary of the expression changes observed is given in Appendix 3.
Figure 4.2 Effect of 50 μM Cd over time on root growth and wali gene expression

(A) Root growth of SS plants over time. Values were calculated as the increase in root length from the starting time point and are the means of 10 root measurements from each treatment. Error bars indicate SE. The graph shows plants that were treated with 50 μM Cd (■), plants that were grown in 50 μM Cd for 24 h followed by recovery in Cd-free media (▲), and plants that were grown in Cd-free media (■). (B) Autoradiographs of northern hybridisations to total RNA extracted from the root tips of the treated plants. Plants were either untreated (0), or treated with 50 μM Cd for 2, 4, 12, 24, 48 or 72 h or treated with Cd for 24 h followed by recovery in Cd-free media for 4 (24/4), 24 (24/24), or 72 h (24/72). Radiolabelled inserts from each of the wali clones and from pTA250.2 (26S rRNA gene) were used to probe the membrane. (C) Relative expression levels. Densitometry was performed and the expression level of each band in (B) was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the untreated (0) sample.
4.2.3. Low Nutrient Conditions

The expression of the wali genes was also assessed in SS plants grown in the absence of one or more of the major plant nutrients, or with very low levels of one nutrient (Fig. 4.4). One set of plants grown under normal conditions and an Al-treated group of plants were included as controls. Low levels of Ca in the growth medium produced the greatest effect on plant growth (Fig. 4.4A), and this treatment also induced transcripts of each of the wali genes except for wali2 (Fig. 4.4B and 4.4C). The expression of wali2 was reduced by the low Ca treatment. These results were also observed in two other independent experiments (both using the cultivar Warigal, see Appendices 2 and 3). Media containing no P, no Mg, no Fe, or containing only CaCl₂ did not have a large effect on expression of wali1, -3, -4 or -5 after 2 d growth. wali4 showed slight induction with these treatments, and wali1, -3 and -5 showed small reductions in the abundance of their transcripts. Some of these smaller changes were not repeatable when the experiment was performed in the cultivar Warigal (Appendices 2 and 3). The expression of wali2 was reduced under most of these conditions, and similar results were obtained when the experiment was repeated in Warigal (Appendix 2). The exception to this occurred when plants were deprived of Mg; in Figure 4.4 this treatment resulted in a reduction of wali2 transcript levels, in Appendix 2 the no Mg treatment resulted in no significant change in transcript levels.

4.2.4. Wounding

Northern analysis was performed on wheat (SS) root and leaf tissue that had been wounded (Ch. 2.7.3), then incubated for 6 h or 2 d (Fig. 4.5). The transcripts of wali1, -3 and -5 all increased in wounded roots, whereas the wali4 transcripts did not show any consistent trends in the time points tested. In leaf tissue, wali1, -3, -4 and -5 all showed increases in their transcript levels with wounding. The expression of wali2 did not show any wound induction in roots, and virtually no expression in leaves was detected at any time point.

4.3. Localisation of Expression of the Wali Genes

To obtain further information about the expression of the wali genes, the patterns of expression of the genes in the root tip (and in leaves for wali1) was investigated using the technique of in situ hybridisation. At the time that this work was commenced, the technique of in situ hybridisation was not established in the laboratory. I decided to use a non-radioactive method to label my RNA probes (Ch. 2.11.9) in preference to radioactive probes for a number of reasons. When ³⁵S- or ³H-labelled probes are used, the photographic emulsion usually requires exposure times of at least several days, and up to several months. ³²P is not generally used to label probes because of its lack of resolution at the microscopic level. Long exposure times can cause considerable delays in optimising a protocol. In addition, the use of photographic emulsions, which are required to detect radioactive emissions, can be the cause of background problems. In contrast, the DIG system of non-radioactive labelling usually results in colour development in less than 24 h, or at most, four days. The non-radioactive system does
Figure 4.3 Effect of various toxic metal ions on root growth and expression of the wali genes.

Plants from the wheat line SS were either not treated (control) or treated for 2 d with Al, Fe, Cd, Zn, Cu, Ga, In or La at the concentrations shown. (A) Increase in root length after the 2 d treatment. Values are the means of 15 root measurements from each treatment. Error bars indicate SE. (B) Autoradiographs of northern hybridisations to total RNA extracted from the root tips of the treated plants. Probes were the inserts from the wali clones or the 26S rRNA gene from pTA250.2. (C) Relative expression levels of the genes. Densitometry was performed on each band of the northern hybridisations, and normalised to those obtained from the rRNA probe. The fold induction of each treatment was calculated relative to the control sample.
not require special handling and disposal conditions as the use of a radioactive probe does, and once DIG-labelled probes are made they will last at least 12 months. A drawback to using the non-radioactive system however, is that counter-staining tissue after hybridisation (which is commonly used with radioactive probes) can obscure the colour obtained from the hybridised probe. For this reason, I did not use any histochemical stains in tissue that had been used for in situ hybridisation.

The in situ hybridisation protocol employed was based on those of Bochenek and Hirsch (1990) and Cox and Goldberg (1988). Paraformaldehyde fixation was chosen because this method appeared to be one of the most commonly used for in situ hybridisation work. Glutaraldehyde fixation is also often used, although this fixative can cause background problems when fluorescently-labelled antibodies are included in detection protocols. Although fluorescently-labelled antibodies were not utilised here, it is possible that they may included in the future. The tissue was embedded in paraffin because paraffin sectioning, in my hands, was more successful than cryosectioning, and it allowed thinner sections to be cut.

The proteinase K digestion step in the protocol was optimised by preliminary work which investigated a range of concentrations (1, 10 and 100 mgL$^{-1}$). The higher concentrations of proteinase K resulted in better accessibility to the mRNA targets, as judged by increased hybridisation. However, at the higher concentration of proteinase K, more tissue loss and damage was evident in the sections. For these reasons, 10 mgL$^{-1}$ proteinase K was used for all the in situ hybridisation experiments shown here. If other fixation protocols are used, this proteinase K digestion step may require optimisation again.

A major difficulty encountered during the optimisation of the protocol was the loss of tissue from the slides. The use of properly treated slides was crucial for the successful adhesion of the tissue. Although more expensive than coating slides by hand, the use of commercially prepared slides (Superfrost® Plus, supplied by Salmond Smith Biolabs) was found to greatly reduce tissue loss. Some damage to the tissue can also occur when removing glass coverslips from the slides. To reduce this damage, pieces of parafilm were instead used, which were more easily removed. A comparison between glass and parafilm coverslips did not show any obvious differences between the signal or background obtained.

Using these general procedures, the root tips from Warigal plants grown in the presence or absence of 10 μM Al for 2 d were used to localise the expression of wali1, -2, -3 and -5. wali4 was not used as a probe because other researchers have investigated the expression patterns of PAL genes in other plant species using both in situ hybridisation (Wu and Hahlbrock, 1992), and fusions of the PAL promoter to the reporter gene β-glucuronidase (Ohl et al., 1990; Shufflebottom et al., 1993).
SS plants were grown for 5 d in Ruakura medium, their roots were rinsed in MilliQ water, and the plants transferred to either Ruakura medium (control), or to media lacking or deficient in one or more nutrients (see Ch. 2.7.2 and 2.7.3 for details), and grown for a further 2 d. (A) Increase in root length after the 2 d treatment. Values are the means of 15 root measurements from each treatment. Error bars indicate SE. (B) Autoradiographs of northern hybridisations to total RNA extracted from the root tips of the treated plants. Probes were the inserts from the wali clones or the 26S rRNA gene from pTA250.2. (C) Relative expression levels of the genes. Densitometry was performed on each band in (B), and normalised to those obtained from the rRNA probe. The fold induction of each treatment was calculated relative to the control sample.
Figure 4.6 shows the results of in situ hybridisations using DIG-labelled antisense probes to wali1, -2, -3 and -5. Results from control (sense) probes are also included to show the background levels of colour development obtained from non-specific hybridisation. As anticipated, root tips from plants treated with Al showed an altered morphology compared to untreated roots. The root tip became thickened due to the enlargement of many cells, in particular the cortical cells. Differentiated cells in Al-treated roots also occurred much closer to the apex of the root tip than in the roots of plants grown in the absence of Al. For example, cells that appear to have thickened secondary walls were visible close to the root apex of some Al-treated plants (Fig. 4.6B and R), but they were never observed in an equivalent position in untreated root tips.

Hybridisation to wali1 transcripts in untreated roots was observed to occur predominantly in the meristematic region of the root tip (Fig. 4.6F). Very little staining was seen in the root cap. In the roots of Al-treated plants, reduced hybridisation to the wali1 transcripts was observed in the enlarged cells of the root apex (such as the cortical cells and cells which appear to be in the quiescent centre, Fig. 4.6G). Further back from the root apex, staining was observed in most cell types, but at a lower level than that found in the root tip (Fig. 4.6H). An increase in hybridisation was seen in cells throughout the root relative to that seen in the root tip. Again, little or no staining was observed in the root cap of Al-treated plants. In leaf tissue, the wali1 probe showed preferential hybridisation to the mesophyll layer of cells (Fig. 4.6E). This pattern of expression was observed in the leaves of both control and Al-treated plants.

Transcripts hybridising to the wali2 probe were observed predominantly in the root cap and the epidermis (Fig. 4.6I and J), though expression was also detected in the meristematic cells in some cases (Fig. 4.6I). In Al-treated plants, the expression patterns were similar, although the expression observed in the epidermal cells was often not as obvious as that seen in the roots of untreated plants (Fig. 4.6K). In general, less hybridisation to the wali2 antisense probe was observed in the root tips of Al-treated plants. Further back from the root tip, wali2 expression was seen predominantly in the outer layers of the root (Fig. 4.6L).

wali3 and wali5 both showed very similar patterns of hybridisation. Very little expression was detected in the root tips of untreated plants (Fig. 4.6M and P). Further back from the root tip, the wali3 probe stained predominantly the cortical cells, with very little staining observed in the stele (vascular tissue). For wali5, very little staining was detected in any cells of untreated roots. In the roots of Al-treated plants, both wali3 and wali5 have similar patterns of hybridisation with transcripts detected predominantly in the outer cortical regions of the root (Fig. 4.6O and S). This pattern of hybridisation was observed much closer to the root apex than was seen for wali3 in untreated plants (Fig. 4.6N). Hybridisation to the wali3 and wali5 probes was also observed in some of the enlarged cortical cells at the root tip (wali3, Fig. 4.6N; wali5, Fig. 4.6Q and R). Hybridisation to the wali5 probe was noted in cells which appear to have secondary cell walls, found in root tip sections of some Al-treated plants (Fig. 4.6R).
4.4. DISCUSSION

4.4.1 VARIABILITY IN RNA LOADINGS IN THE NORTHERN ANALYSES

In several of the northern hybridisation experiments presented here there was considerable variation in the loadings between different lanes, as judged by hybridisation to the 26S rRNA probe. This variation probably occurred because different amounts of RNA were loaded onto these gels. In the Cd concentration experiment, a difference in loading was unavoidable. In this case, the yield of RNA in the 100 μM Cd sample was low and only a small amount of RNA was available for loading (Fig. 4.1). For the other northern hybridisations, it is most likely that uneven amounts of RNA for the varying samples were also applied to the gels. For some samples, aliquots of the RNA appeared to have varying concentrations. The method used to quantify the RNA was measurement of the samples absorbance at OD₂₆₀. Problems were often encountered with the reproducibility of the OD₂₆₀ measurements, with differing values obtained for several measurements of the same sample. In addition, each of the samples was electrophoresed through a TBE agarose gel to check the integrity of the samples, prior to
electrophoresis through the northern gel. These TBE agarose gels also indicated that the loadings of the RNA samples were not even. The cause of this variability is not clear, but may be due to sampling errors, or RNA solutions that were not homogeneous (possibly because of polysaccharide contamination). It is unlikely that the variability seen with the differing amounts of RNA in the northern hybridisations was due to uneven transfer of the RNA. After transfer of the RNA to the nylon membranes, the northern gels were always stained, and transfer of the RNA was usually complete. On occasions when some RNA remained, there was no evidence of places were no transfer had occurred. In addition, when the various wali clones were hybridised to the northern membranes, there was usually no evidence of any hybridisation bubbles, which might indicate uneven transfer. The only case where uneven hybridisation was observed was in the northern analyses presented in Figure A2.2C (in Appendix 2). In this case some of the values obtained from the densitometry may not be as accurate as they would be if the hybridisations had been more even.

4.4.2 Expression of the wali genes in response to stress

Four of the five wali genes (-1, -3, -4 and -5), although isolated because they were induced by Al treatment, appear to form part of a more general plant response to toxic metal stress and to various physical and environmental stresses. There were differences in the responses of these wali genes to the various stresses, both qualitatively (for example, the induction of wali4 expression in roots by Al but not by physical wounding) and quantitatively (for example, compare the relative inductions of wali1 and wali3 after treatment by Cu and Ga in Fig4.3). It is possible that these genes are being induced by different components of the signal transduction pathway for each stress.

Northern analyses presented in Chapter 3 included RNA extracted from the root tips of plants treated with 250 µM Cd for 4 h (Fig. 3.7). There were no increases in transcript abundances for any of these genes under these Cd treatment conditions. However, the concentration of Cd used (250 µM) in the experiment was very high. At this Cd concentration, it was not possible to isolate RNA from plants treated for 2 d, which was the time point used for most of the Al treatments. In the work presented in this chapter I measured induction by 50 µM Cd, a concentration that inhibited root growth to a similar extent as the Al treatments (Fig. 4.3A). Under these conditions, wali1, -3, -4 and -5 showed induction, with a maximum level of expression after 72 h (Fig. 4.2). Similarly, the transcript levels of wali1, -3, -4 and -5 increased when the plants were exposed to toxic levels of all other metal ions tested (Fig. 4.3). These induction results contrast with those reported by de Miranda et al. (1990) for Mimulus guttatus. These workers showed that a reduction of the mRNA encoding a metallothionein-like protein occurred following metal treatments. Direct comparison of the two sets of results is difficult however, since different sampling times (2 d in my experiments, versus 21 d in the M. guttatus experiments) and different metal ion treatments were employed.

Al-induced nutrient deficiencies, including P, Ca or Fe, have been reported (Foy, 1984). The wheat cultivar Warigal shows Mg deficiency after long term Al treatment, and it is known that increasing the
concentration of Mg or Ca in solution can ameliorate Al toxicity in wheat (Edmeades et al., 1991). I therefore examined induction of the wali genes under conditions where various nutrients were omitted from the media, in order to test whether the response to Al stress might be the result of a reduction in nutrient supply. However, with the exception of low Ca (discussed below), the low nutrient treatments did not induce wali1, -3, -4 and -5 (except for a slight induction of wali4 by some of the treatments). Longer periods of low nutrient supply were not investigated. It therefore remains possible that expression of the wali genes may increase in plants grown in nutrient deficient conditions for longer periods of time. Okumura et al. (1992) have shown that 15 d growth in the absence of Fe induced a homologue of wali1 in barley. Similarly, more severe nutrient deficiencies caused by the use of chelating agents might be expected to induce the wali genes. However, the results of the experiments reported in this chapter suggest that induction of wali genes by Al does not result, for example, simply from a cessation of Mg uptake.

Low Ca treatment resulted in the induction of wali1, -3, -4 and -5. While this would be consistent with the idea that Al directly causes an inhibition of Ca uptake, the low level of Ca used was highly inhibitory to root growth. It is possible that induction by low Ca under the conditions used here is part of a more general stress response. For example, the treatment may have caused physical damage to the membranes. It is also possible that the altered ratio of Na:Ca in the treatment may be causing salt stress.

Homologues to wali4 (encoding PAL) have been shown to be wound inducible in other plant species (Lois and Hahlbrock, 1992). The wali4 transcript was induced in wounded leaves, but not in roots. In contrast, wali1, -3, and -5 were all induced in both wounded roots and leaves (Fig. 4.5). The fact that wali4 expression was not affected in wounded roots suggests that the Al-induced increase in expression of this gene is not due to any physical damage of the roots caused by the Al treatment. However, as only two time points were taken after the wounding treatments the possibility cannot be ruled out that the expression levels of wali4 changed more dramatically at some other time after wounding of the root tissue.

Figure 4.6 In situ localisation of wali gene expression

The figure is presented on the following two pages. Root and leaf sections (from the wheat cultivar Warigal) were probed with DIG-labelled RNA probes. Details of the fixation, embedding, sectioning and in situ hybridisation protocols are given in Ch. 2.7.4 and Ch. 2.14.5. The treatment employed, and the probe used are indicated (in blue) on the photographs. Sections (A-D) were probed with sense (control) probes for wali1 (A and D), wali5 (B) and wali3 (C). Sections (E-H) were probed with an antisense wali1 probe; (I-L) with an antisense wali2 probe; (M-O) with an antisense wali3 probe; (P-S) with an antisense wali5 probe. Hybridisation to the probes is indicated by a brown or purple colour. Sections (A, F, I, J, M and P) were from plants grown in Ruakura medium for 7 d (= no Al), and sections (B, C, D, E, G, H, K, L, N, O, Q, R and S) were from plants grown in Ruakura medium for 5 d, then Ruakura medium with 10 μM Al added for a further 2 d (= + Al, Ch. 2.7.3). (A, B, F, G, I, J, K, M, N, P, Q and R) are longitudinal sections of the root apex; (C, H, L, O and S) are longitudinal sections taken approximately 1 to 4 mm from the root apex; and (D and E) are transverse sections of leaf tissue. The magnification of all micrographs is the same. The scale bar (shown in A) represents 0.5 mm. Arrows in B and R indicate a group of cells with secondary cell walls. rc, root cap; s, stelae; c, cortex; q, quiescent centre; e, epidermis.
wali2 gene expression was found to be quite different from that of the other genes studied. The results for wali2 expression were characterised by relatively small changes in expression levels, and often reductions in the transcript levels of wali2 when the other wali genes showed induction. There were also some inconsistencies in terms of whether the wali2 gene was induced. For example, compare the induction of wali2 by 50 μM Cd in Figure 4.2, but not in Figure 4.1 (see also Appendix 3). The variability of expression seen with the wali2 probe may indicate that the wali2 gene is responding to factors other than those tested (for example, root age or root length). Although the plants were grown in a controlled environment chamber and in a standard medium, it is probable that not all conditions were identical between experiments.

4.4.3 IN SITU HYBRIDISATION

The results from the in situ hybridisation experiments indicate that the wali mRNAs accumulated in different areas of the root (Fig. 4.6). Background levels of non-specific hybridisation were generally low. However, further optimisation of the in situ technique might have provided clearer results. The most obvious area for future optimisation work is the fixation method used for the tissue. Both under-fixation and over-fixation of tissue can cause problems with sectioning (leading to unsatisfactory morphology) and can also result in loss of signal or increased background hybridisation (Ausubel et al., 1987; Cox and Goldberg, 1988). The preservation of the tissue examined in this work was not optimal. Details of the tissue located 1 to 4 mm back from the root apex were not always clear, and these regions of the root often appeared to be shrunken in appearance. Another aspect of the in situ method that could be improved by further optimisation of the protocol is the detection of the DIG-labelled probes. Improvements may be made to the colourimetric detection of hybridised transcripts that could further reduce background hybridisation. For example, different blocking reagents could be used prior to the incubation with the anti-DIG antibody. In addition, changes in the incubation times of the blocking step and antibody incubation may result in an alteration of the signal to noise ratio. Alternatively, fluorescently-labelled antibodies could be employed to detect the DIG-labelled probes, which would allow counterstaining of the tissue to better identify the different cell types present in the tissue being examined.

The Al-treated plant tissue used for the in situ analysis showed altered morphology relative to untreated tissue. The changes observed are consistent with those reported previously in wheat (Wheeler et al., 1992b; de Lima and Copeland, 1994) and in Zea mays L. (Bennet et al., 1985a). Changes in the site of expression of the wali genes were observed with Al treatment, although these changes may have resulted from the altered root morphology induced by Al-treatment. For example, reduced hybridisation of the wali1 probe was observed in the enlarged cells of the Al-treated root tip relative to the untreated root tip. Increased vacuolation in the root tips of wheat stressed with Al has been observed previously (de Lima and Copeland, 1994). The presence of large vacuoles would result in less cytoplasm per cell in the sections, and therefore less accessible mRNA. Alternatively, there may be a genuine reduction in transcripts per cell, but the reduction may be an indirect effect of the Al treatment, resulting from the
altered differentiation status of cells near the tip. The majority of the induction of wali1 transcripts in roots may be derived from cells further back from the root apex. These complex differences in expression in different parts of the root make it clear that studies on the regulation of stress-induced genes need to be specific in describing which portions of the root (or any other tissue studied) have been used in the identification of changes of gene expression.

4.4.4 SUMMARY
The changes in gene expression identified as a response to Al stress in root tips are clearly not specific to Al stress, but occur also in response to factors such as toxic levels of other metals, low Ca treatment and physical wounding (with the exception of wali2). Similar results have been reported for a pathogenesis-related protein induced by Al stress (Cruz-Ortega and Ownby, 1993). Most of the wali genes isolated in this work therefore appear to form part of a suite of stress genes that are induced by a range of toxic metal treatments, including treatment with Al.
5.

**INTRODUCTION**

Information on the functions of the wali proteins might be gained in a number of ways. If sequence homology can be detected, it can sometimes lead to the identification of closely related proteins having known functions or enzymatic capabilities (Ch. 3.6 and 3.7.3). Isolating the native protein would enable its biochemical function to be investigated, but the isolation of many proteins is generally not a simple matter, and yields are often low. Furthermore, isolation of the native protein usually requires some specific biochemical knowledge of the protein (for example, the enzymatic function, for which an assay can be developed).

An alternative approach is to produce the protein of interest in a bacterial (or other) expression system. Proteins can usually be readily purified from bacteria, with large yields possible using some types of expression systems. The expressed proteins can then be examined directly for biochemical functions in an *in vitro* assay. Antibodies to the purified (expressed) protein can be used in a variety of ways to determine what (if any) changes in the levels of the protein might occur in response to environmental or developmental cues. An antibody of sufficient specificity and titre can also be used to establish whether or not a protein is located in a specific subcellular location. This type of information may enable the functions or role of a protein to be inferred.

This chapter describes the cloning of the coding regions of each of the wali genes into a bacterial expression vector (pGEX-2T, described in Ch. 5.2.1), the expression of the resulting fusion proteins in *E. coli*, and their purification using affinity chromatography to glutathione S-transferase (GST, Ch. 5.2.2). Once isolated, the wali1-GST fusion protein, was used to raise antibodies for use in western analysis (Ch. 5.3.1). A south-western hybridisation was performed with the wali2 fusion protein, to determine whether any DNA binding activity could be detected (Ch. 5.3.2). The wali3 and wali5 fusion proteins were used in an assay to determine if they could function as proteinase inhibitors (Ch. 5.3.3).

5.2 **EXPRESSION OF THE WALI GENES IN *E. COLI***

The bacterial expression vector pGEX-2T was used in this work. It contains the bacterial protein GST downstream of the inducible tac promoter, with a multiple cloning site allowing insertion of the coding regions of interest in-frame with the GST coding region. The use of the inducible tac promoter enables proteins which might otherwise have a detrimental affect on the growth of the bacterium to be expressed.
in a controlled fashion. The GST moiety of the resulting fusion protein allows easy purification of the recombinant protein, because the fusion protein binds to glutathione sepharose beads, and can be eluted subsequently under mild conditions. The vector possesses a thrombin cleavage site that enables GST sequences to be cleaved from the recombinant protein.

5.2.1 Construction of the pGEX-wali Gene Fusions

GST fusions were generated with each of wali1, -2, -3 and -5. wali4 (encoding the enzyme PAL) was not included as it is a partial clone. The PAL protein has also been studied extensively by other researchers, both in vivo (reviewed in Hahlbrock and Scheel, 1989; Jones, 1984) and also in a bacterial expression system where it was found to retain its catalytic activity (Schulz et al., 1989).

The wali1 coding region contained an in-frame BamH I site that allowed direct cloning of most of the coding region directly into the pGEX-2T vector (the first five amino acids were not included). The 500 bp BamH I fragment was isolated from wali1 cDNA, and ligated into BamH I-digested pGEX-2T DNA. The ligation was transformed into competent E. coli cells, and the resulting recombinant colonies screened for one that contained the wali1 BamH I fragment in the correct orientation. The recombinant plasmid was named 1.4. The 5' end of the insert was sequenced, using a primer homologous to the 3' end of the GST coding region, to confirm that it did indeed contain the correct DNA insert, and that no mutations had been introduced into the DNA during the cloning procedure. A summary of this cloning procedure, including a diagram of the pGEX-2T vector, and which shows which region of the wali1 coding region was included in the fusion, is presented in Figure 5.1.

In wali2, -3 and -5 there were no convenient restriction sites that would allow direct cloning of the coding regions into the pGEX-2T vector so that an in-frame fusion would result. Therefore two oligonucleotides were designed, one homologous to both wali3 and wali5 (KCS1), and one specific to wali2 (KCS2). These oligonucleotides included two (KCS1) or three (KCS2) nucleotides that were different from the wali DNA. In each case, these differences meant that an in-frame BamH I site would be incorporated into the sequence of PCR products amplified using these oligonucleotides. The sequences of the KCS1 and KCS2 oligonucleotides are compared to the homologous sequences of wali2, -3 and -5 in Table 5.1.

The oligonucleotide KCS2 (homologous to wali2) was designed to amplify all of the wali2 coding region. A PCR amplification was performed using the KCS2 and forward primers with wali2 DNA as the template and the conditions described in Chapter 2.11.10. A PCR product of the expected size (1250 bp) was amplified, digested with BamH I and ligated into BamH I-digested pGEX-2T DNA. The ligation mixture was transformed into competent DH5α cells and the resulting recombinant colonies screened for one that included the wali2 PCR product in the correct orientation. The recombinant plasmid obtained was named 2.13. As was done for the wali1-GST fusion, the 5' end of the insert of
2.13 was sequenced to confirm its identity. Figure 5.2 shows a summary of the wali2-GST fusion construction.

**Figure 5.1 Construction of the wali1-GST fusion**

(A) This figure shows a representation of the construction of the 1.4 plasmid, containing the wali1-GST protein fusion (not to scale). Nucleotides underlined within the pGEX-2T sequence shown (the multiple cloning site) are stop codons in each of the three reading frames. GST = glutathione S-transferase coding region under the control of the IPTG-inducible promoter P\text{lac}. The vector also contains the lac I\text{q} gene, required for the control of the P\text{lac} promoter. The Amp\text{R} gene confers ampicillin resistance, and the origin of replication is derived from the pBR322 plasmid. (B) Nucleotides 51 - 118 of the wali1 sequence (as shown in Fig. 3.9) and the deduced protein sequence for the N-terminus of the wali1 protein. The nucleotides (and amino acids) coloured in green are included in the wali1-GST fusion (the more 3' nucleotides and corresponding amino acids are not shown). The initiating ATG codon from the wali1 cDNA is double underlined.
The predicted proteins of wali3 and wali5 each have a hydrophobic leader at their N-terminus. This hydrophobic region has not been found in any mature Bowman-Birk protease inhibitors that have been amino acid sequenced to date (refer to Ch. 3.6). In addition, the inclusion of hydrophobic regions in protein fusions could result in the recombinant proteins becoming associated with bacterial cell membranes, which may hamper isolation. For these reasons, the oligonucleotide KCS1 was designed to be homologous to wali3 and wali5 at a position internal to the putative wali3 and wali5 polypeptides, such that the hydrophobic leader sequences were not included.

PCRs utilising the primers KCS1 and the forward primer, and either wali3 or wali5 DNA were performed (Ch. 2.11.10). In each case a PCR product of the expected size was amplified (570 bp or 510 bp respectively for wali3 and wali5, data not shown). In the case of wali3, a Mun I site was present in the 3' untranslated region of the clone. This Mun I site allowed the wali3 PCR product that had been digested with BamH I and Mun I to be directionally cloned into the BamH I and EcoR I sites of the pGEX-2T vector, so that it was not necessary to screen for inserts cloned in the correct orientation. This Mun I site is not present in wali5, so the wali5 PCR product was cloned using BamH I only. The wali5 PCR product was digested with BamH I and ligated into BamH I-digested pGEX-2T. As before, the ligations were transformed into DH5α and the identities of the recombinant plasmids were confirmed by sequencing the 5' ends of the inserts. The plasmid containing the wali3-GST fusion was named 3.7, and the plasmid containing the wali5-GST fusion was named 5.5. A summary of the construction of 3.7 and 5.5 is presented in Figure 5.2.

### 5.2.2 ISOLATION OF RECOMBINANT PROTEINS

*E. coli* cells containing the appropriate plasmids (1.4, 2.13, 3.7, 5.5 or pGEX-2T), were inoculated into 400 mL cultures and grown at 37° C until the OD<sub>600</sub> reached a value of approximately 1.0. IPTG was then added to the cultures to induce the production of the GST protein or the wali-GST protein fusions. After induction of the proteins, the cells were harvested, lysed and the proteins purified by affinity chromatography on a glutathione sepharose column (see Ch. 2.10.2 for details). Figure 5.3 shows the purification of each of these proteins. Each of the recombinant proteins were of the expected size

<table>
<thead>
<tr>
<th>clone/oligo</th>
<th>nucleotide sequence</th>
<th>mismatches</th>
<th>position</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCS2</td>
<td>CACCAACCAGGATCCATGGGTAT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wali2</td>
<td>CACCAACCAGCTAGCATGGGTAT</td>
<td>3</td>
<td>28 - 51</td>
</tr>
<tr>
<td>KCS1</td>
<td>CTTGTCATGGATCCCTTCACA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wali3</td>
<td>CTTGTCATGGGAATCCTTCACA</td>
<td>2</td>
<td>99 - 122</td>
</tr>
<tr>
<td>wali5</td>
<td>CTTGTCATGGACTCCCTTCACR</td>
<td>2</td>
<td>117 - 140</td>
</tr>
</tbody>
</table>

The nucleotides in each cDNA clone that correspond to the oligonucleotides KCS1 and KCS2 are presented. The number of mismatches refers to how many nucleotides in the wali clones differ to those of the oligonucleotide. The position refers to the location of the nucleotide sequences in the sequences of the wali clones, as shown in Fig. 3.9. The engineered BamH I sites in the oligonucleotides are underlined. oligo = oligonucleotide.
Figure 5.2 Construction of GST fusions with the wali2, -3 and -5 genes

(A) In the representations of the wali clones, the blue portions correspond to the pSPORT I vector, and the green regions correspond to the cDNA inserts. The coding regions of the cDNA clones are represented by the large green arrows. The shaded regions in wali3 and wali5 represent the hydrophobic leaders. The positions of the oligonucleotides KCS1, KCS2 and forward (fwd) are represented by small black arrows. Only the restriction enzyme sites used for cloning the PCR products are shown. (B) Nucleotide sequences of wali2, -3 and -5, corresponding to nucleotides 21 - 90 (wali2), 55 - 124 (wali3) and 73 - 142 (wali5) as shown in Fig. 3.9. Nucleotides changed by the PCR amplification (and any corresponding changes in the amino acid sequence) are shown in blue. The BamHI site used in the cloning of the PCR products is underlined. The initiating ATG codons from the wali cDNAs are double underlined. Nucleotides (and amino acids) coloured in green (or blue) are included in the GST fusions (the more 3' nucleotides and corresponding amino acids are not shown).

This diagram shows the cloning strategy for constructing plasmids 2.13, 3.7 and 5.5 which contain fusions between GST and wali2, wali3 and wali5 respectively in the pGEX-2T vector (not to scale). (A) In the representations of the wali clones, the blue portions correspond to the pSPORT I vector, and the green regions correspond to the cDNA inserts. The coding regions of the cDNA clones are represented by the large green arrows. The shaded regions in wali3 and wali5 represent the hydrophobic leaders. The positions of the oligonucleotides KCS1, KCS2 and forward (fwd) are represented by small black arrows. Only the restriction enzyme sites used for cloning the PCR products are shown. (B) Nucleotide sequences of wali2, -3 and -5, corresponding to nucleotides 21 - 90 (wali2), 55 - 124 (wali3) and 73 - 142 (wali5) as shown in Fig. 3.9. Nucleotides changed by the PCR amplification (and any corresponding changes in the amino acid sequence) are shown in blue. The BamHI site used in the cloning of the PCR products is underlined. The initiating ATG codons from the wali cDNAs are double underlined. Nucleotides (and amino acids) coloured in green (or blue) are included in the GST fusions (the more 3' nucleotides and corresponding amino acids are not shown).

Table 5.2. The amounts of each protein isolated were estimated using an assay based on that of Bradford (1976), and are listed in Table 5.2. For unknown reasons the yields obtained for each of the proteins varied considerably. In the case of wali2, the major peptide isolated was assumed to be a degradation product, but some protein of the expected size (highlighted with an arrow in Fig. 5.3) was
also obtained. Degradation products were also present in the preparations of each of the other fusion proteins. However, this was not entirely unexpected as no protease inhibitors were added to the harvested bacterial cells. Some of the degradation products seen were not observed when the proteins were electrophoresed through other SDS-polyacrylamide gels (not shown), suggesting that some of the degradation evident in the gels shown in Figure 5.3 occurred during the preparation or electrophoresis of the samples.

### Table 5.2 Yields of the wali-GST (and GST) proteins

<table>
<thead>
<tr>
<th>plasmid</th>
<th>protein</th>
<th>expected size</th>
<th>amount isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-2T</td>
<td>GST</td>
<td>26</td>
<td>13.2</td>
</tr>
<tr>
<td>1.4</td>
<td>wali1-GST</td>
<td>33</td>
<td>4.8</td>
</tr>
<tr>
<td>2.13</td>
<td>wali2-GST</td>
<td>63.5</td>
<td>0.2</td>
</tr>
<tr>
<td>3.7</td>
<td>wali3-GST</td>
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</tr>
<tr>
<td>5.5</td>
<td>wali5-GST</td>
<td>33.5</td>
<td>8.6</td>
</tr>
</tbody>
</table>

Amounts of the proteins (mg) recovered from 400 mL cultures of E. coli cells harbouring the plasmids shown. The calculated sizes of the proteins (in kDa, calculated from the amino acid sequence) are also shown.

### 5.3 ANALYSIS OF THE RECOMBINANT PROTEINS

Although one of the purposes of producing the fusion proteins was to generate antibodies, due to time constraints, this was done only for the wali1-GST protein. Antibodies against the wali2, -3 and -5 fusion proteins may be generated in the future. However, some analysis of the functions of the wali2, -3 and -5 fusion proteins was attempted.

#### 5.3.1 WESTERN ANALYSIS OF WALI1

A 1.5 mg aliquot of the wali1-GST fusion protein was used to generate antibodies in a rabbit (kindly performed by Dr Phil L'Huillier of AgResearch, Ruakura). An enzyme-linked immunosorbent assay (ELISA) was performed by Jan Meyer (at the University of Auckland), which indicated that antibodies to GST were present in the rabbit serum. The rabbit serum was used in conjunction with an ECL Western Blotting Analysis System (Amersham) to attempt to detect the presence of wali1 protein in wheat leaf and root samples. However, these western analyses had high background signals (data not shown). To try to eliminate some of the background, the antibodies were immunoaffinity purified by first passing the antiserum over a column containing the GST protein to absorb the anti-GST antibodies. The antiserum was then passed over a column containing the wali1-GST protein to select antibodies specific to the wali1 sequences. The bound antibodies were then eluted off the wali1-GST column. This 'purified' antibody solution was found to still contain some reactivity to the GST portion of the wali1-GST fusion protein. It was passed over the GST column twice more to try and remove anti-GST reactivity. The purified antibody was then used again in a western analysis containing samples from wheat root and leaf tissue from plants that were untreated or treated with 10 μM Al for 2 d (Ch. 2.13.4,
2.14.3 and 2.7.2). Also included in the western analysis was the wali1-GST fusion protein (cleaved with thrombin) to check the specificity of the purified antibodies.

The results obtained from this western analysis are shown in Figure 5.4. The antibody solution still detected the GST protein (26 kDa band), although in lane 3 (Fig. 5.4B) a small (<14 kDa) protein band
was detected that may be the wal1 portion of the fusion protein. Other degradation products of the wal1-GST protein were also detected. In the lanes containing the wheat proteins, faint background hybridisation was seen to many of the plant proteins. No bands of the size expected for the wal1 protein were detected under the conditions employed.

**Figure 5.4 Western analysis with the anti-wal1 antibodies**

Two duplicate gels were electrophoresed (A and B). Aliquots (50 μg) of total proteins from Warigal leaf (L) and root (R) tissue from plants that were untreated (-) or treated with 10 μM AI for 2 d (+) were resolved on the 20% SDS-polyacrylamide gels. Other samples electrophoresed through the gels were the Rainbow protein molecular weight markers (M), and the wal1-GST protein digested with thrombin, at three dilutions: 0.03 μg, 0.3 μg and 3 μg (labelled 0.03, 0.3 and 3 respectively). (A) Coomassie Blue stained gel. (B) Autoradiograph of the western blot of the duplicate gel. The gel was electroblotted to a PVDF membrane, incubated with the anti-wal1 antibodies, and detected using the ECL Western Analysis System (exposure time of 2 min).

### 5.3.2 South-western analysis of the wal12 fusion protein

As no significant homology was found between wal12 and any sequences currently in the databases, a function for the wal12 peptide remained elusive. As shown in Figure 3.9, the putative protein encoded by wal12 has a repeating motif of cysteine amino acids. This motif is similar to, although distinct from, the pattern of cysteine amino acids found in the DNA-binding domains of Zn finger proteins (Ch. 3.6). It seemed possible that the wal12 gene might encode a new type of Zn finger protein. If this was true, the wal12 protein should be capable of binding DNA in the presence of Zn. Sukegawa and Blobel (1993) have expressed a Zn finger protein in *E. coli*, and used a south-western procedure to show that it binds DNA in a Zn-dependent fashion. This procedure does not require large amounts of the fusion protein, and it seemed an ideal method to test whether the wal12 protein might bind DNA.

The south-western technique involved separating proteins under denaturing conditions on an SDS-polyacrylamide gel. The purified wal12-GST protein was included as well as the wal12-GST protein cleaved with thrombin, so that if any DNA-binding activity was detected, it could be assigned to either the wal12 or the GST portions of the fusion protein. Also electrophoresed on the gel were the total *E. coli* proteins from DH5α cells containing the 2.13 plasmid, both with and without induction by IPTG. After electrophoresis, the proteins were transferred to a membrane, then renatured in the
presence or absence of Zn. In the absence of Zn, EDTA and DTT were added to the renaturation buffer to chelate metal ions and reduce disulphide bonds. Total wheat genomic DNA (from Warigal, kindly supplied by Dr Tom Richardson) was sheared to an average length of 800 bp, radioactively labelled, and hybridised to the proteins, again in the presence or absence of Zn. The membrane was then washed and autoradiographed to determine which proteins bound the DNA. The resulting autoradiographs, along with a Coomassie Blue-stained duplicate gel are shown in Figure 5.5.

Several E. coli proteins showed DNA binding activity both in the presence and absence of Zn (Fig. 5.5) with most of these proteins appearing to bind more DNA in the presence of Zn. However, this increase may be attributable to the presence or absence of the DTT or EDTA in the renaturation and reaction buffers, rather than to a direct requirement for Zn. One protein band (arrowed), which appeared to bind DNA only in the presence of Zn (and the absence of EDTA and DTT), showed an increase in DNA-binding activity in cells that had been induced by IPTG relative to the uninduced cells. The identity of this protein is not known. It is also not known whether the increase in DNA-binding activity of this protein is correlated with an increase in the amount of protein present. No DNA-binding activity was detected with the purified wali2-GST fusion protein, under any of the conditions tested.
5.3.3 Do the wali3 and wali5 fusion proteins act as proteinase inhibitors?

As discussed in Chapter 3.6, the putative wali3 and wali5 proteins share some similarities to Bowman-Birk proteinase inhibitors. However, the homology is quite low and I decided to examine whether the E. coli-expressed wali3 and wali5 proteins functioned as proteinase inhibitors experimentally. A proteinase inhibitor assay (Kakade et al., 1969) was adapted to test whether the wali3 and wali5 fusion proteins showed any capability for inhibiting the serine proteinases trypsin or chymotrypsin. One of the active sites in each of the wali3 and wali5 putative protein sequences is predicted to be specific for inhibiting chymotrypsin (Ch. 3.6). The proteinase was diluted in a phosphate buffer and warmed to 37°C in the presence or absence of an inhibitor (or putative inhibitor). A casein substrate was then added to the proteinase mixture, and incubated at 37°C for 20 min. Trichloroacetic acid was used to stop the reaction, by precipitating the intact protein and larger peptide fragments. The smaller degradation products (if any) remained in the supernatant, and were measured spectrophotometrically.

Three experiments were performed in an attempt to detect any proteinase inhibitor activity of the wali3 and wali5 fusion proteins. In the first experiment 20 µg of either the wali3 or wali5 fusion proteins were incubated with the trypsin or chymotrypsin. Three controls were included: a positive control where 20 µg of soybean trypsin inhibitor was added to the proteinases; a negative control where no protein was added to the proteinases; and a second negative control where 20 µg of GST protein was added to the proteinases. The second negative control was included to test whether the GST portions of the wali3 or wali5 fusion proteins had any inhibitor activity. The results obtained from this experiment are presented in Figure 5.6. Only the positive control, containing the soybean trypsin inhibitor, exhibited any inhibitory activity towards the trypsin or chymotrypsin.

![Figure 5.6 Proteinase inhibitor activity of the wali3 and wali5 fusion proteins](image)

Trypsin or chymotrypsin was incubated with the following: NaP = sodium phosphate buffer only; GST = 20 µg GST protein; STI = 20 µg soybean trypsin inhibitor; wali3-GST = 20 µg wali3-GST protein; wali5-GST = 20 µg wali5-GST protein. Casein (4 mg) was added to the mixtures, and after digestion for 20 min, intact proteins were precipitated from the mixture with trichloroacetic acid. The relative amounts of degradation products present in the reaction were measured at OD280 (Ch. 2.12.3).
A second experiment was performed to test whether the GST portions of the wali3 and wali5 fusion proteins might be blocking inhibitory activity otherwise present in the proteins. In this experiment, the wali3-GST, wali5-GST and GST proteins were incubated with thrombin to cleave the GST portions from the fusion proteins. A portion of the digests was then electrophoresed through an SDS-polyacrylamide gel to check that the thrombin cleavage was complete (not shown). The digested proteins were then used in the same assay as described above. The results of this experiment are shown in Figure 5.7. As before, only the soybean trypsin inhibitor exhibited inhibitory activity.

![Figure 5.7 Proteinase inhibitor activity of the wali3 and wali5 fusion proteins cleaved with thrombin](image)

In the third experiment a large excess of the wali3-GST, wali5-GST and GST proteins (400 μg) was added to the chymotrypsin (0 - 2 μg). This experiment was performed to test whether a small proportion of the fusion proteins had any inhibitory activity towards chymotrypsin. Inhibitory activity towards trypsin was not tested due to the large quantities of fusion protein required in this experiment. The results from this experiment are shown in Figure 5.8. Again, only the soybean trypsin inhibitor displayed any inhibitory activity.

### 5.4 DISCUSSION

The results presented in this chapter describe the construction of fusion proteins between GST and four of the wali genes, and the successful expression of these fusions in E. coli. Three of the four genes were expressed to high levels and the proteins were purified. The fourth, wali2, had much lower expression levels and showed degradation products. The preliminary experiments aimed at using the expressed proteins to derive more information about the expression or activities of the wali proteins are discussed below.
Figure 5.8 Chymotrypsin inhibitory activity of the wali3 and wali5 fusion proteins (in excess)

Chymotrypsin was incubated with the following: NaP = sodium phosphate buffer only; GST = 400 µg GST protein; STI = 20 µg soybean trypsin inhibitor; wali3-GST = 400 µg wali3-GST protein; wali5-GST = 400 µg wali5-GST protein. Casein (4 mg) was added to the mixtures, and after digestion for 1 h, intact proteins were precipitated from the mixture with trichloroacetic acid. The relative amounts of degradation products present in the reaction were measured at OD<sub>280</sub> (Ch. 2,12.3).

5.4.1 ANTIBODY GENERATION TO THE WALI1-GST PROTEIN

Antibodies were raised against the wali1-GST fusion. Sufficient quantities of the wali3 and wali5 fusion proteins were isolated so that it should now also be possible to raise antibodies against these proteins in the future. However, yields of the wali2-GST fusion were lower, and conditions for growing DH5α cells containing the 2.13 plasmid will have to be optimised to obtain better yields of the wali2-GST protein for antibody production.

The majority of antibodies raised against the wali1-GST fusion protein appeared to be targeted against epitopes on the GST portion of the protein. This is not surprising as the GST portion makes up 80% of the fusion protein. Attempts to remove these GST-specific antibodies by immunoaffinity purification were not completely successful, and some still remained in the purified antibody solution used in the western analysis. One possibility for this may be that some of the antibodies present in the purified solution were targeted against an epitope located between the GST and wali1 portions of the fusion protein. This antibody would recognise the fusion protein, but might not be efficiently removed by the GST affinity column. Such an antibody would not be specific to the wali1 portion of the fusion protein.

In addition to problems with the purification of the antibodies, there were difficulties in detecting the wali1 portion of the fusion protein. I was not successful in resolving the wali1 portion of the fusion protein on an SDS-polyacrylamide gel. This did not appear to be due to the size of the protein alone, as it was possible to resolve the wali3 and wali5 portions of those fusion proteins, and the wali3 and wali5 fusions are of a similar size to the wali1 fusion (Table 5.2). The following are possible explanations for the failure to detect the wali1 protein (both the fusion, and the native protein in the wheat root and leaf samples):
(a) Some feature of the wali1 protein may make it difficult to detect by Coomassie Blue staining. Silver staining of SDS-polyacrylamide gels also failed to detect the wali1 protein (data not shown). I tried using a method developed by Schägger and von Jagow (1987), which was developed specifically for the resolution of small proteins; however, this method of electrophoresis also failed to resolve the wali1 protein. Schägger and von Jagow (1987) have also reported that some small proteins could not be stained, which they presumed to be because the proteins either eluted from the gel or did not bind the dye used (Serva blue G). If the wali1 protein was not resolved by electrophoresis under the conditions used it may not be present to be transferred to the membrane during the western blotting protocol used.

(b) The wali1 protein may not be retained in the acrylamide gel after electrophoresis, due to its small size, or the protein may not be efficiently retained by the membrane used during the electroblotting procedure. In addition, the wali1 protein may be susceptible to proteolytic degradation.

(c) No (or too few) polyclonal antibodies may have been targeted to the wali1 portion of the fusion protein, or the antibodies present may not recognise the native wali1 protein in wheat (perhaps due to post-translational modifications of the protein).

(d) The antibodies generated may have low affinity for the wali1 protein, and could not detect the protein at the concentrations present in the samples used in the western analysis.

(e) The native wali1 protein in wheat may be processed to smaller peptides, which are difficult to separate by electrophoresis under the conditions used. It has been reported by de Miranda et al. (1990) that the *Mimus guttatus* homologue of wali1 (a metallothionein-like protein) may be processed to remove the central hydrophobic region of the protein. However, the evidence for this processing is circumstantial. Previous authors have experienced problems in working with metallothionein-like proteins (reviewed in Tomsett and Thurman, 1988; Robinson et al., 1993) and prior to the isolation of cDNA clones there was no evidence for the existence of metallothionein-like proteins in plants.

It is worth noting that other researchers working with homologues to wali1 have also experienced difficulties in raising antibodies to these proteins (N. Robinson, personal communication). The problems experienced here therefore are probably specific to the wali1 protein, and there is no reason to believe that similar difficulties would arise with antibodies raised to the other wali fusion proteins.

### 5.4.2 South-Western Analysis of the Wali2-GST Protein

The possibility that the wali2 protein may have the capability of binding DNA was explored using south-western analysis. The binding assay worked quite effectively, and several *E. coli* proteins bound to labelled wheat DNA, both in the presence and absence of Zn. However, no DNA-binding activity of the wali2-GST protein was detected (Fig. 5.5). The most likely explanation is that the wali2 protein can not bind DNA. However, there remain several possible reasons why a negative result could have been obtained with a protein that does in fact bind DNA *in vivo*. For example, the protein may require modifications to become capable of binding DNA, and these modifications would probably not be made
when the protein is synthesised in a prokaryotic system (Marston, 1986). The protein may also only bind DNA under conditions different from those tested here. Alternatively, the wali2 protein may have bound DNA in the south-western, but the specific DNA that it binds may only be present in a few copies in the Warigal genome. Therefore the DNA which (may have) bound to the wali2 protein would only contribute a very small proportion of the total probe DNA, and would not be detectable using autoradiography.

Of the *E. coli* proteins that were found to be capable of binding DNA in the south-western analysis (Fig. 5.5), one protein (approximately 28 kDa in size) appeared to bind more DNA in cells that had been induced with IPTG than in uninduced cells. The 28 kDa protein is too small to be the wali2-GST fusion protein (63.5 kDa), although it may be a degradation product of the fusion protein. The only other proteins whose concentrations should increase with IPTG induction are the proteins of the *lac* operon (a permease, a transacetylase and β-galactosidase, which is truncated in DH5α cells). To date, none of these *lac* operon proteins have been reported to have the capability to bind DNA. The *lac* repressor protein (whose synthesis is not induced by IPTG) is able to bind DNA, however, and in the bacterial cells would be bound to the operator of the *lac* operon, and would also bind to the Ptac promoter in the 2.13 plasmid. After induction with IPTG, the repressor would no longer bind to these regions and instead may be available to bind other DNA, perhaps non-specifically. The 28 kDa protein is probably not the *lac* repressor however, as the *lac* repressor is approximately 38 kDa in size (reviewed in Griffiths *et al.*, 1993). Further experiments are required to determine the identity of the 28 kDa protein.

5.4.3 PROTEINASE INHIBITOR ASSAYS WITH THE WALI3-GST AND WALI5-GST PROTEINS

Due to the homology of the wali3 and wali5 deduced protein sequences to Bowman-Birk proteinase inhibitors, I examined the expressed fusion proteins for the ability to inhibit proteinases in functional assays. Neither of the wali3 and wali5 fusion proteins were capable of inhibiting trypsin or chymotrypsin (Figs. 5.6, 5.7 and 5.8). An experiment was performed to explore the possibility that the GST portion of the proteins was blocking the inhibitory activity of the wali3 and wali5 proteins. However, cleaving the GST moiety from the fusion proteins did not have an effect. Another experiment was performed to test whether a small proportion of the fusion proteins was folded into an active configuration. However, using a large excess of the fusion proteins relative to the proteinase did not reveal any inhibitory activity. In this third experiment an increase in degradation products was observed when either GST, wali3-GST or wali5-GST was added to the chymotrypsin as compared to when only buffer was added (see Fig. 5.8). This increase is probably due to the increase in total protein in these reactions (4.4 mg of protein compared to 4 mg in the buffer control). A similar, though smaller increase was also observed in the second experiment (Fig. 5.7). The increase in degradation products in this experiment was probably due to the presence of thrombin in the digests.
The lack of proteinase inhibitor activity observed for both the wali3 and wali5 fusion proteins could result from three causes:

(a) The native wali3 and wali5 proteins may in fact not function as proteinase inhibitors in vivo. The homology between wali3 and wali5 and other Bowman-Birk proteinase inhibitors is quite low (Ch. 3.6), but key Cys, Ser and Asp residues are conserved. The conservation of the Cys residues indicates that the wali3 and wali5 proteins probably share structural homologies to the Bowman-Birk proteinase inhibitors, although it remains possible that they have different biochemical functions.

(b) The wali3 and wali5 fusion proteins may require different conditions to those tested for inhibitory activity to be detected. A change of pH or temperature may allow activity to be detected. It is also possible that the wali3 and wali5 proteins do not inhibit trypsin or chymotrypsin, but some other serine proteinase.

(c) It is possible that the wali3 and wali5 fusion proteins were not folded or processed correctly in the bacterial expression system utilised here. This may have been for a number of reasons:

(i) The GST portion of the fusion proteins may have interfered with the correct folding of the wali3 and wali5 portions.

(ii) When the oligonucleotide KCS1 was designed for cloning the wali3 and wali5 coding regions into the pGEX-2T vector, the hydrophobic leader of the proteins was not incorporated. It is possible that the proteins require the hydrophobic leader for them to be folded correctly, and also that the leader may have to be processed properly for the proteins to acquire activity.

(iii) Fundamental differences between prokaryotes and eukaryotes may have resulted in incorrectly folded or processed proteins. This is probably the most likely possibility. For example, the wali3 and wali5 proteins are predicted (by homology to Bowman-Birk proteinase inhibitors, see Ch. 3.6) to have five disulphide bonds, and disulphide bonds are not always formed in proteins expressed in the bacterial cytoplasm (Marston, 1986). If this is the case then bacterial expression using a thioredoxin gene fusion system may help (LaVallie et al., 1993), or the proteins could be expressed in a eukaryotic system such as yeast. Alternatively, small amounts of protein may be isolated using a wheat germ in vitro coupled transcription/translation system. It might also be possible to refold the wali3 and wali5 proteins into an active configuration, although the possibility mentioned in (ii) above may not make this a viable proposition.

### 5.4.4 CONCLUSION

Each of the wali1, -2, -3 and -5 proteins have been expressed successfully as fusions with the bacterial GST protein. However, preliminary analyses failed to uncover any biochemical function for wali2, -3 or -5. Successful expression in E. coli can be used as a tool to learn more about the proteins. For example, it should be possible to raise antibodies against the wali2, -3 and -5 fusion proteins, and use them to discover the cellular location of the native proteins. Functional studies of the wali proteins may require additional constructs to be made, or for the proteins to be expressed in other systems which retain biological activity.
CONCLUDING DISCUSSION

The aim of this work was to investigate the changes which might occur in gene expression when plants are subjected to Al stress. The resulting isolation of five different genes whose expression is induced by Al treatment constitutes the first characterisation of genes induced by Al stress in plants. Four of these five genes also showed induction by other stress treatments, indicating that these genes form part of a coordinated response to many types of stresses. The wali, -3 and -5 genes had not previously been identified as genes which respond to a number of different stress conditions. The general implications of the results obtained in this work are discussed below, together with some consideration of possible future experiments that might clarify other aspects of Al toxicity.

6.1 TECHNIQUES USED IN THE ISOLATION AND CHARACTERISATION OF THE WALI CLONES

6.1.1 IDENTIFICATION OF AL-INDUCED CLONES

The strategy followed for this work was to identify genes induced by Al using the technique of differential screening of a cDNA library. Several other methods are available for identifying molecular changes that occur in response to Al stress. In the following section I will discuss some of these techniques, highlighting the advantages and disadvantages compared to the differential screening approach.

Most of the molecular characterisation of Al stress carried out by others prior to this work commencing used two-dimensional polyacrylamide gel electrophoresis to identify induced proteins. Protein-based work has so far been of limited value in that only one protein has been purified and identified, which increases in response to Al stress in plants (Cruz-Ortega and Ownby, 1993). In contrast, the differential screening undertaken in this work quickly identified five genes whose expression is increased by Al stress. Other changes that may be difficult to detect with protein-based techniques can occur if the level of a protein remains static, but its function is activated in some way (e.g. by binding of a cofactor or a change in cellular location). However, many molecular changes in response to Al stress may also be difficult to identify with differential screening of cDNA libraries as used here. For example, the activity of many proteins is controlled post-transcriptionally and no differences in steady state mRNA levels may be detected (reviewed in Kuhlmeier, 1992; Gallie, 1993).

There are a number of general advantages inherent in the differential screening approach. Once a gene has been identified as differentially expressed, information can quickly be gathered concerning its nucleotide sequence and its expression. In contrast, once a protein of interest has been identified,
purification to homogeneity and determining its identity by peptide sequencing is not a simple procedure. Furthermore, some induced proteins may be difficult to extract from plant tissues, or may be secreted by the plant so that changes in protein levels may not be detected using techniques such as two-dimensional polyacrylamide gel electrophoresis.

In spite of the drawbacks inherent in the use of two-dimensional gel electrophoresis for identifying changes in protein abundance with stress, there are advantages in working directly with the native protein. For example, functional studies can be performed, in the hope that a biochemical role for the protein may be identified. This type of approach can also be taken when a cDNA clone has been isolated because the protein encoded by the cDNA can be produced in an expression system - as was done here. Functional studies can then be performed with the fusion protein, although it must be remembered that there may be differences between the native and expressed proteins.

One limitation of both differential screening and two-dimensional polyacrylamide gel electrophoresis is that only relatively abundant mRNAs and proteins can be identified. There are other techniques that can be used to identify less abundantly expressed genes involved in a particular stress event. For example, subtractive probes can be made that enrich the cDNA for genes specific to the stress (see Ch. 1.8). The cDNA library constructed during the course of this research could be used in this way to identify other genes whose expression is induced by Al treatment. However, the preparation of subtractive probes is fraught with technical difficulties. Another technique useful for identifying genes with specific expression patterns is differential display (Liang and Pardee, 1992; Sokolov and Prockop, 1994). In this technique a specific subset of different mRNA populations is amplified using reverse transcription coupled to PCR. The amplified products are compared on acrylamide or agarose gels, and cDNAs that are seen to be amplified preferentially from the tissue of interest can be isolated directly from the gel, amplified and used to probe northerns. These probes can also be used to isolate cDNA or genomic clones from libraries. The major limitation of this technique is the number of primers needed to amplify all the different mRNAs present in Al-stressed tissue.

Another technique for identifying genes that are induced by Al is to screen previously isolated genes, that may have a role in the plants response to Al, using northern analysis. Work previously carried out on the physiology of Al stress suggests likely candidate genes that might be used as probes. For example, genes involved in cell wall biosynthesis, maintenance of the plasma membrane, channel proteins, DNA repair enzymes or possible intracellular targets of Al could be investigated to determine whether their expression changes with Al stress. Richards and Gardner (1994) used this approach to investigate the transcript levels of histone and heat shock genes in response to Al stress (see Ch. 1.7). de Lima and Copeland (1994) showed that the reduction in the number of amyloplasts in the root caps of Al-treated plants is accompanied by an increase in the activity of enzymes involved in starch mobilisation such as α-amylase. They have also showed that enzymes involved in fermentative metabolism, such as alcohol dehydrogenase, are also affected by Al stress (Copeland and de Lima, 1992). It would be
interesting to see if the increases in enzyme activity observed could be related to changes in gene expression, not only of the enzymes studied, but of other related genes in similar biochemical pathways. The work presented in this thesis suggests other genes whose response to Al stress might be worth examining. Since a gene for PAL (wali4) was induced, it would be interesting to determine whether the expression of other enzymes involved in the phenylpropanoid pathway is also affected by Al stress. It may be possible to determine which particular downstream products of PAL are required in Al-stressed tissues, depending on whether, for example, there is induction of enzymes which lead to lignin biosynthesis or enzymes which lead to flavonoid production.

The fact that most of the wali genes were also induced by other stresses suggests that other stress-related genes may be induced by Al. In particular, it would be interesting to test whether genes involved in the protection of cells from oxidative stress, such as those encoding superoxide dismutase, peroxidase and catalase, as well as enzymes involved in glutathione production, are induced by Al treatment. As mentioned in Chapter 1, Cakmak and Horst (1991a) have found that the activity levels of superoxide dismutase and peroxidase (but not catalase) were increased in the roots of soybean plants after treatment with Al.

The differential screening approach used here resulted in the successful isolation of five genes whose expression is induced by Al stress. However, differential screening is useful only for identifying highly expressed genes which respond to a stress. In future a combination of approaches will yield the most information on identifying genes induced by Al. The differential display technique has the potential to identify all genes that show a difference in expression, regardless of their basal levels. In addition, if cloned genes are available for northern analyses, then it would be beneficial to screen these for differential expression.

### 6.1.2 Analysis of the Expression of Al-induced Clones

Northern hybridisations have been used extensively in the work presented in this thesis. They have provided valuable information concerning how the expression of the wali genes respond to a number of different stresses. Quantification of the northern hybridisation results showed a degree of variability, both between and within experiments (Appendix 3). In addition to variability from the northern data, a certain amount of variability is also to be expected when dealing with biological parameters such as root length. The complexities of Al speciation in solution, particularly its sensitivity to pH and organic ions, added an additional layer of variability in these experiments. These uncertainties mean that the northern results presented in this thesis have been interpreted largely qualitatively, and that minor quantitative differences have been ignored.

An alternative procedure to northern analysis for quantifying transcript abundance is the technique of RNase protection. In this procedure, an antisense labelled RNA probe is made from the gene of interest. This probe is then hybridised to RNA isolated from plants that have been treated in varying ways, the
single stranded RNA is degraded, and the remaining double stranded mRNA/probe duplexes are electrophoresed through acrylamide or agarose gels. The radioactive bands can then be excised from the gels and quantified by counting the amount of radioactive isotope present. The amount of isotope present in each band should be directly proportional to the amount of each specific message present in the original RNA population. Thus expression levels in different treatments or tissues can be compared. The size of the duplex regions involved can be controlled, so that several different probes, designed to have varying lengths, can be analysed at one time. Internal controls using rRNA or housekeeping genes can also be included to determine that equal amounts of the different RNA samples are used. RNase protection is reported to provide increased sensitivity, and possibly improved accuracy in quantifying the expression levels of the transcripts tested (Ausubel et al., 1987). However, there are some difficulties associated with this technique. Small amounts of contaminating probe or genomic DNA can lead to false signals. In addition, since degradation of single stranded RNA is highly efficient, any mismatch between probe and target results in production of fragments smaller than the predicted band. Such mismatch would occur if genes from other species were used as probes. Since the extra sensitivity of RNase protection was not needed to examine the expression of the wali genes, the technique of northern hybridisation (already established in the laboratory) was used throughout this work. However, in future, if a large number of northern hybridisations was required using multiple probes then it might be beneficial to use the RNase protection methodology.

6.2 THE WALI CLONES

This section of the discussion will describe the genes isolated in this work, and possible reasons for their induction by the Al treatment. Each of the wali genes (except for wali3 and wali5, which are homologous to each other) will be discussed separately.

wali1 encodes a protein with homology to a group of plant metallothionein-like proteins (MLPs). As well as being induced by Al stress, wali1 was strongly induced by other toxic metals, physical wounding and low levels of Ca in the growth media. This gene was expressed in root and leaf tissue, although Al stress on root tissue did not increase expression of the gene in leaf tissue. Direct stress placed on the leaf tissue (wounding), did however, induce higher levels of expression. In situ hybridisation has shown that the gene is expressed strongly in meristematic tissue of the root, and at lower levels throughout most other cell types in the root, and is expressed in the mesophyll layer of cells in the leaf.

Metallothioneins are thought to have a role in binding and detoxifying metals, but these proteins do not bind Al (Kägi, 1991; Nielson et al., 1985). wali1 is therefore unlikely to be induced to directly detoxify Al present in the cell. Metallothioneins are also thought to have a role in metal ion homeostasis. If this is the case for plant MLPs then it is not surprising that the gene would be expressed in many different cell types throughout the plant. The fact that expression is highest in meristematic cells also fits with this
role, as meristematic cells are known to be highly metabolically active. As wali1 transcript levels are induced by Al treatment, then increasing levels of metallothioneins must be required by the plant, perhaps because Al is disrupting metal ion homeostasis in some way. It would be useful to determine which metal ions the wali1 protein can bind, and whether or not the location or availability of these metals is changed by Al treatment. For example, if Al is displacing metal ions (directly or indirectly) from enzymes that require them as cofactors, then these metal ions may themselves become damaging. The increase in metallothioneins in Al-stressed tissues therefore may merely reflect the fact that they are present to detoxify these displaced ions.

As suggested in Chapter 3.7.3, animal metallothioneins may act as scavengers of damaging free radicals (Thornally and Vasák, 1985). Radicals are known to be generated during many stressful conditions such as wounding and in response to some metal ions. These are conditions where induction of wali1 expression is observed. It is possible that the presence of free radicals at damaging concentrations induces the expression of wali1 to some extent. It would be worth testing whether or not treatments known to directly cause radical production (for example, UV irradiation or treatment with methyl viologen, Foyer et al., 1994) can also induce the expression of wali1.

wali3 and wali5 are induced by the same range of stresses as wali1, although there are some differences in the relative levels of induction observed. wali3 and wali5 encode proteins with similarities to Bowman-Birk proteinase inhibitors. Proteinase inhibitors, like metallothioneins, have been implicated in the detoxification of damaging free radicals (Frenkel et al., 1987; Ch. 3.7.3). It is possible that wali3 and wali5 are also induced by the presence of radicals in stressed plant cells. The differences in induction of expression of wali1 compared to wali3 and wali5 suggest that there are differences in the mechanisms by which these genes are regulated. For example, wali1 expression may be directly regulated by the presence of some metal ions, perhaps by direct binding to the wali1 protein. wali3 and wali5 are found in a different location in plant roots from wali1, with expression of these genes observed primarily in the cortical tissue of the root. If these genes really do function as proteinase inhibitors, then it is not surprising that expression is observed in the outer cell layers of the root, where they may form a defence against invading pests. This difference in the location of expression of these genes, compared to wali1, may be responsible for the different levels of induction observed for some stresses, as each stress may affect different cell types in the root with different severities.

wali4 is a clone encoding the enzyme phenylalanine ammonia-lyase (PAL). Like the clones previously discussed, it is induced not only by Al, but also by toxic metals, low Ca levels and by wounding in leaves. Wounding in root tissue was not observed to induce the expression of wali4 at the time points tested. Another difference in the expression observed for wali4 (compared to wali1, -3 and -5) is that some induction of expression was observed after only 0.5 h of Al stress. PAL genes in other plant species are known to be induced by a range of different stresses (Ohl et al., 1990; Schuch, 1992), although at this stage it is unknown what component of the Al stress is inducing this gene. As discussed
in Chapter 3.7.3, a requirement for the enzymatic products of PAL, and also the downstream products of other enzymes in the phenylpropanoid pathway, may result in the induction of wali4.

wali2 has no significant homology to any sequences currently in the database. Experiments that I conducted to test whether or not the wali2 gene product was capable of binding DNA, were inconclusive at best. A function for wali2 has remained elusive. The expression of wali2 is also quite different to any of the other wali genes. Although induced by low concentrations of Al, higher concentrations resulted in a reduction of expression of wali2. wali2 expression is unlikely to be important in protecting against Al stress. Although induction of expression was observed in one Al-tolerant wheat line (RR), no induction was observed in the other tolerant wheat cultivar tested (Waalt).

wali2 expression was often reduced by many of the other stresses observed, or was variable in its induction. In the in situ hybridisation experiments wali2 was generally found to be expressed in the epidermal cell layer and the root cap, but expression in other areas (for example, meristematic tissue) was more variable. The differences in wali2 expression may be attributable to developmental factors, or to small differences in the media prepared from experiment to experiment. For example, as Al solution chemistry is very complex, it is possible that small differences in the way batches of media were made could effect the toxicity of Al between experiments. The same may also be true for some of the other stress treatments used. As differing severities of Al treatment could have quite different effects on the expression of wali2, slight differences in other stresses between experiments may also result in quite different changes in wali2 expression. The results gained from the in situ hybridisation experiments provide a possible rationale for down-regulation of wali2 at high Al levels. As expression of wali2 was observed primarily in the outer layers of the root, low levels of stress that affect these cells may result in the induction of wali2. However, under more damaging stress conditions, the epidermal cells may be damaged or destroyed (as occurs with Al stress), and wali2 expression would then be observed to decrease. Some of the more severe stress treatments (for example the low Ca treatment) did result in a strong decrease in the expression of wali2.

The induction of wali2 expression in response to Al stress was bimodal, with the first increase seen after only 0.5 h of Al treatment. This early induction of wali2 expression to Al stress may have occurred because the cells in which wali2 is expressed (the epidermal and root cap cells) are the first to encounter Al. There may be increases seen in wali2 expression to more severe stresses that occur early after the application of the stress. However, this possibility was not fully tested in this study. Further experiments are required to determine exactly how and when wali2 responds to most stresses.

At this stage, the genes that I have isolated do not directly support any one theory of how Al damages plant root tips. The differences in the response of most of the wali genes to Al stress, for example, as shown in the time course of Al treatment, suggest that there may be more than one component of Al stress that is inducing this group of genes. It should be remembered that wali1, wali3 and wali5 were
induced only after 24 h of Al treatment. At this time the effects observed are probably not a direct response to the damage incurred by the primary target of Al toxicity, but may instead be in response to other damage caused by the Al stress. For example, oxidative damage (the presence of damaging radicals) is known to occur in Al treated root tissue, and some of the wali genes may be able to protect against oxidative damage. Thus one possibility is that the wali genes are induced after root growth is inhibited, in order to protect the plant against Al-induced oxidative stress. However, alternative explanations are possible. For example, the experiments of Ryan et al. (1993) have shown that when areas of the root more distal to the apex are treated with Al, the roots continued to grow, but damaged cells are observed. So some of the changes in gene expression observed here may be in response to this sort of wounding damage. The fact that many cells are observed to be physically damaged by Al treatment, and that wali1, -3 and -5 are also induced by physical wounding supports this argument. However, this is not the case for all of the wali genes since wali2 and wali4 are not induced by wounding of root tissue.

Some hypotheses which identify particular targets of Al toxicity lead me to expect that certain types of genes may be induced. If the cell wall is heavily damaged, genes involved in the repair and construction of the wall may be induced. PAL may qualify as a gene indirectly associated with cell wall biosynthesis, although the other walis are probably not involved in this sort of role. If membrane proteins are damaged by Al, then their expression may be increased, though none of the wali genes isolated so far appear to be membrane proteins. Similarly, if the cell membrane is directly affected, then perhaps enzymes involved with lipid biosynthesis, or repair of the membrane may be induced. Although these types of genes were not detected in this work, we can not at this stage rule out the possibility that Al is affecting these processes.

6.3 FUTURE WORK

The work described in this thesis has been undertaken at a very early stage in the characterisation of the molecular response of plants to Al stress. There are probably many other genes whose expression is induced by Al, that could be isolated using the techniques mentioned in Chapter 6.1.1. In addition, the genes characterised here are mostly induced only after 24 h of Al stress. As some effects of Al are known to occur after shorter periods of Al stress, such as inhibition of root elongation after 30 min of Al treatment (Günsé et al., 1992; Ch. 1.3), it would be interesting to identify the earliest changes in gene expression that are occurring. Research of this type is currently being undertaken in this laboratory by Dr Eric Schott and Keith Richards, who have isolated a number of genes that have changes in gene expression within 2 h (and sometimes within as little as 15 min) of the introduction of Al stress. The number of different genes already isolated using these approaches suggests that the work presented in this thesis has only scratched the surface of the molecular changes occurring in response to Al stress.
Besides identifying the changes in gene expression that occur in response to Al stress, we need to characterise the functions of the induced genes. Sequence information is valuable in detecting homology to a known gene, because it may suggest both possible functions for a protein, and possible experiments to test for function. A number of different experimental approaches can be used to determine the function of the genes. In this thesis, the approach used was to express the proteins in a bacterial system, so that direct experiments could be designed to test the proteins for biochemical activities. A similar approach may be taken with newly isolated Al-induced genes.

Once genes have been identified as being induced by Al stress, it would also be interesting to determine whether or not the changes in expression observed in wheat (as was characterised here) are similar to the changes that occur in other plant species. This information may provide a way of detecting differences between plants in response to Al stress, since some differences have been observed in the effects of Al on different species (for example, see Ch. 1.5c). Changes in gene expression that occur in all Al-susceptible plant species will provide information on the similarities which occur between the effects created by Al in these plants, and perhaps indicate processes which are important in the manifestation of Al toxicity.

Once putative functions of Al-induced proteins are established it may be worth determining if Al can have a direct effect on these proteins. In vitro binding studies would establish whether the proteins isolated could bind Al, and if biochemical functions were known for the proteins other experiments could determine whether Al has an adverse effect on the properties of the proteins of interest. If a protein that is affected by Al can be modified so that it no longer binds Al, without affecting its biochemical functions, then this protein may be a candidate for improving Al tolerance in plants. This approach is only likely to be successful however, if the protein of interest is of primary importance in the affect of Al on plant roots. Binding studies have been carried out for proteins such as calmodulin (Siegel and Haug, 1983). However, it must be noted that evidence for an effect in vitro does not prove that the protein is affected in vivo. It must also be established whether or not Al reaches the particular sub-compartment of the cell where the protein of interest is located, and whether the Al is in a form and concentration capable of binding, and thereby affecting, the protein in this location.

Determining the functions of the proteins whose expression is induced by Al may lead us to predicting that some of these proteins have a protective role against the stress. As discussed in Chapter 1.8, these proteins may subsequently be useful in providing the means of improving the tolerance of plants to Al stress. In addition, proteins that protect against other stresses may also be of use in protecting plants from Al stress. The fact that the wali genes were induced by stresses other than Al suggests that there are similarities between these stresses. For example, as oxidative stress may play a role in Al toxicity, the production of plants with greater resistance to this sort of stress may also be more resistant to Al. Transgenic plants with increased levels of antioxidant proteins such as superoxide dismutase and peroxidase are already available (see Ch. 1.8) and these should be tested for their response to Al stress.
The use of transgenic technologies may also help in establishing the function of a gene. Techniques to reduce (for example, through antisense or co-suppression) or increase (through overexpression) the amount of a protein present in a plant may result in a phenotype that can suggest a biochemical function for a gene. In addition, it is possible that changing the amount of specific proteins present in the plant will result in an increase in Al tolerance. This sort of approach was not investigated with the wali genes isolated here for a number of reasons. The wali clones were isolated from wheat, which is a plant that was not particularly amenable to transformation until recently (Nehra et al., 1994; Becker et al., 1994). However, studying the effect of modifying expression levels of homologues to the wali genes in a plant more amenable to transformation may prove to be informative. During this investigation I did attempt, without success, to detect homologues of the walis in the model plant *Arabidopsis thaliana* (data not shown). In addition, Dr Andreas Königstorfer of this laboratory tried to isolate homologues of the wali genes from an *Arabidopsis thaliana* library, again with little success. Using the nucleotide sequence of the *Arabidopsis* homologue of the wali1 gene already present in the database, he did successfully isolate the gene using a PCR-based approach. The current research effort in this laboratory to identify early Al effects has focused on genes induced by Al in *Arabidopsis*. Although little Al research has involved *Arabidopsis* to date, it is useful as a model plant for a number of reasons. For example, it can be routinely transformed, and the small size of the plant and the rapid generation time make it an attractive plant for studies involving transformation. In addition, much genetic research now utilises *Arabidopsis*, so that plants that may be generated in the study of Al-induced genes may have similar phenotypes to previously characterised mutant plants, which may make it easier to interpret the results of down- or up-regulation of Al-induced genes.

The promoters of Al-induced genes could also be examined in transgenic plants as fusions with reporter genes such as β-glucuronidase or luciferase. The regions of the promoters that control induction by Al stress could then be determined, and the elements and transcription factors which control this induction identified. It may then be possible to determine what part of the Al stress is controlling the induction of these genes, and whether there are any promoter regions in common with other Al-induced genes. If a common promoter element is identified, it could be used to identify other genes that are likely to be induced by Al stress. The promoters of Al-induced genes could also be scrutinised for elements known to respond to other stresses, and if they are present, their role in gene induction by Al stress investigated.

Once genes are identified that may afford plants protection from Al stress, it would be advantageous to express these genes in the cells most affected by Al toxicity. The identification of promoters specific to these regions would be useful. The wali2 gene appears to be a gene that is expressed in the cells that are first affected by Al (the root cap and epidermal cells). Although some variability in the expression of wali2 was observed, it is possible that isolation of a promoter that directs expression in similar cell types...
to wali2 would be beneficial to future projects directed at increasing Al tolerance through transgenic technologies.

6.4 SUMMARY

This research represents the first major attempt to determine the changes in gene expression that occur with Al stress. Al toxicity affects many agriculturally important crop species, and may also be involved in the forest dieback that is occurring in many areas of the world. Because of the large amount of acidic soil present in many areas, Al has the potential to be one of the most significant growth limiting factors in agricultural production today. A large amount of work has been performed on the physiology of plants suffering from Al stress, but prior to this work virtually nothing was known of the molecular changes occurring in these plants.

Five genes have been identified in this work whose expression increases with Al stress. There are undoubtedly more genes induced by Al toxicity. The identification of these genes, followed by the elucidation of their functions, should increase our knowledge on the effects of Al on plants. This information may in turn suggest possible routes for improving the Al tolerance of plants. The increasing world demand for food means that the use of soils that do not provide optimal growing conditions will be required for agricultural production in the future. Development of plants, that are capable of high yields on acidic soils where Al toxicity is a problem, is a goal that has ever increasing importance.


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Frank RL and Vodkin LO (1991) Sequence and structure of phenylalanine ammonia-lyase gene from Glycine max. DNA Sequence 1: 335-346


Hilder VA, Barker RF, Samour RA, Gatehouse AMR, Gatehouse JA and Boulter D (1989) Protein and cDNA sequences of Bowman-Birk protease inhibitors from the cowpea (Vigna unguiculata Walp.). Plant Mol. Biol. 13: 701-710


Joudrier PE, Foard DE, Floener LA and Larkins BA (1987) Isolation and sequence of cDNA encoding the soybean protease inhibitors PI IV and C-II. Plant Mol. Biol. 10: 35-42


Sasaki M, Kasai M, Yamamoto Y and Matsumoto H (1992) Root elongation and ion flux of wheat varieties differing in aluminum tolerance. *In* Plant cell walls as biopolymers with physiological functions, Yamada Science Foundation, Osaka, Japan pp 401-403


Details of the subclones generated from wal1 - wal5 for the purpose of nucleotide sequencing are given below. The map of each clone shows the positions of the restriction enzyme sites used in generating subclones. The arrows below the maps represent the amount of sequence obtained that resulted in contiguous sequence in each clone in both directions. Additional sequence was often generated from other subclones (not represented in the diagrams) which resulted in further redundancy. The tables summarise the information about each subclone, including which plasmid vector the fragments were cloned into, the restriction enzymes used to isolate each fragment for subcloning (these enzyme sites may not have been regenerated during the cloning procedure), or whether the subclone was generated using the DNase I deletion procedure described in Chapter 2.11.7. Also included in the table are the nucleotide coordinates of the subcloned cDNA fragments within the original wal1 clones (relative to the numbering given in Fig. 3.9).

**Key:**
- ■ multiple cloning site from pSPORT 1 (not to scale)
- □ 3' and 5' untranslated regions
- □ open reading frame
- □ poly A tail

### wali1

<table>
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<th>subclone</th>
<th>vector</th>
<th>DNA fragment</th>
<th>position</th>
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<td>pSPORT 1</td>
<td>SalI - NotI</td>
<td>1 - 575</td>
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<tr>
<td>1.2</td>
<td>pBC</td>
<td>SalI - NotI</td>
<td>1 - 575</td>
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<tr>
<td>1.3</td>
<td>pBluescript</td>
<td>PstI - HindIII</td>
<td>247 - 575</td>
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<tr>
<td>1.5</td>
<td>pBluescript</td>
<td>PstI</td>
<td>1 - 250</td>
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</table>

The map of each clone shows the positions of the restriction enzyme sites used in generating subclones. The arrows below the maps represent the amount of sequence obtained that resulted in contiguous sequence in each clone in both directions. Additional sequence was often generated from other subclones (not represented in the diagrams) which resulted in further redundancy. The tables summarise the information about each subclone, including which plasmid vector the fragments were cloned into, the restriction enzymes used to isolate each fragment for subcloning (these enzyme sites may not have been regenerated during the cloning procedure), or whether the subclone was generated using the DNase I deletion procedure described in Chapter 2.11.7. Also included in the table are the nucleotide coordinates of the subcloned cDNA fragments within the original wal1 clones (relative to the numbering given in Fig. 3.9).
wali2

subclone | vector | DNA fragment | position
---------|--------|--------------|----------
wali2  | pSPORT 1 | Sal I - Not I | 1 - 1190 |
2.2    | pBC    | EcoR I - BamH I | 1 - 1190 |
2.3    | pBluescript | Sal I | 1 - 128 |
2.4    | pBluescript | Sal I - Not I | 125 - 1190 |
2.5    | pSPORT 1 | Pst I - Not I | 591 - 1190 |
2.6    | pBC    | Smal I - Pvu II | 1 - 316 |
2.7    | pBluescript | Pst I | 1 - 594 |
2.8    | pBluescript | Nco I | 42 - 517 |
2.9    | pSPORT 1 | Nco I - Not I | 514 - 1190 |
2.10   | pBluescript | Rsa I - BamH I | 929 - 1190 |
2.11   | pBluescript | Rsa I | 662 - 928 |
2.12   | pBluescript | Rsa I | 260 - 661 |

wali3

subclone | vector | DNA fragment | position
---------|--------|--------------|----------
wali3  | pSPORT 1 | Sal I - Not I | 1 - 582 |
3.2    | pBC    | Sal I - Not I | 1 - 582 |
3.3    | pBluescript | Pst I - Sst II | 156 - 406 |
3.4    | pSPORT 1 | Sst II - Not I | 405 - 582 |
3.5    | pBluescript | Hind III | 39 - 582 |
3.6    | pBluescript | Pst I | 1 - 159 |
### wali4

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### wali5

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Duplicates of some of the northern analyses shown in Chapter 4 are presented here. In addition, the results of northern analyses containing samples from plants that had been grown in deoxygenated media, or grown continually in the dark for 2 d are also shown. Appendix 3 summarises all the northern data presented here and elsewhere in this thesis that were duplicated.

Figure A2.1 shows the root growth of the plants during a range of 2 d treatments. The experiments shown in parts A and C of Figure A2.1 used plants of the cultivar Warigal, and the experiment shown in part B used SS plants. The oxygen levels of the media in the low \( O_2 \) treatments (and also the controls) from these experiments were monitored before the plants were placed in the media, and also after 24 h growth in the media. These oxygen measurements, as well as the temperature of the media at the time of the measurements, are shown in Table A2.1.

| Figure A2.1 Root growth of Warigal and SS plants under various stress conditions |
|-----------------------------------|---------------------------------|
| A Warigal                         | B SS                            |
| control                           | control                         |
| 10 \( \mu M \) Al                 | low \( O_2 \)                   |
| Ca only / 6d                       | dark                            |
| Ca only / 2d                       |                                |
| no P                               |                                |
| no Mg                              |                                |
| no Fe                              |                                |
| low \( O_2 \)                      |                                |
| low Zn                             |                                |
| low Cu                             |                                |
| 1 \( \mu M \) Ca                  |                                |
| 50 \( \mu M \) Ca                 |                                |
| 50 \( \mu M \) Cd                 |                                |
| 2.5 \( \mu M \) La                |                                |

Three separate experiments were performed (A, B and C). Plants were grown in Ruakura medium for 5 d after germination, then transferred to media lacking one or more nutrients, or to media containing the amounts shown of various metals, or to deoxygenated media, or to Ruakura medium and placed in a dark chamber. The plants were then grown for a further 2 d. The exception to this was the Ca only / 6d treatment, where the plants were grown in Ruakura medium for only 1 d, then the plants were transferred to medium containing only CaCl\(_2\) and grown for a further 6 d. Root growth values were calculated as the increase in root length during the 2 d treatments (or during the final 2 d of growth for the Ca only / 6d treatment) \(+/-\ SE\) (see Ch. 2.7.2 and 2.7.3 for details on treatments). Values are the means of 10 (A and C) or 15 (B) root measurements from each treatment.
Total RNA was isolated from the root tips of the plants in each treatment shown in Figure A2.1 (Ch. 2.9). Northern blots were prepared using these RNA samples, and were hybridised to probes from each of the wali clones. The membranes were also hybridised to a rRNA probe (prepared from pTA250.2 insert DNA, containing a wheat 26S rRNA gene). Figure A2.2 shows the results from these northern analyses. Due to the uneven loading of some of the RNA samples (as judged by hybridisation to the rRNA probe), densitometry was performed and the expression level of each band in Figure A2.2 was determined relative to that of the rRNA band. The densitometry results are shown in Figure A2.3.

Some of the concentrations of metals used in these experiments are different to those shown in Chapter 4. This is due to the fact that these represent the earlier experiments performed, where some optimisation of the different treatments was performed. For example, the Cu and Zn treatments shown in Figure A2.1A did not have a large effect on root growth in comparison to the untreated control. The Cu treatments were subsequently increased to 2.5 μM (from 1 μM) in the treatments shown in Figure A2.1C and Figure 4.3. Similarly, the Zn treatment was increased from 50 μM in Figure A2.1A to 150 μM in Figure A2.1C and to 200 μM in Figure 4.3. In addition, the La treatment (at 2.5 μM in Fig. A2.1C) was increased to 5 μM in the experiment shown in Figure 4.3. This means that differences in the expression levels of some of the wali clones in response to these lower concentrations of toxic metals (compared to the results shown in Ch. 4) may be due to differing amounts of stress suffered by the plants.

In general, the toxic metal treatments used here resulted in increased expression of each of wali1, -3, -4 and -5 (see Fig. A2.2 and A2.3). However, some of the less toxic treatments (e.g. the 2.5 μM La treatment, as mentioned above) did not result in clear induction of gene expression. Because of the differences in the amounts of some of these metals used, it may not be valid to compare these treatments.

As occurred in the experiment shown in Figure 4.3, the expression of wali2 generally was not induced by the toxic metal treatments. The major qualitative difference between the different experiments is that
the expression of wali2 was greatly reduced by the 2.5 μM Cu treatment shown in Figure 4.3, but was slightly increased in the 2.5 μM Cu treatment shown in Figure A2.2C and Figure A2.3C. In addition, the 1 μM Cu treatment (Figs. A2.2A and A2.3A) slightly reduced the expression of wali2. As noted previously (Ch. 4.4.1) the response of wali2 appears to be highly variable (for unknown reasons).

The effects of low concentrations of nutrients on the expression of the wali genes was also examined in the experiments presented here. These experiments repeat the work shown in Figure 4.4 in Chapter 4. An additional treatment, the Ca only treatment of 6 d duration is also shown here. As this longer treatment did not have a much greater effect on gene expression of the walis than the Ca only treatment of 2 d duration, it was not repeated in other experiments. In general, the trends observed in the experiment shown in Figure 4.4 were similar to that which occurred here. For example, each of wali1, -3, -4 and -5 showed an increase in expression with the low Ca treatments (Figs. A2.2A, C, A2.3A and C). Similarly, the expression of wali2 decreased in response to the low Ca treatment. The expression of wali1, -3, -4 and -5 were not greatly affected by the other low nutrient treatments. wali2 expression decreased in most of the low nutrient treatments, except for the no Mg treatment, where expression levels were similar to the untreated control (this is different to the result shown in Fig. 4.4).

There were some inconsistencies in the results obtained from the low O2 and dark treatments. For example, in the experiment shown in part B of Figures A2.2 and A2.3, these treatments often did not result in very large changes in gene expression. For example, most of the induction or repression in gene expression that occurred, yielded changes of approximately two-fold over controls. The exceptions to this were the induction of wali1 in the low O2 treatment (a four-fold increase) and the induction of wali5 in the dark treatment (a six-fold increase). However when this experiment was repeated (included in Figs. A2.2A and A2.3A), wali1 was not induced by the low O2 treatment, and also showed greater induction in the dark treated plants. wali5 also showed differences in the repeat of this experiment, although there was induction by the dark treatment again, and the low O2 treatment caused a small reduction in transcript levels, where previously there was a nearly three-fold increase in expression induced by this treatment.

The reasons for the differences between the two experiments are not clear, but there are several possibilities. The differences observed between the experiments may indicate a real difference in response to these stresses between Warigal and SS. Alternatively, when the media was degassed to reduce the O2 content, the temperature of the medium also decreased to a different degree in the two experiments (Table A2.1). It is possible that differences in the temperature of the medium in the two experiments had an effect on gene expression. In the dark treatments, the plants were placed in a wire cage surrounded by aluminium foil to block out light. Although the temperature within the cage was checked throughout the two experiments (it was always within 1° C of the rest of the chamber), the humidity in the cage may have varied during the experiment. In view of the difficulty in reproducing
Total RNA was isolated (Ch. 2.9) from the root tips of Warigal (A and C) or SS (B) plants after 2 d of the indicated treatments (or 6 d of Ca only treatment). The treatments were as described in the legend to figure A2.1. The RNA was electrophoresed through 1.2% agarose gels and blotted to Hybond-N+ membranes (Ch. 2.11.1 and 2.13.3). The membranes were successively hybridised to labelled inserts from each of the wali clones and also to a 26S rRNA probe (Ch. 2.14.2). In C, some intervening lanes on the northern hybridisations have been removed, but all the samples shown for each probe were electrophoresed through the same gel and came from the same autoradiograph.
conditions accurately for these two experimental treatments, they were not included in the data presented in Chapter 4.

In general, it was observed through the course of these northern hybridisation experiments that small changes in gene expression (approximately two-fold or less) were difficult to reproduce and may represent pot-to-pot variation. As much of the induction and repression of wali2 observed was in this sort of range, the changes observed may not be significant.

**Figure A2.3** Densitometry analysis of northern hybridisations

The graphs contain the densitometry data obtained from the northern analysis shown in Fig. A2.2. Parts (A, B and C) correspond to the northern hybridisation (A, B and C) in Fig. A2.2. The northern hybridisation in (A) of Fig. A2.2 with the wali4 probe was not included in this figure, as there was too much background hybridisation present. After densitometry, the expression level of each band in Fig. A2.2 was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the control samples.
Table A3.1 summarises the level of expression of each of the wali clones under various treatments. The information presented was derived from the densitometry data of the northern hybridisations presented in Chapters 3 and 4 and in Appendix 2. Only those treatments that were repeated at least once are included.

When comparing the level of induction obtained in the same treatment in different experiments, the following should be kept in mind:

(a) Some treatments used different cultivars, and these cultivars are known to react differently to Al, and possibly also have differences in their reactions to other stresses.

(b) Some of the amounts of the metal ions used differ between treatments, for example 2.5 μM Cu versus 1 μM Cu.

(c) Densitometry was used to quantify the changes observed in the expression of the wali genes. However, there are a number of different variables that may affect the values obtained with densitometry. For example, the autoradiographs from the northern hybridisations were scanned one-dimensionally, so that the intensity of the signal was measured in a line through each band, rather than measuring the whole of each band. This may result in biased values if the RNA distribution is not even within a band, or if the width of the bands within a northern gel vary. For example, some of the bands in the northern hybridisations shown in Fig. A2.2C are uneven. In addition, some bands are more dispersed than others, so that it can become difficult to decide exactly where a band begins and ends, especially if there is a wide range of expression levels within one northern hybridisation. For example, compare the sizes of the bands obtained with the wali1 probe in Figure 3.7B. Besides the errors that may be introduced during the densitometry itself, other factors may affect the values obtained. For example, the film used for autoradiography reacts to the radioactive signal linearly over a certain range, but eventually the film is 'saturated' and little increase in signal is observed with longer exposure times. This may result in an underestimate of the signal obtained for some of the darker bands. In addition, where very faint bands were scanned, or even areas where no band was visible, the background hybridisation present will add to the value obtained from densitometry, so that the band may be given a higher value. For example, no visible bands were observed for any of the three leaf samples in the autoradiograph of the northern hybridisation with the wali4 probe shown in Figure 3.7B. However, in the densitometry data shown for this probe in Figure 3.7C, it appears that some expression was detected in the leaf tissue, and that it varied for the different treatments shown. The reason for this variation is that, even though a similar background value was obtained for each of the three leaf samples, the values obtained for the control rRNA probe differed, and this probe was used to normalise the results obtained.
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<td>54.5</td>
</tr>
<tr>
<td>2.5 μM Ga</td>
<td>SS</td>
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<td>38</td>
<td>44.3</td>
<td>1.1</td>
<td>47.7</td>
<td>3.8</td>
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<tr>
<td>dark</td>
<td>Wg</td>
<td>A2.3A</td>
<td>83</td>
<td>4.5</td>
<td>0.7</td>
<td>4.1</td>
<td>-</td>
<td>8.6</td>
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<tr>
<td>dark</td>
<td>SS</td>
<td>4.3</td>
<td>66</td>
<td>1.2</td>
<td>0.4</td>
<td>1.6</td>
<td>1.9</td>
<td>6.3</td>
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<tr>
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<td>Wg</td>
<td>A2.3A</td>
<td>51</td>
<td>0.5</td>
<td>0.4</td>
<td>0.5</td>
<td>-</td>
<td>0.6</td>
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<tr>
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<td>16</td>
<td>3.9</td>
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<td>0.6</td>
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<td>-</td>
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<tr>
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<td>A2.3C</td>
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<td>1.7</td>
<td>2.1</td>
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<td>14.1</td>
<td>2.3</td>
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<tr>
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<tr>
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<td>11.1</td>
<td>1.3</td>
<td>10.3</td>
<td>1.5</td>
<td>14.0</td>
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The numbers refer to the fold induction over the untreated controls, which are given values of 1.0 in each case. The column 'Fig. ref' refers to the Figure within this thesis where the northern results were presented. The ‘% growth’ column describes the effect the treatment had on root growth, expressed as a percentage of the untreated root growth (a value of 100% represents growth equal to the untreated plants). Each of the treatments presented was of 2 d duration, except for one of the Ca only treatments. All the results shown come from treated root tips (i.e. not from leaf tissue). The plants used were Warrigal (Wg), Waalt (Wt), SS and RR. The 10 μM Al and 50 μM Cd treatments marked with asterisks (*) denote treatments that were part of a time course. In these two treatments, the relative level of expression calculated used control plants that were two days younger than the treated plants as the base line expression (i.e. the value denoted as having an expression level of 1.0). All expression values have been rounded to one decimal place. The 10 μM Al and 100 μM Al treatments marked with a † denote separate northern analyses using the same RNA samples. Details on the growth conditions and the treatments used are given in the Figure legends referred to and also in Chapter 2.7.2 and 2.7.3.
A direct comparison of the errors inherent in the system adopted can be made by comparing the daggered (†) samples in the Table. In these cases the same samples were used in two different northerns, and the values obtained should be equivalent.

In spite of these caveats, some clear trends can be observed in the induction of the genes to various treatments. In Figure A3.1, the level of induction for each of the wali genes during different Al treatments is plotted against the amount of root growth observed for these treatments.

![Figure A3.1 Comparison of the effects of Al on induction of wali gene expression and root growth.](image)

This figure includes only the results (from Table A3.1) for the Al treatments where root growth of the plants was measured. The data from all cultivars at 0, 10 or 100 μM Al are shown. The lines of best fit for the data were drawn using the Cricket Graph 'curve fit' command.

It can be seen from this comparison that there is a relationship between the amount of root growth measured and the induction of the wali genes. For each of wali1, -3, -4 and -5, higher levels of induction of each genes' expression is correlated with greater reductions in root growth. In contrast, wali2 shows the opposite effect, with the treatments that had a more severe effect on root growth, reducing the level of wali2 expression. However, induction of wali2 expression is observed in some of the less severe Al treatments. wali2 induction by 10 μM Al was observed only in Warigal and RR (see Table A3.1), and never in Waalt or SS. Of the five times that changes in wali2 expression by 10 μM Al treatment was quantified, wali2 expression was increased in four cases. In two of these four cases, no root growth measurements were made, so that the graph showing the response of wali2 to Al may be biased against showing the induction by 10 μM Al.

Although the overall relationships between wali gene induction and inhibition of root growth are clear, there is a lot of variability in the data summarised in Figure A3.1. The data represent only three concentrations of Al (0, 10 and 100 μM), but showed considerable differences in root growth under these conditions. These differences are due in part to the comparison of different cultivars to different levels of Al, though some differences were observed when comparing the same cultivar at one level of
Al (Table A3.1). These differences may be caused by inaccuracies in the measurement of the roots during the experiment, or to slight differences in the concentrations of soluble toxic Al in each experiment. As was discussed in Chapter 1.2, slight variations in the pH of the growth medium can result in large differences in the amount of soluble Al present. Other differences between experiments, such as temperature, plant developmental stage, light levels or density of plants might also have affected the level of induction of the wali genes.

Two treatments (one for 10 μM Al, and one for 50 μM Cd) are highlighted with asterisks in Table A3.1. These two treatments came from the 2 d point of time courses of Al or Cd treatment, where the untreated plants in each case were sampled 2 d before the treated plants. In contrast, the other 10 μM Al and 50 μM Cd treatments shown in Table A3.1 had their untreated plants harvested at the same time as the treated plants. In the asterisked treatments, the induction of the wali gene expression was often greater than the induction observed for the other 10 μM Al or 50 μM Cd treatments. One explanation for the higher induction values in these two experiments may be that the untreated controls had lower uninduced levels of expression of the wali genes (with the exception of wali4). This interpretation would suggest that there may be changes in the expression of these genes during the growth and development of the plants, in addition to the induction observed by the stress treatment. The validity of this hypothesis would have to be tested with further experiments.
FIGURE LEGENDS

Figure 1
Effect of differing concentrations of Cd on root elongation in the wheat line SS. Plants were grown in Ruakura medium for 5 d after germination, then transferred to Ruakura media containing 0 to 200 μM Cd for 48 h. Root growth was calculated as final root length minus root length before the addition of Cd. Values are the means of 10 root measurements from different plants in each treatment +/- SE.

Figure 2
Effect of 50 μM Cd on root elongation and expression of the wali genes. (A) Root growth of the plants. Values were calculated as the increase in root length from the starting time point and are the means of 10 root measurements from each treatment +/- SE. The graph shows plants that were treated with 50 μM Cd (■), plants that were grown in 50 μM Cd for 24 h followed by recovery in Cd-free media (▲), and plants that were grown in Cd-free media (●). (B) Autoradiographs of northern hybridisations to total RNA extracted from the root tips of the treated plants. Plants from the wheat line SS were either untreated (0), or treated with 50 μM Cd for 2, 4, 12, 24, 48 or 72 h or treated with Cd for 24 h followed by recovery in Cd-free media for 4 (24/4), 24 (24/24), or 72 h (24/72). (C) Relative expression levels. Densitometry was performed and the expression level of each band in (B) was determined relative to that of the rRNA band. The fold induction of each treatment was calculated relative to the untreated (0) sample.

Figure 3
Effect of various toxic metal ions on root growth and the expression of the wali genes. Plants from the wheat line SS were either untreated (control) or treated for 2 d with Al, Fe, Cd, Zn, Cu, Ga, In or La at the concentrations shown. (A) Increase in root length after the 2 d treatment. Values are the means of 15 root measurements from each treatment +/- SE. (B) Relative expression levels of the genes (original autoradiographs not shown). Densitometry was performed on each band of the northern hybridisations, and normalised to those obtained from a rRNA probe. The fold induction of each treatment was calculated relative to the control sample.

Figure 4
Effect of low nutrient media on root growth and expression of the wali genes. SS plants were grown for 5 d in Ruakura medium, then their roots were rinsed in MilliQ water, and the plants were transferred to either Ruakura medium (control), or to media lacking or deficient in one or more nutrients (see Methods for details), and grown for a further 2 d. (A) Increase in root length after the 2 d treatment. Values are the means of 15 root measurements from each treatment +/- SE. (B) Relative expression levels of the genes (original autoradiographs not shown). Densitometry was performed on each band, and normalised to those obtained from a rRNA probe. The fold induction of each treatment was calculated relative to the control sample.
Figure 5
Effect of wounding on the expression of the wali genes in root and leaf tissue from SS plants. After 5 d growth in Ruakura medium root and leaf tissue was wounded and incubated as described in methods. Tissue was collected 6 h or 2 d after wounding. Control plants (c) were grown for a total of 7 d in Ruakura medium before harvesting. Each graph shows the quantitation of the expression of each of the wali genes after northern hybridisation (original autoradiographs not shown). Densitometry was performed on each band, and normalised to those obtained from a rRNA probe. The fold induction of each treatment was calculated relative to the root control sample (as some genes have very low levels of expression in leaf tissue).

Figure 6
Localisation of the wali genes mRNA by in situ hybridisation to longitudinal root sections from the wheat cultivar Warigal. Sections were probed with DIG-labelled RNA probes. Sections (A-C) were probed with an antisense wali1 probe, (D-F) with an antisense wali3 probe, (G-I) with an antisense wali5 probe, (J-L) with sense probes for wali1, wali5 and wali3 respectively. Hybridisation to the probes is seen as a brown or purple colour. Sections (A, D, G and J) were from plants grown in Ruakura medium for 7 d, and sections (B, C, E, F, H, I, K and L) were from plants grown in Ruakura medium for 5 d, then Ruakura medium with 10 μM AI added for a further 2 d. Sections (C, F, I and L) are from approximately 1 to 4 mm from the root apex. The magnification of all micrographs is the same except for H. Scale bar, 0.5 mm. Arrows in H and K indicate a group of cells with secondary cell walls. rc, root cap; v, vascular tissue (stele); c, cortex; q, quiescent centre.
Figure 1
Figure 2
Figure 3

(A) Bar graph showing root growth (cm) for different treatments. Treatments include control, 10 µM Al, 100 µM Al, 50 µM Fe, 200 µM Cd, 2.5 µM Cu, 2.5 µM Ga, and 2.5 µM In.

(B) Histograms showing fold induction for wali1, wali3, wali4, and wali5 across various treatments. Treatments include control, 10 µM Al, 100 µM Al, 50 µM Fe, 200 µM Cd, 2.5 µM Cu, 2.5 µM Ga, and 2.5 µM In.
Figure 4
Figure 5
Figure 6