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Induction of global changes in *Nicotiana tabacum* chromatin following nutritional stress

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This thesis was submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Molecular Medicine and Pathology

November 2012
Abstract

Cultures of tobacco (*Nicotiana tabacum*) BY-2 cells provide a good model for studying plant cell growth under different nutrient conditions. A previous study reported changes in apparent DNA content, as measured with flow cytometry of propidium iodide (PI) stained cell nuclei, during the course of culture. The basis of these changes was the main impetus for this investigation.

While fluorescence of BY-2 nuclei stained with PI (which binds DNA by intercalation) increased then decreased with culture time, that of cells stained with Hoechst 33342 (which binds DNA without intercalation) did not fluctuate, suggesting that changes in cellular DNA content were not occurring. Addition of PI, but not of Hoechst 33342, caused swelling of nuclei, implying that intercalation induced a change in chromatin conformation. Since decreased PI staining accompanied depletion of sucrose in the culture medium, the effects of nutritional stress were also investigated.

Deprivation of sucrose caused severe reduction of growth, cell shrinkage, vacuolation and formation of monodansylcadaverine positive acidic vesicles, indicative of autophagy. Nitrate deprivation caused very different morphological changes, with cellular swelling and formation of starch granules. Interestingly, starch granules were packaged within a double membrane, usually indicative of autophagosomes. These changes were not related to alterations in PI staining.

The mechanism for induced alteration in PI staining was investigated by examination of chromatin. Modifications in DNA methylation were observed both through differences in the DNA fragmentation pattern following treatment with methylation-sensitive restriction endonucleases and by a decrease in total 5-methylcytosine content. Changes in histone H1 staining were also measured using fluorescent antibodies and mirrored PI staining by decreasing then increasing with culture time.

It is hypothesised that cellular energy metabolism controls a process that partially removes histone H1, a nucleosome linking histone, from DNA. This allows a DNA glycosylase, an enzyme found in plants but not animals, to cut the N-glycosidic link between the DNA backbone and 5-methylcytosine, effectively acting as a demethylase. The chromatin alteration allows access to PI, explaining the increased staining. Depletion of an energy source at later stages of culture allows reattachment of histone H1 and restoration of DNA methylation.
Acknowledgments

I would like to acknowledge everyone who contributed to the fulfilment of this research project and first on my list is of course my supervisor Professor Bruce Baguley. Thank you for your patience, support, guidance, wisdom and dedication to my cause. You have been an excellent mentor and your efforts are very much appreciated.

Much gratitude must go out to Dr. Peter Wigley and Dr. Andrew Broadwell, who provided the facilities at BioDiscovery and the finances needed to achieve the goal of my studies. Without their encouragement and generosity, none of this research would have been possible.

The initiator of this research and the person who encouraged me to begin a PhD is Dr. Iona Weir. Your passion for science and the art of flow cytometry sparked a great interest in me. Thank you so much for giving me my first real job, believing in me and setting up the basis for my studies.

The BY-2 culture was gifted by Associate Professor Jo Putterill at the School of Biological Sciences at the University of Auckland. Dr. Adrian Turner with the School of Biological Sciences was very gracious to give me a crash course in sample preparation and use of the TEM. Quantification of DNA methylation using HPLC was achieved by Pam Turner with the Auckland Cancer Society Research Centre at the University of Auckland. HPLC results for 2,4-D quantification were gained from the efforts of Dr. Stephen Bloor at BioDiscovery’s Wellington laboratory. Many thanks to you all!

Practical advice and general lab know-how was provided by Dr. Damian Wright, tēnā rawa atu koe! Thanks also to Dr. Phillippa Rhodes for the occasional useful tip, but mainly just the nagging! To all of the team at BioDiscovery, thank you for providing such a great working environment, and of special note, Andrej Brummer, Stella Veeracone and Lisa Benson in the CyTonyx lab. Cheers guys!

To all of my family and friends, your understanding, support and interest during my studies has made all of the difference. I love you all and count myself very privileged to be amongst such great people.

This thesis is dedicated to the memory of my beautiful sister Saacha.
Table of contents

Abstract ................................................................. i
Acknowledgments ..................................................... ii
Table of contents ....................................................... iii
Abbreviations ........................................................... xii

1. Introduction and literature review ......................... 1
  1.1 Nicotiana tabacum BY-2 as a model plant system ....... 1
  1.2 Apparent changes in tobacco cell DNA content .......... 1
  1.3 Chromatin structure and function ......................... 2
    1.3.1 DNA conformations ........................................... 2
    1.3.2 Histones ......................................................... 3
      1.3.2.1 Core histones ............................................... 3
      1.3.2.2 Linker histones ............................................. 5
    1.3.3 The nucleosome and histone H1 positioning .......... 7
    1.3.4 Chromatin ....................................................... 9
      1.3.4.1 Euchromatin ................................................. 10
      1.3.4.2 Heterochromatin .......................................... 10
    1.3.5 Core histone modifications and gene expression .... 12
      1.3.5.1 Histone methylation ..................................... 14
      1.3.5.2 Histone acetylation ...................................... 14
      1.3.5.3 Histone ubiquitination .................................. 14
      1.3.5.4 Histone phosphorylation ................................ 15
      1.3.5.5 Other histone modifications ......................... 15
    1.3.6 DNA methylation and transcription .................... 15
  1.4 Programmed cell death ......................................... 18
    1.4.1 Categories of cells that undergo programmed cell death 18
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4.2 Programmed cell death in plants</td>
<td>19</td>
</tr>
<tr>
<td>1.5 Autophagy</td>
<td>20</td>
</tr>
<tr>
<td>1.6 Autophagy and apoptosis</td>
<td>22</td>
</tr>
<tr>
<td>1.7 Autophagy in plants</td>
<td>24</td>
</tr>
<tr>
<td>1.8 The cell cycle in plants</td>
<td>25</td>
</tr>
<tr>
<td>1.8.1 Autophagic cell death and the cell cycle</td>
<td>26</td>
</tr>
<tr>
<td>1.9 Nutrient withdrawal and autophagy</td>
<td>27</td>
</tr>
<tr>
<td>1.9.1 BY-2 cell nutritional requirements</td>
<td>28</td>
</tr>
<tr>
<td>1.9.1.1 Sucrose starvation in BY-2 cells</td>
<td>28</td>
</tr>
<tr>
<td>1.9.1.2 Nitrate starvation in BY-2 cells</td>
<td>28</td>
</tr>
<tr>
<td>1.10 Background, scope and aims of this thesis</td>
<td>28</td>
</tr>
</tbody>
</table>

2. Methods .............................................................................. 31

2.1 General methods ................................................................... 31
2.1.1 Plant cell culture growth and maintenance .......................... 31
2.1.1.1 Growth conditions .................................................... 31
2.1.1.2 *Nicotiana tabacum* liquid cultures .............................. 31
2.1.2 Production of BY-2 protoplasts ........................................ 32
2.1.3 DNA content measurement of BY-2 nuclei .............................. 33
2.1.4 Microscopy ....................................................................... 33
2.1.5 Flow cytometry .................................................................. 34
2.1.5.1 Instrument specifications and setup .............................. 34
2.1.5.2 Instrument daily maintenance ...................................... 34
2.1.5.3 Acquisition and analysis software ............................... 35
2.1.5.4 Data gating procedure ............................................... 35
2.2 Chapter 3 methods .................................................................. 35
2.2.1 The effect of propidium iodide loading on the DNA peak positions of BY-2 nuclei ................................................. 35
2.2.2 The effect of propidium iodide loading on the DNA peak positions of *P. sativum* nuclei .............................................. 36

---

iv
Table of contents

2.2.3 $G_0/G_1$ peak position changes during growth of BY-2 cells in complete medium ................................................................. 36
2.2.4 Fluorescence microscopy of PI and Hoechst 33342 stained BY-2 nuclei ................................................................................. 37
2.2.5 Mixing of PI stained $P. sativum$ nuclei with BY-2 nuclei ........ 37
2.2.6 Mixing of PI stained BY-2 nuclei from different time points .... 37

2.3 Chapter 4 and chapter 5 methods ........................................... 38
2.3.1 Carbon and nitrogen starvation medium preparations ........ 38
2.3.2 DNA peak position changes during growth of BY-2 cells in sucrose and nitrate starved media .................................................... 38
2.3.3 DNA peak position during growth of BY-2 cells in different media using Hoechst 33342 fluorescence ..................................... 38
2.3.4 The effect of 2,4-dichlorophenoxyacetic acid supplementation on DNA peak position ................................................................. 39
2.3.5 Growth curves of BY-2 cells grown in 12 well plates .......... 39
2.3.6 Cell viability assessment during growth of BY-2 cells in various media .............................................................................. 39
2.3.7 Changes in pH during the growth of BY-2 cells ................ 40
2.3.8 Sucrose concentration in BY-2 12 well cultures grown in various media ................................................................. 40
2.3.9 Protein content in the different BY-2 media from 12 well BY-2 cultures .............................................................................. 41
2.3.10 Depletion of 2,4-D in different BY-2 media from 12 well BY-2 cultures .............................................................................. 42
2.3.11 BY-2 cell morphology ......................................................... 43
2.3.12 Starch granule development in nitrate deprived media ....... 43
2.3.13 Transmission electron microscopy of starch granules in nitrate starved BY-2 cells ................................................................. 44
2.3.14 Formation of spherical bodies in response to protease inhibition ......................................................................................... 45
# Table of contents

2.3.15 Staining of sucrose deprived BY-2 cells with monodansylcadaverine ................................................................. 46
2.3.16 BY-2 cell area measured in two dimensions .................. 46
2.3.17 Total protein content estimation within BY-2 cells during growth in various media ......................................................... 46
2.3.18 Nitric oxide levels in BY-2 cells grown in various media ...... 47
2.3.19 Intracellular calcium levels in BY-2 protoplasts ............... 47

2.4 Chapter 6 methods ................................................................ 49

2.4.1 Methylation-sensitive restriction fingerprinting of BY-2 genomic DNA ........................................................................ 49

2.4.1.1 DNA extraction from BY-2 cells ............................................................ 49
2.4.1.2 DNA quantitation and quality ................................................................. 50
2.4.1.3 Restriction enzyme digestion ................................................................. 50
2.4.1.4 PCR conditions ....................................................................................... 50
2.4.1.5 Gel electrophoresis and visualisation ...................................................... 51

2.4.2 Total 5-methylcytosine content of BY-2 cells during growth and starvation ............................................................... 51

2.4.2.1 DNA extraction from BY-2 cells ............................................................ 51
2.4.2.2 Preparation for HPLC analysis of 5-methyl-cytosine ............................. 51
2.4.2.3 HPLC analysis of 5-methyl-cytosine ...................................................... 52

2.4.3 Anti-histone H3 mono-methyl K4 and anti-histone H1 antibody analysis .............................................................................. 53

3. Analysis of BY-2 nuclei staining using flow cytometry ......................... 54

3.1 Introduction ........................................................................ 54

3.2 Results ................................................................................. 56

3.2.1 The effect of propidium iodide loading on the DNA peak positions of BY-2 nuclei .............................................................. 56
Table of contents

3.2.2 The effect of propidium iodide loading on the DNA peak positions of *P. sativum* nuclei .............................................................. 58
3.2.3 *G₀/G₁* peak position changes during growth of BY-2 cells in complete medium ................................................................................. 59
3.2.4 Fluorescence microscopy of PI and Hoechst 33342 stained BY-2 nuclei ........................................................................................................ 63
3.2.5 Mixing of PI stained *P. sativum* nuclei with BY-2 nuclei .......... 65
3.2.6 Mixing of PI stained BY-2 nuclei from different time points .... 67
3.3 Discussion .................................................................................................................. 69

4. Carbon source availability and its effect on BY-2 cell growth and chromatin state ................................................................. 72

4.1 Introduction ............................................................................................................. 72
4.2 Results ..................................................................................................................... 75
  4.2.1 DNA peak position changes during growth of BY-2 cells in various media ................................................................. 75
  4.2.2 DNA peak position during growth of BY-2 cells in different media using Hoechst 33342 fluorescence ........................................... 79
  4.2.3 The effect of 2,4-dichlorophenoxyacetic acid supplementation on DNA peak position ......................................................... 80
  4.2.4 Growth curves of BY-2 cells grown in 12 well plates ............... 81
  4.2.5 Cell viability assessment during growth of BY-2 cells in various media ................................................................................................. 83
    4.2.5.1 Live and dead cell proportions when grown in complete medium ......... 84
    4.2.5.2 Live and dead cell proportions when grown in sucrose depleted medium ................................................................. 85
    4.2.5.3 Live and dead cell proportions when grown in sucrose and nitrate depleted medium ................................................................. 87
  4.2.6 Changes in pH during the growth of BY-2 cells .............. 88
Table of contents

4.2.7 Sucrose concentration in BY-2 12 well cultures grown in various media ................................................................. 89
4.2.8 Protein content in the different BY-2 media from 12 well BY-2 cultures ................................................................................................................................. 90
4.2.9 Depletion of 2,4-D in different BY-2 media from 12 well BY-2 cultures ................................................................................................................................. 91
4.2.10 BY-2 cell morphology .................................................................................................................................................. 92
4.2.11 Formation of spherical bodies in response to protease inhibition .................................................................................. 94
4.2.12 Staining of sucrose deprived BY-2 cells with monodansylcadaverine .............................................................................. 96
4.2.13 BY-2 cell area measured in two dimensions ................................................................................................................. 97
4.2.14 Total protein content estimation within BY-2 cells during growth in various media ................................................................. 98
4.2.15 Nitric oxide levels in BY-2 cells grown in various media..... 100
4.2.16 Intracellular calcium levels in BY-2 protoplasts ............... 104
    4.2.16.1 Intracellular calcium levels in freshly subcultured BY-2 protoplasts. 104
    4.2.16.2 Intracellular calcium levels in BY-2 cells grown in complete medium ...................................................................................................................................................... 105
    4.2.16.3 Intracellular calcium levels in BY-2 cells grown in sorbitol substituted medium ...................................................................................................................................................... 107
4.3 Discussion ................................................................................................................................................................................. 109

5. The impact of nitrogen withdrawal on BY-2 cell growth and chromatin state ................................................................. 115
5.1 Introduction .................................................................................................................................................................................. 115
5.2 Results ....................................................................................................................................................................................... 116
    5.2.1 DNA peak position changes during growth of BY-2 cells in nitrate starved media ................................................................. 116
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.2 DNA peak position during growth of BY-2 cells in different media using Hoechst 33342 fluorescence</td>
<td>119</td>
</tr>
<tr>
<td>5.2.3 The effect of 2,4-dichlorophenoxyacetic acid supplementation on DNA peak position</td>
<td>120</td>
</tr>
<tr>
<td>5.2.4 Growth curves of BY-2 cells grown in 12 well plates</td>
<td>121</td>
</tr>
<tr>
<td>5.2.5 Cell viability assessment during growth of BY-2 cells in nitrate deprived cultures</td>
<td>123</td>
</tr>
<tr>
<td>5.2.5.1 BY-2 cells grown in medium lacking nitrates</td>
<td>123</td>
</tr>
<tr>
<td>5.2.5.2 BY-2 cells grown in KCl substituted medium</td>
<td>124</td>
</tr>
<tr>
<td>5.2.6 The pH during growth of BY-2 cells in media lacking nitrates</td>
<td>125</td>
</tr>
<tr>
<td>5.2.7 Sucrose concentration in BY-2 12 well cultures grown in nitrate starved media</td>
<td>126</td>
</tr>
<tr>
<td>5.2.8 Protein content in the different BY-2 media from 12 well BY-2 cultures</td>
<td>127</td>
</tr>
<tr>
<td>5.2.9 Depletion of 2,4-D in different BY-2 media from 12 well BY-2 cultures</td>
<td>128</td>
</tr>
<tr>
<td>5.2.10 BY-2 cell morphology during nitrate deprivation</td>
<td>129</td>
</tr>
<tr>
<td>5.2.11 Starch granule development in nitrate deprived media</td>
<td>130</td>
</tr>
<tr>
<td>5.2.12 Transmission electron microscopy of starch granules in nitrate starved BY-2 cells</td>
<td>131</td>
</tr>
<tr>
<td>5.2.13 Formation of spherical bodies in response to protease inhibition</td>
<td>133</td>
</tr>
<tr>
<td>5.2.14 Staining of nitrate deprived BY-2 cells with monodansylcadaverine</td>
<td>134</td>
</tr>
<tr>
<td>5.2.15 BY-2 cell area measured in two dimensions</td>
<td>135</td>
</tr>
<tr>
<td>5.2.16 Total protein content within BY-2 cells during growth in nitrate deprived media</td>
<td>136</td>
</tr>
<tr>
<td>5.2.17 Nitric oxide levels in nitrate deprived BY-2 cells</td>
<td>138</td>
</tr>
<tr>
<td>5.2.18 Intracellular calcium levels in BY-2 cells grown in KCl substituted medium</td>
<td>141</td>
</tr>
</tbody>
</table>
## 6. Chromatin alterations during the growth cycle of BY-2 cell cultures

6.1 Introduction ................................................................. 148
6.2 Results ............................................................................. 151
   6.2.1 Methylation-sensitive restriction fingerprinting of BY-2 genomic DNA ................................................................. 151
   6.2.2 Total 5-methylcytosine content of BY-2 cells during growth and starvation ................................................................. 152
   6.2.3 Anti-histone H3 mono-methyl K4 and anti-histone H1 antibody analysis .................................................................. 154
6.3 Discussion ....................................................................... 161

## 7. Overview and concluding discussion

7.1 Overview ......................................................................... 164
7.2 Nuclear staining changes during BY-2 cell growth ........ 165
7.3 Response to carbon deprivation in BY-2 cells .......... 166
7.4 The effect of nitrogen withdrawal in BY-2 cells .......... 168
7.5 Chromatin remodelling .......... ......................................... 171
7.6 Problems inherent to this study and possible solutions .... 172
7.7 Implications and future directions ................................. 175

### Appendices

Appendix 1: Plant liquid culture media ..................................... 177
   1.1 Murashige and Skoog (MS) micronutrient stock solution (200X) ................................................................. 177
| 1.2  | Murashige and Skoog (MS) macronutrient and micronutrient stock solution (20X) | 177 |
| 1.3  | Murashige and Skoog (MS) macronutrient (KCl substituted) and micronutrient stock solution (20X) | 178 |
| 1.4  | BY2 medium (complete) | 178 |
| 1.5  | BY2 medium (sorbitol substituted) | 178 |
| 1.6  | BY2 medium (KCl substituted) | 179 |
| 1.7  | BY2 medium (KCl and sorbitol substituted) | 179 |
| 2.1  | BY2 protoplast test buffer | 179 |
| 2.2  | BY2 protoplast enzyme solution | 180 |
| 3.1  | Reynolds lead citrate solution | 180 |
| 4.1  | Galbraith’s buffer | 180 |
| 4.2  | PBS/Triton X-100 solution | 181 |
| 5.1  | CTAB buffer | 181 |
| 5.2  | CTAB extraction buffer | 181 |
| 5.3  | TE buffer | 181 |
| 5.4  | RNase solution | 181 |

**References** | 182
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>µg</td>
<td>microgram(s)</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre(s)</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer(s)</td>
</tr>
<tr>
<td>µM</td>
<td>micromoles per litre</td>
</tr>
<tr>
<td>ACD</td>
<td>autophagic cell death</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchroninic acid</td>
</tr>
<tr>
<td>BF</td>
<td>bright field</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
</tr>
<tr>
<td>bp</td>
<td>DNA base pair(s)</td>
</tr>
<tr>
<td>BY-2</td>
<td>Nicotiana tabacum cv. Bright Yellow 2</td>
</tr>
<tr>
<td>CTAB</td>
<td>hexadecyltrimethylammonium bromide</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>DAF-FM</td>
<td>4-amino-5-methylamino-2',7'-difluorofluorescein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole, dihydrochloride</td>
</tr>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>dNTPs</td>
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<td>FS</td>
<td>forward scatter</td>
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<td>g</td>
<td>acceleration of gravity</td>
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<tr>
<td>g</td>
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<td>G</td>
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<tr>
<td>HPCV</td>
<td>half-peak coefficient of variance</td>
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<td>HPLC</td>
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<tr>
<td>kDa</td>
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</tr>
<tr>
<td>L</td>
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<td>LC3</td>
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</tr>
<tr>
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<tr>
<td>Mbp</td>
<td>mega base pair(s)</td>
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<td>monodansylcadaverine</td>
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<td>mM</td>
<td>millimoles per litre</td>
</tr>
<tr>
<td>MS</td>
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<td>nuclear DNA</td>
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<td>nanomoles per litre</td>
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<td>photo multiplier tube</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
</tbody>
</table>
Abbreviations

RNase .............................................................................................................. ribonuclease
rpm .................................................................................................. revolutions per minute
RT ................................................................. room temperature (ambient temperature)
SDS ......................................................................................................... sodium dodecyl sulphate
SDS-PAGE ......................................................... SDS-polyacrylamide gel electrophoresis
sec ........................................................................................................... second(s)
SNAP ............................................................... S-nitroso-N-acetylpenicillamine
SPE ................................................................................................... solid phase extraction
SS ................................................................................................... side scatter
ssDNA ................................................................. single stranded deoxyribonucleic acid
TAE ........................................................................... tris-acetate acid EDTA buffer
TBE ........................................................................................ tris-boric acid EDTA buffer
TE ........................................................................... tris-hydrochloric acid EDTA buffer
TEM .......................................................... transmission electron microscopy (microscope)
tris ................................................................... 2-amino-2-hydroxymethyl-1,3-propanediol
tris-HCl ........................................................................ tris solution, pH adjusted with HCl
Triton X-100 .............................................................. iso-octylphenoxypolyethoxyethanol
U ................................................................................................... ultraviolet fluorescence filter set-up
USA ............................................................................................. United States of America
UV ................................................................................................ ultraviolet light
1. Introduction and literature review

1.1 *Nicotiana tabacum* BY-2 as a model plant system

The BY-2 cell line has become an important model system in the plant science world. It was developed in 1968 by Dr. Nobumaro Kawashima at the Hatano Tobacco Experimental Station of the Japan Tobacco and Salt Public Corporation and was derived from a *Nicotiana tabacum* cv. bright yellow-2 seedling callus (Kato et al., 1972). Although several groups obtained and published work on it, unrestricted use and distribution of the BY-2 cell line was not granted by the company until 1999 (Nagata et al., 2004). The popularity of this cell line as a model system of higher plants is due to its ability to replicate rapidly (doubling time is approximately 13 h), its ability to undergo cell cycle synchronisation (for instance following addition of drugs such as aphidicolin), its high cell homogeneity and its high competency of transformation by *Agrobacterium tumefaciens* (Kato et al., 1972; Nagata et al., 1992; Nagata et al., 2004).

1.2 Apparent changes in tobacco cell DNA content

Previous studies investigating programmed cell death (PCD) in tobacco (*N. plumbaginifolia*) cells revealed changes in propidium iodide (PI) staining of DNA during the course of culturing (O'Brien et al., 1998a; O'Brien et al., 1998b). The fluorescence intensity of PI is linked to cellular DNA content and would be expected to remain stable, but increasing fluorescence intensities were seen during the growth phase of the tobacco cultures (O'Brien et al., 1998a; O'Brien et al., 1998b). The changes were hypothesised to be a result of chromatin changes rather than of changes in cellular DNA content. One of the main aims of this thesis was to investigate the causes and mechanistic basis for these changes and the next section therefore reviews chromatin structure.
1.3 Chromatin structure and function

1.3.1 DNA conformations

In nature, three DNA conformations have been described, known as A-, B- and Z-DNA (Sinden, 1994). Other conformations such as cruciform, triplex and H-DNA have also been postulated, with evidence to support their existence in vivo investigated in various studies (Rahmouni and Wells, 1989; Karlovsky et al., 1990; McClellan et al., 1990). A- and B-DNA form right hand helices, with A-DNA likely to occur in dehydrated samples and B-DNA believed to be the predominant form present in living cells (Sinden, 1994). Z-DNA forms a very different left handed helix (Wang et al., 1979) and is associated with methylation and possibly DNA-protein complexes (Sinden, 1994) (figure 1.1).

Figure 1.1. Naturally occurring DNA conformations, A-DNA (a), B-DNA (b) and Z-DNA (c). Image courtesy of Richard Wheeler, Sir William Dunn School of Pathology, University of Oxford.
1.3.2 Histones

Intimately associated with eukaryotic DNA, the histone proteins were discovered over a century ago (Kossel, 1884), but their role in the structural organization of DNA was not elucidated until much later (Kornberg, 1974; Kornberg and Thomas, 1974). It is now known that histones not only allow compaction of DNA, but also play a crucial role in the regulation of gene transcription. Histones can be divided into two categories; core histones and linker histones.

1.3.2.1 Core histones

Highly conserved amongst eukaryotes, the core histones are small, basic proteins that are rich in lysine and arginine content. The basic structure of all core histones is a long hydrophobic alpha-helix bordered by a pair of short alpha-helices. In order for the core histones to fulfil their role in DNA organization, an octamer is assembled through recruitment of four different histone proteins; H2A, H2B, H3 and H4. A tetramer of two H3 and two H4 proteins forms a scaffold onto which two H2A-H2B dimmers associate, forming a core histone octamer (Jorcano and Ruiz-Carrillo, 1979) (figure 1.2).
When comparing the core histones present in animals to those in plants, there is much similarity, especially with histones H3 and H4 being amongst the most highly conserved proteins known. An example of this is the difference of only two amino acid residues between calf and pea histone H4 (DeLange et al., 1969). Four amino acid residues are different in the H3 protein of several plant species when compared to animal sequences and it is proposed that this may affect interactions within the histone
octamer (Smith et al., 1995). The H3.2 variant in alfalfa is analogous to H3.3 in animals and is produced in higher amounts in alfalfa than H3.3 is in animals (Waterborg, 1993). When compared to the main variant H3.1, H3.2 is produced twice as fast and has a shorter half life, therefore it has been suggested that there is more dissolution of plant nucleosomes during transcription than in animals (Smith et al., 1995).

There is greater difference between the H2 proteins in plants and animals, with three H2A and six H2B variants found in wheat alone (Spiker, 1976). In general, the plant H2 variants have higher molecular weights and differ in their N-terminal tails (Smith et al., 1995). One such N-terminal tail difference is the Ser-Pro-Lys-Lys (SPKK) motif present in plant H2A (Koning et al., 1991), suggesting a role for plant H2A in the stabilization of the nucleosome, as this motif is postulated to stabilize DNA as it enters/exits the nucleosome (Churchill and Travers, 1991) and is present in sea urchin linker histone H1 (Lindsey and Thompson, 1992).

### 1.3.2.2 Linker histones

Less conserved than the core histones, the linker histones encompass the H1 subtypes, and variants such as H5 found in avian erythrocytes (Aviles et al., 1978). The basic structure comprises of a globular domain of approximately 80 amino acids, with N- and C-terminal tails. The globular domain belongs to the ‘winged helix’ family of DNA-binding proteins and consists of three alpha-helices and three beta-strands as surmised from crystallographic studies of histone H5 (Ramakrishnan et al., 1993).

While the linker histones appear to have a role in DNA stabilization and higher order structure (Allan et al., 1986), they may not be absolutely necessary for DNA compaction and higher order structure under certain conditions (Schwarz and Hansen, 1994). Beyond their tentative role in higher order structure of DNA, linker histones are also known to function as regulators of gene expression (Shen and Gorovsky, 1996; Folco et al., 2003), perhaps through displacement of transcription factors (Zlatanova, 1990).

The globular region amino acid residues that are involved in DNA binding of H1 are all conserved between plant and animal protein sequences (Crane-Robinson and Ptitsyn, 1989), although overall, there appears to be greater variability in plant H1 proteins than animals (Ivanchenko et al., 1987), with larger molecular weights and more variation in the longer N- and C-terminal tails (Smith et al., 1995) (figure 1.3). Also of note, is the absence of a five-residue segment near the C-terminal end of the globular region in
plant H1 variants (Ivanchenko et al., 1987). This may indicate less specificity for positioning of H1 on the plant nucleosome and if so, some functions of the plant H1 protein may be assumed by H2A (Smith et al., 1995) as mentioned in the previous section.

Figure 1.3. A comparison of calf thymus histone H1 reversible modifications with Arabidopsis H1-1, maize H1 and pea H1. Sites of reversible modifications are indicated for calf; where these amino acids are conserved in plants, the signifying letter is in a filled ellipse. Note that G indicates the central globular region of the H1 protein and P indicates a phosphorylated amino acid. The complete sequence of calf thymus H1 is unavailable. Typically however, a mammalian H1 variant has four to five S(T)PXK motifs, usually distributed in the C-terminal domain (rat H1d has five sites and trout H1 has four sites). The positions of plant S(T)PXK motifs have been indicated. Adapted from Smith et al. (1995).
Chapter 1: Introduction and literature review

The role H1 plays in DNA organization and gene expression was analysed when *Arabidopsis* histone H1 was overexpressed in tobacco plants (2.3-2.8 times the levels present in the wild-type). Phenotypic changes ranged from mild to severe and there was an early occurrence of cells with abnormally heterochromatinized nuclei. Regardless, these cells were still able to perform basic physiological functions such as differentiation and this led to the suggestion that chromatin structural changes are dependent on H1 stoichiometry, but have limited effect on genes that control basal cellular functions (Prymakowska-Bosak *et al.*, 1996).

1.3.3 The nucleosome and histone H1 positioning

Packaging of DNA within eukaryotic cells is achieved at the primary level by coiling it around the core histone octamer. The resulting structure is known as a nucleosome (figure 1.4), and encompasses 147 bp of DNA wound 1.7 times around each histone octamer (Luger *et al.*, 1997). Multiple nucleosomes along the length of a DNA strand form a ‘beads on a string’ type configuration that can be observed *in vitro* (VanHolde and Zlatanova, 2007). The DNA is positioned along ‘ramps’ that are structural components of the histone octamer, forming both direct protein-DNA interactions and water-mediated interactions. The majority of histone-DNA interactions are water-mediated, which allows for non-sequence-specific binding of DNA along the phosphate backbone. Direct protein-DNA interactions occur between the α1 helices of two adjacent histones and at a site formed by the L1 and L2 loops (Davey *et al.*, 2002).
Figure 1.4. Model for the nucleosome. The N-terminal regions of the core histones lie free of the core particle and probably interact at higher orders of chromatin structure. The route of the DNA as it enters/leaves the structure is unknown. Histone H1 has been placed in its probable position, and the limit of the linker DNA that emerges from the chromatosome (168 bp) has been indicated by a dashed line, although in the chromatin fibre this DNA would have a defined structural position. Adapted from Smith et al. (1995).

The positioning of the H1 globular section on the nucleosome is still debated, with several models proposed (figure 1.5). The symmetric model assumed the globular domain was positioned centrally on the axis of symmetry of the core octamer, interacting with about 10 bp of DNA entering and exiting the nucleosome (Allan et al., 1980). More recently, an asymmetric model placed the globular domain of H5 between the central gyre of DNA and where the DNA exits the nucleosome i.e. off centre from the core octamer axis of symmetry (Zhou et al., 1998). Another model also places the H1 globular domain in an asymmetric position, binding to a single DNA gyre and between the DNA and the core octamer (Pruss et al., 1996).
1.3.4 Chromatin

Beyond coiling around the histone octamer, further compaction of eukaryotic DNA is achieved through the formation of chromatin. Folding of the ‘beads on a string’ type configuration of nucleosomes and linker DNA has long been assumed to result in the formation of a 30 nm fibre (Finch and Klug, 1976). *In vitro* observation of nucleosomal arrays using electron microscopy reveals a zigzag pattern of the nucleosomes and linker DNA at low ionic strengths. As the salt concentration is increased, further folding into flat ribbons is observed, before the appearance of irregular rod-like structures at ionic strengths of physiological relevance (100 mM NaCl) (Thoma *et al.*, 1979). These rod-like structures have a diameter of 30 nm and appear similar to the chromatin fibres observed in nuclei preparations (Wolffe, 1995). While many models have been proposed as to how the nucleosomes are organised into these 30 nm fibres, most fall into two categories; single-start helices and two-start helices. The single-start helix model involves wrapping a tightly packed chain of nucleosomes into a helix, with stability provided by internucleosomal interactions. In the two-start helix model, straight linker DNA sections connect between two helices of nucleosomes i.e. a zigzag of nucleosomes is organized into a helix, stacked atop a repeated structure and connected by linker DNA (VanHolde and Zlatanova, 2007). While the organization of the 30 nm fibre within mitotic chromosomes has been debated for some time, its existence has recently been refuted. In a study of human mitotic HeLa cells, it was concluded that the chromosomes consisted of irregularly folded nucleosome fibres, with no regular structure >11 nm (Nishino *et al.*, 2012). Based on early microscopic observations, and later due to functional differences, chromatin can divided into two major categories; euchromatin and heterochromatin.
Chapter 1: Introduction and literature review

1.3.4.1 Euchromatin

Cells undergoing mitosis, arrested during metaphase by application of colchicine and stained using dyes such as Giemsa show banding (Lavappa, 1978). Euchromatin generally appears as light coloured bands and represents a lightly packed form of chromatin. These DNA sections are often rich in gene content and are therefore sites of active transcription. This loose chromatin structure allows better access for gene regulatory proteins and RNA polymerase to bind the DNA and initiate transcription (Wolffe, 1995) (figure 1.6).

In Arabidopsis, the LHP1 protein has been associated with areas of euchromatic DNA, whereas in BY-2, LHP1 association with euchromatin is less clear and perhaps confounded by the differing genomic organization and large genome size (Libault et al., 2005). The LHP1 protein is the only known plant analogue of the heterochromatin protein 1 (HP1) family found in mammals, Drosophila and yeast (Gaudin et al., 2001). HP1 binding is normally associated with heterochromatin formation (Wreggett et al., 1994), and while it does bind to euchromatic sites (correlating with gene repression) (Hwang et al., 2001), it is interesting that in Arabidopsis LHP1 is mainly associated with actively transcribed regions (Libault et al., 2005).

1.3.4.2 Heterochromatin

Tightly packed, dark staining sections of the metaphase chromatid are known as heterochromatin. Containing many satellite DNA sequences (genetically inactive DNA repeats) and genes that are suppressed, the densely packed nature of heterochromatin makes DNA less accessible to transcriptional factors and RNA polymerase (Wolffe, 1995). Heterochromatin is often associated with hypoacetylation (Casas-Delucchi et al., 2012) and di- or tri-methylation of the K9 residue of the histone H3 tail (H3K9) (Rosenfeldt et al., 2009) (figure 1.6).

There are two subtypes of heterochromatin, constitutive and facultative. Constitutive heterochromatin is often found occurring around the centromeres and telomeres, where it performs a structural function and contains few genes that are often poorly expressed. Facultative heterochromatin differs by an absence of repetitive elements and has the ability, when cued to become less condensed, therefore allowing access to genes for transcription (Grewal and Jia, 2007). Genes within facultative heterochromatin are often silenced through mechanisms such as siRNA (Onodera et al., 2005) and histone methylation (Doerfler and Bohm, 2006).
Chapter 1: Introduction and literature review

Figure 1.6. Properties of euchromatic and heterochromatic regions. The important properties of heterochromatin can be specified, but there are exceptions in every instance. The characteristics listed in the figure are most consistently observed in pericentromeric heterochromatin. These regions are rich in remnants of transposable elements. It should be noted that little is known about either the stoichiometry of HP1 or the folding of the chromatin fibre in heterochromatin; the figure is meant to convey only the association of HP1 and the condensation of the chromatin fibre. Adapted from Grewal and Elgin (2007).

Chromatin in plants can be much more compacted than in animal cells, due to large amounts of repetitive DNA and polyploid genomes. The functional demands of chromatin in plants may therefore differ from animals, with a smaller proportion of DNA actively transcribed in plants. Also, with most animal cells being terminally differentiated, and most plant cells being totipotent, modifications to plant chromatin that influence transcription are likely more flexible and reversible (Smith et al., 1995). Some of the molecular markers of mammalian heterochromatin appear to be present in plants, with examples being the methylation of H3K9 (Jackson et al., 2002) and the tobacco SET protein (ntSET1) that facilitates this methylation (Yu et al., 2004b).
1.3.5 Core histone modifications and gene expression

Regulation of gene expression is in part regulated by chromatin alterations, of which, modifications to the core histones are among the best defined. A summary of histone modifications in *Arabidopsis* and their effect on chromatin structure and transcription is presented in table 1.1.
**Histone modifications in Arabidopsis**

<table>
<thead>
<tr>
<th>Modification</th>
<th>Location</th>
<th>Effect</th>
<th>Transcription</th>
<th>Established by</th>
<th>Removed by</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K4me*</td>
<td>Euchromatin</td>
<td>Open</td>
<td>Activation</td>
<td>trxG class of histone methyltransferases (HMTs)</td>
<td>LSD1-type of histone demethylases (HDMs) FLD</td>
</tr>
<tr>
<td>H3K4me1</td>
<td>Euchromatin</td>
<td>Open</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K4me2</td>
<td>Euchromatin</td>
<td>Open</td>
<td>Activation intron(^1)</td>
<td>ATX1</td>
<td></td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Euchromatin</td>
<td>Open</td>
<td>Activation proximal promoter; S' end gene</td>
<td>ATX1 EFS/SDG8</td>
<td></td>
</tr>
<tr>
<td>H3K9me*</td>
<td>Both</td>
<td>Closed</td>
<td></td>
<td>Su(var) class of HMTs</td>
<td>JmjC-domain and LSD1-type HDMs</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>Heterochromatin</td>
<td>Closed</td>
<td></td>
<td>SUVH2</td>
<td>SUVH5</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>Heterochromatin</td>
<td>Closed</td>
<td></td>
<td>SUVH2</td>
<td>SUVH4</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Euchromatin</td>
<td>Closed</td>
<td>Repression proximal promoter, S' end gene, gene</td>
<td>CLF, SWN, MEA</td>
<td></td>
</tr>
<tr>
<td>H3K27me*</td>
<td>Both</td>
<td>Closed</td>
<td>Repression</td>
<td>E(Z) class of HMTs (PRC2 complex)</td>
<td></td>
</tr>
<tr>
<td>H3K27me1</td>
<td>Heterochromatin</td>
<td>Closed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K27me2</td>
<td>Heterochromatin</td>
<td>Closed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Euchromatin</td>
<td>Closed</td>
<td>Repression promoter, S' end gene, gene</td>
<td>CLF, SWN, MEA</td>
<td></td>
</tr>
<tr>
<td>H3K36me*</td>
<td>Euchromatin</td>
<td>Open</td>
<td>Activation</td>
<td>Set domain HMTs</td>
<td>JmjC-domain HDMs Possibly REF6</td>
</tr>
<tr>
<td>H3K36me1</td>
<td>Euchromatin</td>
<td>Open</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K36me2</td>
<td>Both</td>
<td>Open</td>
<td>Activation intron(^1)</td>
<td>EFS/SDG8</td>
<td></td>
</tr>
<tr>
<td>H3K36me3</td>
<td>Euchromatin</td>
<td>Open</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4K20me*</td>
<td>Closed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4K20me1</td>
<td>Heterochromatin</td>
<td></td>
<td></td>
<td>SUVH2</td>
<td></td>
</tr>
<tr>
<td>H4K20me2</td>
<td>Euchromatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4K20me3</td>
<td>Euchromatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4R3sme2</td>
<td>Euchromatin</td>
<td>Closed</td>
<td>Repression promoter</td>
<td>Arginine methyltransferases SKB1/AIPRMT5</td>
<td>Deimination</td>
</tr>
<tr>
<td><strong>Acetylation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3Kac/H4K**</td>
<td>Both</td>
<td>Open</td>
<td>Activation promoter, S' end gene, 5' end gene</td>
<td>Histone acetyltransferases (HATs) GNAT family: GCN5/HAG1 CBP/p300 family: HAC1, HAC5, HAC12 TAFII family: HAF2/TAF1</td>
<td>Histone deacetylases (HDACs) RPD3 family: HDA19, HDA6 HDA1 family: HDA18 HDA2 family: HDA2A, HDA2B, HDA2C</td>
</tr>
<tr>
<td><strong>Ubiquitination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2BK143ub1</td>
<td>Euchromatin</td>
<td>Open</td>
<td>Activation</td>
<td>Ring-type E3 ligases HUB1, HUB2</td>
<td>Deubiquitinases SUP32/UBP26</td>
</tr>
<tr>
<td><strong>Phosphorylation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3S10ph</td>
<td>Both</td>
<td>Open</td>
<td>Activation</td>
<td>Kinases</td>
<td>Phosphatases</td>
</tr>
<tr>
<td>H3S28ph</td>
<td>Both</td>
<td>Open</td>
<td>Activation</td>
<td>Kinases</td>
<td>Phosphatases</td>
</tr>
<tr>
<td>H2T11ph</td>
<td>Both</td>
<td>Open</td>
<td>Activation</td>
<td>Kinases</td>
<td>Phosphatases</td>
</tr>
</tbody>
</table>

Table 1.1. Histone modifications, their locations in the genome and their effects on chromatin (open/closed) as well as on transcription (where known) are listed. Also listed are the enzymes known to add and remove each modification. Plant histone modifiers are listed in bold type. *Denotes all modifications of a certain type, for example, H3K9me* denotes mono, di, and trimethylation of lysine nine of histone H3, while H3*ac denotes general acetylation of lysines in histone H3. \(^1\)These introns contain cis regulatory elements important for transcriptional regulation. \(^2\)The role of histone phosphorylation in the cell cycle is described elsewhere. Adapted from Pfluger and Wagner (2007).
1.3.5.1 Histone methylation

By far the most common form of histone modification so far described; addition of methyl groups to specific amino acids within core histone proteins is usually associated with transcriptional suppression, although in some cases activation of transcription has been noted (Chandler and Vaucheret, 2001). This process was thought to be irreversible, but demethylation enzymes have since been discovered (Shi et al., 2004; Tsukada et al., 2006). Along with the previously mentioned methylation of H3K9, other histone tail residues have been identified, that once methylated either activate or repress transcription, including H3K4, H3K27, H3K36, H4K20 and H4R3 (Pfluger and Wagner, 2007). An example of histone methylation affecting gene expression is the binding of LHP1 to the H3K27me3 residue, causing a repression of gene expression in Arabidopsis euchromatin (Libault et al., 2005; Nakahigashi et al., 2005).

1.3.5.2 Histone acetylation

Further modification of histones can occur through acetylation or deacetylation of lysine residues on the N-terminal end of the histone proteins. The enzyme families of histone acetyltransferases (HATs) and histone deacetylases (HDACs) are responsible for these modifications, forming the basis of gene expression regulatory processes. Acetylation is thought to cause a decrease in contact between the histone proteins and DNA, resulting in a relaxed chromatin structure that is associated with higher levels of gene expression (Kimura et al., 2005). In eukaryotes, it is known that H4K5, H4K8 H4K12 and H4K16 undergo acetylation to activate transcription, but in alfalfa, acetylation also occurs at a fifth site, H4K20 (Waterborg, 1992). It is thought that this site is methylated in other eukaryotes and therefore not available for acetylation (Waterborg, 1993).

1.3.5.3 Histone ubiquitination

The ubiquitin polypeptide has been shown to be covalently bound to H2A (Goldknoff et al., 1975) at K119 (Nickel and Davie, 1989) and H2B (West and Bonner, 1980) at K120 in humans or K123 in yeast (Thorne et al., 1987). Polyubiquitination is possible on H2A, but only monoubiquitinated H2B has been reported. Ubiquitination of H3 and H1 have also been described, with the site of attachment not yet known (Zhang, 2012). Histone H2B monoubiquitination has been reported in Arabidopsis, where it thought to be involved in flowering time regulation through the actions of the E3 ligases HUB1 and HUB2 on the chromatin of FLOWERING LOCUS C (a known
1.3.5.4 Histone phosphorylation

This type of post-translational modification involves attachment of a phosphate group to serine and threonine residues on the N-terminal histone tails. Sites known to undergo phosphorylation are present on all four core histones (Banerjee and Chakravarti, 2011) and the linker histone H1 (Zheng et al., 2010). While many studies have investigated phosphorylation of mammalian histones, less is known about the role of histone phosphorylation in plant chromatin maintenance and gene expression. Sites of phosphorylation in Arabidopsis include H3S10, H3S28 and H2T11 (Pfluger and Wagner, 2007), with the two known H3 sites also undergoing phosphorylation in BY-2 cells (Kurihara et al., 2006). It is thought that the Aurora kinase enzyme is responsible for phosphorylation of these sites in BY-2 cells, and inhibition of this enzyme resulted in an increase in the ratio of metaphase cells to anaphase/telophase cells. This suggested to the authors that H3S10ph and H3S28ph may have a role in chromosome segregation and metaphase to anaphase transition (Kurihara et al., 2006).

1.3.5.5 Other histone modifications

Various other post-transcriptional modifications of histones have also been revealed and play vital roles in gene expression and regulation. Lysine sumoylation (Shiio and Elsenman, 2003), arginine citrullination (Tanikawa et al., 2012) and ADP-ribosylation of glutamic acid (Ogata et al., 1980) have been identified as switches for the control of chromatin structure and function (Strahl and Allis, 2000).

1.3.6 DNA methylation and transcription

Gene expression patterns are also controlled by DNA methylation; the selective addition of a methyl group to the cytosine pyrimidine ring at position 5, or the adenine purine ring at position 6. DNA methylation is important during development and cell differentiation, establishing stable gene expression patterns that differ between tissue types (Song et al., 2005). Other functions include the suppression of detrimental elements incorporated into the genome (i.e. viral genes) (Okano et al., 1998), alteration of the structure of chromatin (Lorincz et al., 2004) and a role in the development of most cancers (Davis and Uthus, 2004).

While methylation of mammalian DNA mainly occurs on a cytosine that is followed by a guanine (CG), plant DNA can be methylated on cytosine in the CHG and CHH
contexts (where H is A, C or T), as well as on adenine at GATC sites (Finnegan et al., 1998). Adenine methylation in plants is much less common than cytosine methylation and may represent a separate or interdependent system of genome modification (Vanyushin, 2006). When comparing the genome wide level of methylation, approximately 82% of plant CG dinucleotides are thought to be methylated (Gruenbaum et al., 1981), whereas mammalian cells have a lower rate of approximately 70% CG methylation (Robertson and Wolffe, 2000). This difference in the incidence of cytosine methylation may be explained by the large amount of so called ‘junk’ DNA in plants. Harmful elements such as retrotransposons and pseudogenes can be suppressed via methylation (Fukuda et al., 2004) and plant polyploidy may account for the greater propensity toward DNA methylation in plants (Rabinowicz et al., 2010).

The mechanisms employed in methylation of plant DNA involve three classes of DNA methyltransferases; methyltransferase 1 (MET1) which mediates CG methylation, domains rearranged methyltransferase 1 (DRM2) which mediates CHH methylation and chromomethylase 3 (CMT3) which mediates CHG methylation (Meyer, 2011) (figure 1.7). While MET1 and DRM2 have mammalian homologues, CMT3 is unique to plants (Lindroth et al., 2001). All three methyltransferases are involved in maintenance of methylation during DNA replication (epigenetic maintenance), but only DRM2 has been implicated in de novo DNA methylation (Henderson et al., 2010).

RNA-directed DNA methylation (RdDM) (figure 1.7) has been suggested as the mechanism that enables de novo methylation in plants and was first described in tobacco viroid suppression (Wassenegger et al., 1994). RdDM utilises small non-coding RNAs (sRNAs), which are classified into hairpin folded microRNAs (miRNAs) and small interfering RNAs (siRNAs) derived from double-stranded RNA (Chellappan et al., 2010). Methylation occurs to most cytosine residues within the region of sequence identity between the triggering sRNA and the target DNA and this leads to suppression of transcription at the target site (Aufsatz et al., 2002).
Figure 1.7. Schematic illustration of changes in chromatin structure via DNA methylation and small RNA-directed DNA methylation (RdDM). Repression of gene transcription is associated with methylation of DNA by maintenance and de novo DNA methyltransferases. CG methylation ($^{m}$CG) is maintained by MET1 but it is also controlled by VIM1 and the chromatin remodeler DDM1. CHG methylation ($^{m}$CHG) is maintained by CMT3 and the HMT KYP, and at some loci is redundantly controlled by CMT3 and DRM2. CHH methylation ($^{m}$CHH) is maintained by the RdDM pathway, and de novo methylation of DNA in all of these sequence contexts is generally established by DRM2. Upon DNA demethylation by the glycosylase/lyase proteins ROS1, DME, DML2, and DML3, chromatin is modified and gene transcription may be activated. Transposable elements are kept silent by the RdDM pathway involving the generation of transcripts by PolIV that are converted into dsRNAs by RDR2 and cleaved by DCL3 into 24 nucleotide long siRNAs. Antisense siRNAs are loaded onto AGO4, which guide PolIV to homologous DNA, which is then methylated in all cytosine sequence contexts by de novo DNA methyltransferases (DRM2). Adapted from Miguel and Marum (2011).
1.4 Programmed cell death

1.4.1 Categories of cells that undergo programmed cell death

Cells that are committed to undergo PCD do so for a number of reasons, which can be divided into eight different functional categories (Krisnamurthy et al., 2000).

(1) Cells that have served their function

Both plant and animal examples are present in this category and include the death of tadpole tail cells during development (Little and Flores, 1996), root cap cells and cells involved in abscission (Biswal and Biswal, 1999). The identity, function and morphology of cells in this category are lost, but while animal cells are removed, plant cell remnants or corpses remain.

(2) Cells that are unwanted from the beginning

Certain cell types are specific to gender and are removed early on in development. Removal of non-functional megaspore and microspore cells created during plant development (Bell, 1996) and primordial stamen/carpal cells are an example of this category (Gray, 2004). In animals, the Mullerian duct cells undergo PCD in males, but are required in females (Price et al., 1977).

(3) Cells that die during redifferentiation into specialised cell types

Xylem tracheary elements, trichomes, thorns/spines, cork cells and sclerenchyma all start their functional role after death and undergo significant morphological and chemical changes in their cell walls as a result (Beers, 1997). Vertebrate keratinocytes extrude their nuclei as they reach the skin surface and by doing so undergo PCD and attain the function of the outer skin barrier (Allombert-Blaise et al., 2003).

(4) Cells that are subjected to the hypersensitive response (HR)

In plants this is a very important mode of cell death, often associated with pathogenesis and the avoidance of infection by formation of HR lesions. Host cells surrounding a site of attempted infection from a pathological organism may undergo PCD to limit further spread of the invading pathogen (Morel and Dangl, 1997). HR lesions due to infection do not occur in animal cells, but a HR can also result from environmental and abiotic stresses in both plants and animals. Stresses can arise from osmotic changes, oxidative state changes, chemicals/toxins, UV radiation, heavy metals...
(5) Cells that are damaged and unable to function further

Removal of damaged cells is important for the prevention and spread of cancerous cells in animals (Jacobsen et al., 1997). Plant cell death in this category seems to occur, but it is difficult to distinguish from cell death caused by the HR and may or may not be programmed (Krisnamurthy et al., 2000).

(6) Cells that are present in the wrong places

During the development of both plants and animals, PCD occurs in the formation of physical structures such as removal of the cells present between developing digits in mammals (McCall, 2005) and between leaf lobes in some plants such as in the genus Monstera (Greenberg, 1996).

(7) Cells that are produced in excess

There are no examples of plant cells being produced in excess and subsequently undergoing PCD, but the death of vertebrate neurons over a life span is one such example in the animal kingdom (Deshmukh and Johnson, 1997).

(8) Cells that through their death give rise to disease

Diseases of animals that stem from PCD include AIDS (death of helper T cells of the immune system), Parkinson’s, Huntington’s, Alzheimer’s and Lou Gehrig’s diseases (death of certain neurons in the brain). Again, there have been no reported examples of plant cells that fall into this category (Krisnamurthy et al., 2000).

1.4.2 Programmed cell death in plants

With so much information pertaining to apoptosis in mammalian cells, there is surprisingly scant knowledge on the analogous mechanism in plant cells. There are several morphological and biochemical similarities between apoptosis in mammalian cells and PCD in plant cells. The most obvious common features are morphological; cytoplasm and nuclear condensation/shrinkage, formation of DNA-containing apoptotic-like bodies and DNA fragmentation (DellaMea et al., 2007). Biochemical similarities include the regulation of PCD by caspase-like proteolytic enzymes (meta- and para-caspases) (Bonneau et al., 2007), release of cytochrome c from the mitochondrion (Eckardt, 2006), evidence of Bcl-2-like proteins involved in cell death.
(Chen and Dickman, 2004), an increase in cytosolic calcium and ROS triggered cell death (Tiwari et al., 2002). Although these similarities to mammalian apoptosis have been noted, the majority of PCD occurring in plants is likely to involve autophagy and subsequent autophagic cell death (ACD).

1.5 Autophagy

Protein and organelle turnover within the cell is achieved through the processes of autophagy and subsequent proteosome-mediated degradation. These processes allow the cell to remove and recycle unwanted or superfluous organelles and play an essential role during cell death (Guillon-Munos et al., 2006), starvation (Moriyasu and Ohsumi, 1996), differentiation (Beers, 1997), aging (Cuervo, 2003) and prevention of some cancers (Levine, 2007). The target organelle for autophagic pathways is the lysosome in mammalian cells or the vacuole in plants cells, where a large range of hydrolases achieve the breakdown of cellular components. Before targeting to the lysosome, cytoplasmic constituents (including organelles) are enclosed by a membrane to form autophagosomes. There are several theories surrounding the origins of the autophagosome membrane including synthesis by the endoplasmic reticulum and/or Golgi, and de novo nucleation (Yorimitsu and Klionsky, 2005). Much research has been done elucidating the mechanisms involved in yeast autophagy, with several distinct pathways proposed for the processing of cargo material (figure 1.8). Autophagy is conserved among yeast, plant and animal cells, and there is overlap in macroautophagy machinery with that used in the cytoplasm-to-vacuole targeting (Cvt) pathway and peroxisome degradation (pexophagy) (Reggiori and Klionsky, 2002).
Chapter 1: Introduction and literature review

Figure 1.8. Models for macroautophagy, pexophagy and the Cvt pathway in the yeast *Saccharomyces cerevisiae*. The basic mechanism in macroautophagy, macropexophagy and the Cvt pathway is the sequestration of cargo material (bulk cytoplasm, peroxisomes or prApe1 and Ams1 respectively) by a cytosolic double-membrane vesicle. Upon completion, the sequestering vesicle docks with the vacuole and then fuses with it. In this way, the inner vesicle is liberated inside the vacuole, where it is finally consumed by hydrolases. In micropexophagy, sequestration occurs directly at the vacuole surface, again resulting in the release of a vesicle within the vacuolar lumen. The small circles representing prApe1 or Ams1 are monomeric forms and the medium circles indicate the oligomeric forms of these hydrolases. Adapted from Reggiori and Klionsky (2002).

Microautophagy is not well understood and involves the direct uptake of cytoplasm from around the vacuole/lysosome. Macroautophagy operates by membrane engulfment of targeted organelles at sites distant from the vacuole/lysosome and is most often initiated during starvation of the cell. In contrast, the Cvt pathway is biosynthetic, targeting specific components, and operates under nutrient rich conditions. Peroxisomes are organelles containing oxidative enzymes such as catalase, ureate oxidase and amino acid oxidases. Once assigned for removal, peroxisomes are enwrapped in the cytosol with a membrane of unknown origin and targeted to the vacuole/lysosome, in a process known as macropexophagy (Klionsky, 2005).
The process of macroautophagy can be broken down into four major steps: (1) induction, (2) autophagosome formation, (3) docking and fusion of the autophagosome with the vacuole/lysosome and (4) autophagic body breakdown (Reggiori and Klionsky, 2002). Induction of autophagy is thought to be achieved through Tor regulation of the autophagy-related \((APG, \text{recently unified and renamed } ATG)\) genes (Kamada \textit{et al.}, 2000; Kamada \textit{et al.}, 2010). Most of the \(ATG\) genes are constitutively expressed, due to their overlapping role in the Cvt pathway (Klionsky and Emr, 2000), but it appears that Tor regulates the transition from the Cvt pathway to autophagy during times of nutritional stress (Kamada \textit{et al.}, 2000). The downstream effectors of yeast Tor are thought to be the Apg13 protein, a complex that includes the serine threonine kinase Apg1. Apg1 is also essential for induction of autophagy in higher eukaryotes (Klionsky, 2003).

Sequestration of cytoplasmic components is achieved through formation of a double membrane vesicle known as the autophagosome. In mammalian cells, this membrane is thought to originate from the endoplasmic reticulum, but in other model systems, its origin is not known. In yeast, a protein conjugation system involving Apg12 and Apg5 is required for autophagosome formation, with the process requiring the enzymatic actions of Apg7, Apg10 and Apg16 (Deretic, 2008).

Once the autophagosome is complete, targeting and fusion to the lysosome in mammals depends on the regulation of the acidic environment and microtubule action. In yeast, the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) machinery is required for autophagosome docking and fusion (Deretic, 2008).

After fusion of the autophagosome, the outer membrane is incorporated into the vacuolar membrane and the single membrane bound vesicle (autophagic body) enters the lysosome/vacuole. Lipases and various hydrolases then begin to breakdown the autophagic body, with efficient degradation achieved through luminal acidification and presence of proteinase B and the candidate lipase Cvt17 (Deretic, 2008).

1.6 Autophagy and apoptosis

The connection between autophagy and apoptosis remains unclear, but recent observations suggest that the two processes are not mutually exclusive (Thorburn, 2008). ACD (type II cell death) has been proposed as an alternative mechanism of PCD (Clarke, 1990), but its existence as an executor of cell death is still an issue for debate (Kroemer and Levine, 2008; Shen and Codogno, 2011). Evidence such as the presence
of autophagic vesicles in dying cells, delay of death by 3-methyladenine (an inhibitor of autophagy), the absence of caspase activation and delayed DNA fragmentation suggest that autophagy is a type of cell death distinct from apoptosis (type I cell death) (Lockshin and Zakeri, 2004b). But it may also be true that autophagy is activated as a defence mechanism, a ‘last ditch’ effort of the cell to avoid death via apoptosis, or perhaps just a consequence of apoptosis (Thorburn, 2008). The outcome may also depend on environmental conditions and the type of PCD stimulus being applied to the cell. It is therefore likely that type I and type II cell death are related and there is some overlap or ‘crosstalk’ between the two mechanisms (Thorburn, 2008).

A recent publication has elucidated a new pathway to autophagy, showing that an inhibitor of caspase-8 mediated apoptosis, benzylxycarbonylvalyl-alanyl-aspartic acid (O-methyl)-fluoro-methylketone (zVAD) stimulates autophagy in mammalian cells. The genes \textit{ATG7} and \textit{beclin-1}, along with jun amino-terminal kinase were required for the zVAD induced ACD (Yu \textit{et al.}, 2004a). This raises questions about the efficacy of clinical therapies involving caspase inhibition, as they may have the effect of promoting autophagy.

Death-associated protein kinase (DAPk) has been identified as positive mediator of cell death, responding to stimuli such as the activation of the FasR, interferon-\(\gamma\), TNF-\(\alpha\) and detachment from the extracellular matrix. While DAPk seems to be involved in apoptosis, along with a related protein, DAPk-related protein-1 (DRP-1), it is also required for autophagic activity. Depending on the cellular environment, DAPk appears capable of inducing both apoptosis and autophagy (Gozuacik and Kimchi, 2006).

The function of the Bcl-2 family of proteins is well characterised with respect to their role in apoptosis, but several recent publications have provided evidence to support their interaction in autophagy (Shimizu \textit{et al.}, 2004). Transformed human leukemic HL60 cells conditionally expressing the full length Bcl-2 antisense message, showed massive cell death upon induction. The cells died by autophagy and not apoptosis, exhibiting intact mitochondria, lack of AIF translocation and caspase inhibitors did not rescue the cells (Saeki \textit{et al.}, 2000). Beclin 1 is a mammalian ortholog of the yeast autophagy gene \textit{APG6} and its encoded protein was discovered through a yeast two-hybrid screen to interact with Bcl-2 (Liang \textit{et al.}, 1999). The implications of this interaction are still not fully understood, but provide further evidence toward the hypothesis that apoptosis and autophagy are competing and interlinked PCD mechanisms (figure 1.9). (Tsukada and Ohsumi, 1993)
Figure 1.9. An overview of possible cell death scenarios. A cell death signal may induce autophagy, which is required for apoptotic cell death (a), or alternatively, autophagy may antagonise apoptosis (b). Apoptosis and autophagy may also occur independently and competitively, with one process inhibiting the other (and vice versa) (c). Adapted from Gozuacik and Kimchi (2004).

1.7 Autophagy in plants

The genes required for autophagy are highly conserved within the eukaryotes and many of the early molecular experiments were in performed in yeast. With the discovery of the \textit{ATG} genes (Tsukada and Ohsumi, 1993), yeast became a popular model system to study autophagy. To date, twenty-five homologues to the yeast \textit{ATG} genes have been discovered in \textit{Arabidopsis} (Inoue \textit{et al.}, 2006). One of these homologues, \textit{AtAPG9} (homologous to \textit{APG9} in yeast), was altered by a T-DNA insertion, providing an effective \textit{AtAPG9} knockout. The phenotype of this mutant under
Chapter 1: Introduction and literature review

nitrogen and carbon-starvation conditions was an early onset of leaf chlorosis as compared to wild type plants. Acceleration of senescence was also observed in these mutants even under nutrient rich conditions (Hanaoka et al., 2002).

While widespread DNA degradation is observed in apoptosis, it is not a feature of ACD until late in the process (Gozuacik and Kimchi, 2004). PCD in plant cells has been observed that presents a slow degradation of DNA which seems to be associated with changes in DNA conformation (O'Brien et al., 1998b). This observation may be consistent with ACD and warrants further investigation.

1.8 The cell cycle in plants

In order for a plant to grow, differentiate and repair damaged tissue, cell growth and division must occur. The mitotic cell cycle in eukaryotes is the process in which cells replicate their DNA, divide, grow and prepare to divide again. It consists of four distinct phases; the gap 1 phase (G\textsubscript{1} phase), the synthesis phase (S phase), the gap 2 phase (G\textsubscript{2} phase) and the mitotic phase (M phase). M phase is subdivided into five phases; prophase, prometaphase, metaphase, anaphase and telophase, before cytokinesis physically splits the resultant daughter cells. Cells can also enter a quiescent state called the G\textsubscript{0} phase when they temporarily stop dividing (Inze, 2007). The cell cycle is highly regulated and controlled by three major checkpoints; the decision to proceed from G\textsubscript{1} to S phase (G\textsubscript{1} or restriction checkpoint), from G\textsubscript{2} to M phase (G\textsubscript{2} checkpoint) and from metaphase to telophase (metaphase checkpoint) (Francis, 2001). Interestingly, plants can also enter an alternative cell cycle called the endoreduplication cycle (endocycle), where cells repeat DNA replication without mitosis, thereby increasing the ploidy of the cell and is important in development of various organs (Sugimoto-Shirasu and Roberst, 2003). At the basic level, the cell cycle mechanisms that allow cell proliferation are similar in plants and animals, but there are features that set plants apart.

Like other eukaryotes, the plant cell cycle is driven by activation of a variety of cyclin-dependent kinase (CDK) proteins through binding of various cyclins (CYCs) and thereby triggering progression through the G\textsubscript{1} and G\textsubscript{2} checkpoints (Francis, 2001). Plant cells employ many more CDKs and CYCs than animals and fungi, with thirty one CYCs currently described and several classes of CDKs (Inze and DeVeylder, 2006; Inagaki and Umeda, 2011), of which CDKB is unique to the plant kingdom (Hirt et al., 1991; Magyar et al., 1997). Upon activation, the CYCD-CDKA complex is responsible for phosphorylating the retinoblastoma-related (RBR) protein, which releases the associated E2F-DP complex, thereby allowing transcription of target genes required for
transition through the G₁ checkpoint (Komaki and Sugimoto, 2012). After DNA replication (S phase), there is an increase in expression of CYCA, CYCB and CYCD, with these cyclins binding to either CDKA or CDKB. The various CYC-CDK complexes are then thought to be activated upon release of phosphate groups by the CDC25-like phosphatase protein (Komaki and Sugimoto, 2012). The target of these activated CYC-CDK complexes are the three MYB repeat transcription factors in promoter regions of M phase specific genes (Pereira et al., 2012) (figure 1.10).

Figure 1.10. Plant cell cycle overview. Schematic representation of the events that characterize the G₁/S-phase and the G₂/M-phase transitions are visualized, along with illustrations of the mechanisms that take place during genome duplication and cytokinesis. The hormonal control of the cell cycle is also represented in this scheme. Adapted from Pereira et al. (2012).

1.8.1 Autophagic cell death and the cell cycle

Cell cycle arrest induced by various means can result in ACD. In one such example, ablation of the Akt2 protein using siRNA, caused cell cycle arrest in MDA-MB231 breast cancer cells and subsequent ACD (Santi and Lee, 2011). Akt2 is a member of the Akt family of serine/threonine kinases and has a role in overcoming cell cycle arrest in
both the $G_1$ (Ramaswamy et al., 1999) and $G_2$ phases (Kandel et al., 2002). In a temperature sensitive yeast mutant, cell cycle arrest was induced by growing at restrictive temperatures, with microscopy revealing autophagic body-like structures and the suggestion that the cells underwent ACD (Motizuki et al., 1995). PCD caused by various drugs such as those used for chemotherapy, can also be cell cycle specific. A flow cytometric study on a number of human tumour cell lines exposed to the mitotic poison paclitaxel has indicated that the DNA content of individual cells decreased progressively to a point where the content was less than 5% over a period of approximately three days (Parmar et al., 2001). Because cells were cycling, with some cells increasing their DNA content with time and others decreasing it through cell death, the development of a mathematical model was necessary to interpret the flow cytometry results (Basse et al., 2004b; Basse et al., 2004a). The slow rate of DNA degradation with time, as well as the degree of vacuolation of individual cells, is also consistent with the process of autophagy and ACD.

### 1.9 Nutrient withdrawal and autophagy

The autophagic condition is most often initiated and investigated by withdrawal of nutrients, such as carbon, by way of glucose starvation in mammalian cells (Hoyer-Hansen and Jaattela, 2007) and sucrose starvation in plant cells (Moriyasu and Ohsumi, 1996). Limiting the amount of available sugar in mammalian cells results in reduced protein glycosylation and has the downstream effect of endoplasmic reticulum (ER) stress. ER stress triggers the release of $\text{Ca}^{2+}$, the unfolded protein response (UPR) and ER-associated degradation (ERAD), which in turn, trigger a reduction of protein synthesis, regulation of the UPR genes and PCD and/or autophagy (Hoyer-Hansen and Jaattela, 2007).

Carbohydrate starvation in sycamore cells (*Acer pseudoplatanus*) leads to a degradation of membrane polar lipids and formation of double membrane-bound vacuoles that are expelled into the vacuole (Aubert et al., 1996). The regulation process of carbohydrate-induced autophagy in plants remains little understood. Protein breakdown during autophagy may be triggered by sugar levels, as many plant genes are regulated by sugars via catabolite suppression (James et al., 1993).

Nitrogen limitation has been shown to induce autophagy in yeast (Kohda et al., 2007) and *Arabidopsis* cells (Bassham et al., 2006). The mechanism in *Arabidopsis* that induces autophagy during periods of nitrogen limitation is not well described, but seems to involve the nine *AtATG8* genes and two *AtATG4* genes (homologues of yeast
genes ATG8 and ATG4 respectively). As a consequence of nitrogen limitation and autophagy, the *Arabidopsis* root system was promoted, perhaps as a physiological response to seek more nitrogen in the soil (Yoshimoto *et al.*, 2004).

1.9.1 BY-2 cell nutritional requirements

1.9.1.1 Sucrose starvation in BY-2 cells

Some of the physiological and many of the morphological changes that occur during sucrose starvation in BY-2 cells have been previously reported (Moriyasu and Ohsumi, 1996; Bolduc and Brisson, 2002; Yano *et al.*, 2004). The formation of autolysosomes (as assessed by their accumulation in the presence of cysteine protease inhibitors) (Moriyasu and Ohsumi, 1996), degradation of membrane phospholipids (Inoue and Moriyasu, 2006a) and the involvement of the NtBI-1 protein (tobacco homolog of the Bax inhibitor-1 protein, a PCD suppressor) (Bolduc and Brisson, 2002) have provided useful insights into the mechanisms pertaining to sucrose starvation, autophagy and ACD in tobacco cells.

1.9.1.2 Nitrate starvation in BY-2 cells

Currently, there has been no research focussed on the role nitrate starvation plays in autophagy and ACD using the BY-2 cell line as a model system. Since nitrogen limitation is a known trigger of autophagy in fission yeast (Kohda *et al.*, 2007) and *Arabidopsis* (Contento *et al.*, 2005), it is likely that exclusion of nitrates from the growth medium can also activate autophagy and possibly ACD in BY-2 cells. Nitrate starvation will be investigated in subsequent chapters, with its effect on cell morphology, physiology and chromatin remodelling examined.

1.10 Background, scope and aims of this thesis

The background of this thesis was the observation that PI staining of nuclei from cultured tobacco cells varied widely according to the length of time in culture (O’Brien *et al.*, 1998a; O’Brien *et al.*, 1998b). At that time these observations of PI staining changes were interpreted to indicate the induction of plant cell apoptosis. Since then, there has been considerable debate as to whether the pathways leading to plant cell death involve apoptotic (Cronje *et al.*, 2004), autophagic (Bassham, 2007) or other mechanisms (VanDoorn *et al.*, 2011). There is certainly evidence to support the occurrence of different processes (Jan *et al.*, 2008) and perhaps like mammalian cells,
the categories of plant PCD are not mutually exclusive (Thorburn, 2008), requiring investigation on a case by case basis.

In the case of *N. tabacum* (BY-2) cells, preliminary experiments indicated that the phenomenon of changing nuclear PI fluorescence was similar to that seen in *N. plumbaginifolia* cells (O'Brien et al., 1998a; O'Brien et al., 1998b) and it was decided to investigate these changing states of chromatin conformation further. As many of the genes involved in mammalian apoptosis do not have orthologues present in plant genomes and many PCD events in plants do not bear similarity to mammalian apoptosis, the idea that plants undergo apoptosis was not well supported in the literature. It seemed that focus had shifted away from proving the existence of apoptosis in plants and evidence had emerged that supported a role of autophagy in plant PCD. Since autophagy is a highly conserved process and most of the contributing genes are likely present in all eukaryotes (*ATG* genes have been identified in yeast, plant and animal genomes), it was theorised that BY-2 cells may undergo PCD through the actions of autophagy.

In a batch culture, cell proliferation continues until one or more environmental parameters limit the ability of the cells to divide and they reach a stationary phase. Nutritional availability is one such determining parameter and depletion of macronutrients is known to trigger autophagy in plant cells (Moriyasu and Ohsumi, 1996; Toyooka et al., 2006). This raised speculation as to whether the condensation of BY-2 chromatin following the cultures proliferative phase was the result of depleted nutrient availability, leading to autophagy and starvation-induced ACD.

To investigate this theory, experiments were undertaken to define the sequence of chromatin changes in the standard growth medium, in order to provide a comparative platform for treatments of nutrient limitation. It was postulated that by depriving the BY-2 culture of either carbon (sucrose) or nitrogen (nitrates), the time frame of chromatin changes would shift, as autophagy would be induced at an earlier stage. Accordingly, various media were formulated to initiate nutrient starvation, and to allow direct comparison of results, many of the experiments were performed simultaneously on all of the medium treatments. These parallel experiments included investigations of cell proliferation, morphology, physiology and alterations in medium components, with a particular focus on detecting and describing the emergence of autophagic processes. Later experiments then concentrated on determining the underlying mechanism for the switch in chromatin conformation, involving investigation of BY-2 DNA methylation status and histone composition.
Chapter 1: Introduction and literature review

With the BY-2 cells grown in up to six different medium formulations for each experiment, data presentation became an issue. Two options for the thesis structure were contemplated; either to present the results of carbon and nitrogen starvation in a single large chapter with an additional chapter to describe other aspects of cell morphology and physiology, or to split the results of the carbon and nitrogen starvation experiment into two chapters, each with associated data on cell morphology and physiology. The latter option was adopted because it provided greater clarity and a better logical progression to the experiments on chromatin structure.

The overall objectives of this thesis were as follows:

1. To develop robust methods of measuring and interpreting changes in nuclear PI staining, providing a platform for further investigations into potential chromatin alterations during BY-2 cell growth and nutrient deprivation.

2. To measure the effect of carbon starvation on nuclear PI staining of plant cells, comparing these effects with those on plant cell morphology and physiology, with particular reference to autophagy.

3. To measure the effect of nitrogen starvation on nuclear PI staining of plant cells, comparing these effects with those on plant cell morphology and physiology, with particular reference to autophagy.

4. To develop a model that attempts to explain the observed changes in nuclear PI staining based on chromatin modifications and the resulting conformational changes.
2. Methods

2.1 General methods

2.1.1 Plant cell culture growth and maintenance

2.1.1.1 Growth conditions

Plant cell cultures were grown in an insulated growth room at an average temperature of 25°C (± 4°C) (figure 2.1).

![Growth room temperatures](image)

Figure 2.1. Minimum and maximum growth room temperatures measured over a one year time frame.

2.1.1.2 *Nicotiana tabacum* liquid cultures

*Nicotiana tabacum* cv. Bright Yellow-2 (BY-2) was obtained from Dr. Jo Putterill at the School of Biological Sciences, the University of Auckland. It was maintained in BY-2 medium (appendix 1.4) in a liquid shake culture. Sterile flasks of 500 mL capacity and capped with aluminium foil were partly filled with 95 mL of sterile BY-2 medium, to which 5 mL of BY-2 inoculum was added. The added BY-2 cells were from a 7 day old culture, with an absorbance of approximately 0.8 (620 nm) i.e. during late log phase.
Once inoculated, the flasks were protected from light and placed on a shaker unit (130 rpm). Cultures were harvested or used to set up experiments 7 days after subculturing, when in their late log phase (figure 2.2).

![BY-2 density](image)

Figure 2.2. BY-2 liquid culture optical densities measured at 620 nm, following the subculturing of three liquid cultures.

### 2.1.2 Production of BY-2 protoplasts

BY-2 protoplasts were created by enzymatic degradation of the BY-2 cell wall, using the enzymes cellulase and pectolyase. To achieve this, 5 mL of BY-2 liquid cell culture was added to a glass test tube and the cells left to settle out for 10 min. The supernatant was then pipetted off and replaced with 5 mL of BY-2 protoplast test buffer (appendix 2.1). After centrifuging at 300 g for 2 min, the supernatant was pipetted off and replaced with 5 mL of BY-2 protoplast enzyme solution (appendix 2.2). The resuspended cells were then transferred to a glass Petri dish and incubated at 25°C for up to 4 h (in darkness) on a very gentle shaker. Alternatively, the BY-2 protoplast enzyme solution was diluted 4X with BY-2 protoplast test buffer and incubated overnight (approximately 15 hrs). Protoplast formation was monitored during the incubation period under a microscope. Once the cell wall was fully degraded, the protoplasts were transferred to a glass test tube and centrifuged at 300 g for 2 min. The supernatant was then replaced with BY-2 protoplast test buffer, centrifuged at 300 g for 2 min and repeated. The protoplasts were then ready for use in experiments (figure 2.3).
2.1.3 DNA content measurement of BY-2 nuclei

Previously prepared BY-2 protoplasts (section 2.1.2) were centrifuged at 300 g for 2 min and the test buffer replaced with either Galbraith’s buffer (appendix 4.1) or PBS/Triton X-100 solution (appendix 4.2) (both containing freshly added PI stock solution). The cells were then incubated at RT in darkness for 60 min, before flow cytometric analysis (section 2.1.5).

2.1.4 Microscopy

Cell cultures were viewed using a Nikon Eclipse TS2000-S inverted microscope with bright field, phase contrast and epifluorescence. Fluorescence filter cubes used included; ultra violet (Nikon UV-2A, EX 330-380, DM 400, BA 420), interference blue (Nikon B-2A, EX 450-490, DM 505, BA 520) and interference green (Nikon G-2A, EX 510-560, DM 575, BA 590). Images were taken using a Nikon Coolpix 4300 digital camera, recording 4 megapixel (2272 x 1704 pixels) JPG image files in the ‘fine’ detail mode.
2.1.5 Flow cytometry

2.1.5.1 Instrument specifications and setup

Flow cytometric analysis of cell suspensions was achieved using a Coulter Epics Elite flow cytometer (Epics Division of Coulter Corporation, Hialeah, FL, USA) with Isoton II diluent (Beckman Coulter, Brea, CA, USA) used as the sheath fluid. The flow cytometer was fitted with a Uniphase 106-2 air cooled 633 nm Helium-Neon (HeNe) laser, a Uniphase 2201-20SLE air cooled 488 nm Argon laser (Uniphase, San Jose, CA, USA) and a water cooled Coherent Innova 90 visible/UV laser (Coherent, Palo Alto, CA, USA) running in UV mode (347 nm). Depending on the probes used, different lasers were activated, in different combinations (but only if required i.e. unused lasers were switched off), with the same rational applied to the filters used to measure light intensities (figure 2.4).

Figure 2.4. The standard side scatter light filter set used on the Coulter Epics Elite flow cytometer. Mixed frequency light scattered at 90° from passing cells was filtered by dichroic long pass (DL) mirrors and band pass (BP) filters before detection by photo multiplier tubes (PMTs). A 488 nm blocker (BK) prevented any Argon laser side scatter from mixing with fluorescent light produced by the probe(s) of choice. Note; different filter setups were required depending on the lasers in operation and the probes used.

2.1.5.2 Instrument daily maintenance

Alignment of the flow cell tip was achieved with the use of Flow-Check Fluorospheres (Coulter Corporation, Miami, FL, USA). These fluorescent beads are of uniform size and retain uniform fluorescence intensity, thereby allowing daily
verification of the flow cytometer’s optical alignment and fluidics system. When running them, the sample pressure was adjusted to facilitate a rate of approximately 100 events (beads) per second. Once this data rate was stabilised, knobs adjusting the x-, y- and z-axes of both the flow cell tip and beam shaper were turned as required to gain half-peak coefficient of variance (HPCV) values of <2% for forward scatter (FS) and at PMT2-PMT5 after capturing 2000 events. A more detailed procedure can be found in the Flow-Check Fluorospheres instruction manual (Beckman Coulter Incorporated, Brea, CA, USA).

2.1.5.3 Acquisition and analysis software

Expo32 v1.2B Cytometer and Analysis v1.1C software (Applied Cytometry Systems, Sacramento, CA, USA) was used to acquire and analyse list mode files generated from samples run on the flow cytometer. Depending on the experiment, various data parameters were acquired, for example weakly fluorescent dyes required measurement on a logarithmic scale, whereas brightly fluorescent dyes were measured on a linear scale. Further analysis of list mode files was achieved using FlowJo v7.2.1 (Microsoft Windows XP) or v8.5.1 (Macintosh OSX) software (Tree Star Incorporated, Ashland, OR, USA).

2.1.5.4 Data gating procedure

Very small, non-fluorescent cellular debris was excluded from analysis by way of placing a gate on the scatter plot of FS versus side scatter (SS). Also, when analysing BY-2 nuclei stained with DNA specific dyes, doublet (or triplet, quadruplet etc.) events were excluded from examination by way of a gate placed on the scatter plot of dye linear fluorescence versus dye peak fluorescence i.e. PMT3 vs. PMT3 peak in the case of PI stained nuclei. Therefore events outside of the linear diagonal pattern formed, were perceived as multiple events and excluded from analysis.

2.2 Chapter 3 methods

2.2.1 The effect of propidium iodide loading on the DNA peak positions of BY-2 nuclei

Protoplasts were prepared (section 2.1.2) from 2 d old BY-2 cell cultures, with protoplast density adjusted to approximately $1 \times 10^5$ protoplasts/mL. RNase A (50
µg/mL) was added to the protoplasts, enzymatically degrading the RNA present and preventing PI from binding to it. The protoplasts were then incubated at RT for 20 min, before being centrifuged at 300 g for 2 min. The supernatant was discarded and the protoplast pellet resuspended in a PBS buffer containing differing volumes of a PI stock solution (1 mg/mL) and 0.1% Triton X-100 (allowing release of the nuclei), to obtain final PI concentrations of 3, 6, 9, 12, 15, 18, 21 and 24 µM. PI staining was analysed by flow cytometry (section 2.1.5) at subsequent time points of 0, 10, 20, 30 and 40 min post PI addition. The positions of the G0/G1 and G2/M population peaks were noted in each sample. Comparisons of PI loading concentrations were also compared over time.

2.2.2 The effect of propidium iodide loading on the DNA peak positions of *P. sativum* nuclei

Pea (*Pisum sativum* var. *macrocarpon* L. cv. sugar snap) seeds were germinated and grown in potting soil for between 1 and 3 weeks, during which time the apical shoots were harvested. Each shoot was placed in a glass Petri dish (placed on ice) and chopped very finely with a razor blade in 2 mL of Galbraith’s buffer (appendix 4.1). The chopped shoot slurry was then passed through a nylon mesh strainer (35 µm) into a 12 x 75 mm tube (Becton Dickinson, Franklin Lakes, NJ, USA) and stained with PI (18 µM). After incubation on ice for 5 min (since PI staining was very quick in BY-2 nuclei), the *P. sativum* nuclei were analysed via flow cytometry (section 2.15).

2.2.3 G0/G1 peak position changes during growth of BY-2 cells in complete medium

BY-2 cells were grown as 2 mL liquid cultures in Falcon 12 well polystyrene, flat bottomed tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA). To achieve this, cells were harvested from 7 day old, 100 mL shaker flask cultures (section 2.1.1.2), centrifuged at 1000 g for 5 min, with the supernatant discarded and replaced with a double volume of fresh medium. The resuspended cell pellet (approximately 5 x 10⁵ cells) was then distributed in 2 mL aliquots into each well of the 12 well plate. The plate was then covered with BREATHseal (Greiner Bio-One, Frickenhausen, Germany) to minimise evaporation. After the lid was replaced and the plate loosely covered in aluminium foil to exclude light, the cells were grown in a similar fashion to the shaker flask cultures (section 2.1.1.2). Cells were harvested for production of protoplasts (section 2.1.2) and protoplasting concentration was adjusted to
approximately 1 x 10^5 protoplasts/mL. The protoplasts were then centrifuged at 300 g for 2 min and the resulting supernatant decanted off. The remaining pellet was gently resuspended in PBS containing 0.1% Triton X-100 and PI (18 µM), and incubated for 5 min. PI fluorescence was analysed by flow cytometry, with relative peak positions validated on a day-to-day basis by the use of Flow-Check Fluorospheres (Coulter Corporation, Miami, FL, USA). Six replicates were analysed each day over the course of 10 d, for BY-2 cells grown in complete medium. An average of G_0/G_1 peak channel numbers was obtained from the six replicates and plotted over time. *P. sativum* nuclei were also prepared as in section 2.2.2 for comparison of the DNA histograms.

2.2.4 Fluorescence microscopy of PI and Hoechst 33342 stained BY-2 nuclei

Nuclei from the previous experiment (section 2.2.3), were stained with PI (18 µM) and/or Hoechst 33342 (H342) (16 µM) and observed using the appropriate fluorescence filters (UV-2A for H342 and G-2A for PI) in a Nikon Eclipse TS2000-S inverted microscope. H342 was added in addition to or substituted for PI in duplicate samples and treated in the same manner as PI stained samples as outlined in section 2.2.3; except rather than analysing using flow cytometry, the nuclei were imaged using fluorescence microscopy.

2.2.5 Mixing of PI stained *P. sativum* nuclei with BY-2 nuclei

BY-2 nuclei were prepared as in section 2.2.3 and *P. sativum* nuclei were prepared as in section 2.2.2, before they were mixed and analysed via flow cytometry (section 2.1.5).

2.2.6 Mixing of PI stained BY-2 nuclei from different time points

Nuclei prepared as in section 2.2.3, from different time points during culture growth were mixed before flow cytometric analysis. To achieve this, subculturing of the BY-2 cells into 12 well plates was staggered to allow mixing of samples of different age on the same day.
2.3 Chapter 4 and chapter 5 methods

2.3.1 Carbon and nitrogen starvation medium preparations

BY-2 cells acting as negative controls were grown in complete BY-2 medium, with the constituents as listed in appendix 1.4.

Two modifications were made to the complete BY-2 medium in order to achieve carbon source starvation in the cells (i.e. experiments presented in chapter 4).
1. Medium lacking addition of sucrose.
2. Substitution of sucrose with sorbitol (appendix 1.5). Substitution with sorbitol was on a molar equivalent to sucrose.

A third BY-2 medium was also formulated to cause both carbon and nitrogen starvation, with sorbitol substituted for sucrose and potassium chloride substituted for both nitrates and ammonium sources on a mole for mole basis (appendix 1.7).

Two further modifications were made to complete BY-2 medium in order to achieve nitrogen source starvation in the cells (i.e. experiments presented in chapter 5).
1. Medium lacking addition of nitrates (NH$_4$NO$_3$ and KNO$_3$) and thiamine.
2. Substitution of nitrates with chloride and omission of thiamine (appendix 1.6).

Substitution with potassium chloride was of an equivalent molar basis to the nitrates.

2.3.2 DNA peak position changes during growth of BY-2 cells in sucrose and nitrate starved media

These experiments were conducted in parallel with and used the same methodology as presented in section 2.2.3, except that instead of growing the BY-2 cells in complete medium, the media types detailed in section 2.3.1 were used.

2.3.3 DNA peak position during growth of BY-2 cells in different media using Hoechst 33342 fluorescence

Duplicates of the samples generated in section 2.3.2 were also stained with H342 (16 µM) before analysis using flow cytometry (section 2.1.5).
2.3.4 The effect of 2,4-dichlorophenoxyacetic acid supplementation on DNA peak position

BY-2 cells were grown as 2 mL cultures in 12 well plates and the various media treatments (section 2.3.1) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) on a daily basis. Supplementation was designed to maintain a steady level of approximately 900 nM of 2,4-D in the medium. Therefore, based on the average depletion of 2,4-D in the medium types studied (section 2.3.10), levels were boosted from ~200 nM to ~900 nM, by addition of 10 µL of 2,4-D (141 µM). Protoplasts were then prepared from BY-2 cells at different stages of culture growth (section 2.1.2) and nuclei prepared, stained with PI and analysed via flow cytometry (as per section 2.2.3).

2.3.5 Growth curves of BY-2 cells grown in 12 well plates

Growth curves were plotted using two methods for estimating cell population dynamics in the 12 well cultures. The first method required the counting of cells that resided within five 1 mm² (0.1 µL) sections of a haemocytometer grid, with the average number of cells in those sections (replicated six times) used to estimate cell concentration (cells/mL). Cultures of high density were diluted with medium to allow easier observation, with the dilution factor taken into consideration when calculating cell concentration.

Counting the BY-2 cell chains accurately was error prone and time consuming due to the chain formation of BY-2 cells, so a second method was employed, looking at the absorbance of light at 620 nm by culture samples. These density measurements took place in Falcon 96 well polystyrene, flat bottomed tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and were read by an OpsysMR absorbance reader (Dynex Technologies, Chantilly, VA, USA). Each well was loaded with 150 µL of sample volume and the average of six replicates was used for each time point.

2.3.6 Cell viability assessment during growth of BY-2 cells in various media

Samples were taken from 12 well, 2 mL BY-2 cultures each day, over the course of 20 days and subjected to co-staining with fluorescein diacetate (FDA) and propidium iodide (PI). FDA and PI were added to approximately 1 x 10⁶ cells to give a final concentration of 2.4 µM and 3.0 µM respectively. Cells were then incubated at RT for
10 min, before counts were performed using a haemocytometer chamber (as in section 2.3.5). Epifluorescence microscopy utilising an interference blue filter cube for FDA fluorescence and an interference green filter cube for PI fluorescence allowed differentiation of live and dead cells. At each time point, counts of live (FDA positive), dead (PI positive) and unknown status (FDA and PI negative) cells were taken from five 1 mm$^2$ (0.1 µL) sections of the haemocytometer grid, averaged and expressed as a percentage of the total cells present.

2.3.7 Changes in pH during the growth of BY-2 cells

BY-2 cells were grown as 2 mL cultures in 12 well plates utilising the media types listed in section 2.3.1. Medium containing cells was harvested at different time points, with the cells centrifuged to form a pellet and the supernatant removed for pH analysis. Measurements of pH levels were taken using an Orion PerpHect 310 pH meter (Thermo Electron Corporation, Beverly, MA, USA).

2.3.8 Sucrose concentration in BY-2 12 well cultures grown in various media

Sucrose levels were measured indirectly, by first converting any sucrose present in the cell free medium to glucose and fructose using the enzyme invertase (Sigma-Aldrich, Saint Louis, MO, USA), with one unit of invertase able to hydrolyse 1.0 µmole of sucrose per minute at pH 4.6 (25°C). A slight excess of invertase and an extended incubation time ensured maximal conversion of sucrose to glucose (and fructose). A peroxidase/glucose oxidase (PGO) enzyme preparation (Sigma-Aldrich, Saint Louis, MO, USA) was then used to quantify glucose levels via a colour change as per the manufacturer’s instructions. The procedure was based on the following coupled enzymatic reactions:

\[
\text{Sucrose} + H_2O \xrightarrow{\text{Invertase}} \text{Fructose} + \text{Glucose} \\
\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2 \\
H_2O_2 + \text{o-Dianisidine (colourless)} \xrightarrow{\text{Peroxidase}} \text{Oxidised o-Dianisidine (brown)}
\]

Absorbance of the brown oxidised o-dianisidine was measured at 450 nm using an OpsysMR absorbance reader (Dynex Technologies, Chantilly, VA, USA) and was proportional to the glucose concentration present in the sample. Both glucose and
sucrose (with subsequent conversion to glucose) standards were measured (figure 2.5), with the sucrose standard used for quantification purposes.

Figure 2.5. A comparison between glucose and sucrose standard curves, with the sucrose derived standard curve used for sucrose level quantification.

2.3.9 Protein content in the different BY-2 media from 12 well BY-2 cultures

Total protein content was measured using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA) using the manufacturer’s instructions (microplate procedure). This kit utilises the Biuret reaction (reduction of Cu$^{2+}$ to Cu$^{+}$ by protein in an alkaline medium) coupled to the colourmetric detection of Cu$^{+}$ by BCA. The chelation of Cu$^{+}$ by two BCA molecules results in a purple coloured water soluble complex with a strong absorbance at 562 nm. Absorbance was measured at 550 nm using an OpsysMR absorbance reader (Dynex Technologies, Chantilly, VA, USA). Blank absorbance measurements were taken from distilled water samples and were subtracted from BSA standard and medium sample measurements taken in duplicate. A BSA standard curve (figure 2.6) was used for the estimation of total protein content in samples. Medium samples were prepared from 12 well plate cultures that were centrifuged at 1000 g for 5 minutes, with 25 µL of the resulting cell-free medium incorporated into the BCA protein assay.
Chapter 2: Methods

Figure 2.6. An example of a BSA standard curve that was used for the estimation of total protein content in medium samples.

Initial absorbance measurements taken from sucrose containing medium (cell free) were unusually high (even when the standard was corrected with an appropriate sucrose concentration) and it was therefore concluded that sucrose was interfering with the BCA assay. To remove as much sucrose from the medium samples as possible, they were dialysed against swirling distilled water overnight (15 h), using 6-8 kDa cut-off dialysis tubing.

2.3.10 Depletion of 2,4-D in different BY-2 media from 12 well BY-2 cultures

Shaker cultures (100 mL) were harvested at different time points during BY-2 cell growth. The cell suspension was placed into two 50 mL plastic tubes, centrifuged at 4500 g for 10 min and the supernatant transferred into new 50 mL tubes. This was repeated three times to reduce the particulate matter in the culture medium. The remaining supernatant was then passed through a 0.2 μm filter in order to keep it sterile and prevent clogging of solid phase extraction (SPE) columns.

Samples were then sent to BioDiscovery (Wellington laboratory), to be analysed by Dr. Stephen Bloor. Each sample of 20 mL was passed through an IST ENV+ 100 mg sorbent mass SPE column (Argonaut Technologies Inc, Redwood City, CA, USA), washed with 10 mL of distilled water and then eluted with 500 μL of methanol. The methanol eluate was then analysed using an Agilent 1100 series liquid chromatographer coupled to an Agilent G1946B mass spectrometer (Agilent Technologies Inc, Santa Clara, CA, USA). Comparative standards comprised of weighed amounts of 2,4-D dissolved in methanol, which were diluted appropriately and analysed in an identical fashion to the medium samples. Once the 2,4-D peak was identified and this identity
confirmed with mass spectroscopy (figure 2.7), the peak integral was used to approximate 2,4-D quantities in the samples.

![Figure 2.7](image)

Figure 2.7. Chromatographs used in the quantification of 2,4-D levels present in BY-2 medium. The arrow distinguishes the peak positively identified as 2,4-D in the spectra read at 210 nm, with an elution time of approximately 8 min and 47 s.

**2.3.11 BY-2 cell morphology**

The morphologies of the BY-2 cultures were observed during growth in the various media formulations (section 2.3.1) using the microscope set-up described in section 2.1.4. Phase contrast images were recorded showing typical cells present in a 12 well plate culture (2 mL) at days 0, 4, 8 and 12.

**2.3.12 Starch granule development in nitrate deprived media**

Granules that formed in nitrate starved BY-2 cells (KCl substituted medium) were
tested for the presence of starch by application of an iodine solution comprised of 0.1% (w/v) iodine and 1.0% (w/v) potassium iodide dissolved in distilled water. It was added to the cell suspensions at a rate of 1 part iodine solution to 5 parts cell suspension.

2.3.13 Transmission electron microscopy of starch granules in nitrate starved BY-2 cells

Cells were centrifuged at 1000 g, with the supernatant removed and the remaining cell pellet resuspended in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. After 3 h of incubation at RT, the cells were centrifuged at 1000 g, with the glutaraldehyde-containing supernatant deactivated in a glycine solution and the cell pellet washed 3 times in 0.1 M cacodylate buffer for 10 min at each wash step. The washed cell pellet was then resuspended in 0.1 M cacodylate buffer (pH 7.2) containing 1.0% osmium tetroxide and incubated at RT for 1 h.

Once staining with the osmium tetroxide was complete, the cells underwent dehydration in an ethanol series of 30%, 50%, 70%, 90% and 2 x 100% ethanol. A further two incubations with 100% acetone completed the dehydration series. At each step, the cells were incubated for 10 min, before being centrifuged at 1000 g, the supernatant discarded and the cell pellet resuspended in the next series solution. The dehydrated cells were then infiltrated with equal parts of 812 epoxy resin and acetone for 1 h. After centrifugation at 1000 g, the 812 epoxy/acetone mix was replaced with 100% 812 epoxy resin and incubated overnight (16-24 h).

The infiltrated cells were then deposited at the bottom of bullet-shaped resin moulds (beam capsules) and fresh 812 epoxy resin layered overtop, taking care not to disturb the cells too much. Curing of the resin occurred at 60°C over 48 h. The resulting resin pellets were then removed from the mould casings and prepared for sectioning.

Ultra thin sections were achieved using a LKB Bromma ultramicrotome (LKB Instruments, Bromma, Sweden) equipped with a diamond knife. Refracted light from sections floating on water appeared silver or gold when cut to the desired thickness of approximately 70 nm. Sections were then scooped up with pre-charged (to make hydrophilic) copper 100 mesh grids and allowed to dry.

The grids were then stained with 5% uranyl acetate (in butanol saturated water) for 40 min, before washing 5 times for 30 sec in distilled water. Excess water was then mopped up with filter paper and the grids were then stained in Reynolds lead citrate solution (appendix 3.1) for 3 min. After a quick wash in distilled water, the grids were washed a further 4 times for 30 s each time (figure 2.8).
Chapter 2: Methods

Figure 2.8. Steps used to stain sections in preparation for TEM. The sections were bound to copper mesh, which underwent staining with uranyl acetate, rinsing with water, staining with lead citrate and a final rinsing with water. These steps were performed in droplets placed on a Parafilm sheet.

Once dry, the grids were loaded into a FEI/Philips CM12 TEM (FEI Company, Hillsboro, OR, USA), with images of the sections captured with a 1024 x 1024 pixel Gatan 792 Bioscan CCD camera and processed using Digital Micrograph 3 software (Gatan, Pleasanton, CA, USA).

2.3.14 Formation of spherical bodies in response to protease inhibition

BY-2 cell suspensions were harvested after 4 days, with 1 mL (approx. $2 \times 10^5$ cells) centrifuged at 1000 g and the supernatant discarded. The remaining cells were then resuspended in 4 mL of fresh medium and 2 mL aliquots were dispensed into each well of a 12 well plate. Complete mini protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Nonnenwald, Penzberg, Germany) were dissolved in distilled water and added to the BY-2 cells at a final concentration of 2X, 1X, 0.5X and 0.25X the manufacturers recommended dosage (equivalent to 28 µM, 14 µM, 7 µM and 3.5 µM papain – the main protease inhibitor present in the cocktail). The wells were then covered with BREATHseal (Greiner Bio-One, Frickenhausen, Germany) to minimise evaporation, the lid replaced and covered in aluminium foil, before the plate was placed on an orbital shaker in the growth room. Samples were taken every day post treatment and analysed visually (representative images recorded) using phase contrast microscopy (section 2.1.4).
Chapter 2: Methods

2.3.15 Staining of sucrose deprived BY-2 cells with monodansylcadaverine

BY-2 protoplasts were prepared (section 2.1.2) from BY-2 shaker cultures (100 mL) grown in complete medium, sorbitol substituted medium or KCl substituted medium. Monodansylcadaverine (MDC) (40 µM) was added to protoplasts created at different time points during the cultures growth and allowed to incubate at RT for 10 min. The protoplasts were then washed in BY-2 protoplast test buffer (appendix 2.1), before analysis using fluorescence microscopy (interference blue filter cube).

2.3.16 BY-2 cell area measured in two dimensions

Representative images of BY-2 cell cultures grown in 12 well plates on different media were taken using a Nikon COOLPIX 4300 digital camera, recording 4 megapixel (2272 x 1704 pixels) JPG image files in the ‘fine’ detail mode. Scale bars were developed from images of a haemocytometer grid, using Adobe Photoshop 6.0 software (Adobe Systems, San Jose, California, USA) and used to measure the width and length of the BY-2 cells. Four cells were measured in each image and six images were taken at each time point over the course of 10 days. From width and length measurements, a two dimensional cell area was formulated, with the average cell area plotted over time.

2.3.17 Total protein content estimation within BY-2 cells during growth in various media

BY-2 cells were grown in various medium formulations (described in section 2.3.1) as 2 mL, 12 well plate cultures. Samples of 1 mL were taken every day over a 10 day period and placed in 1.5 mL Eppendorf tubes. Because sampling removed half of the culture volume in each well, each treatment was replicated five times, allowing a total of 10 samples to be taken. After centrifugation at 1000 g for 5 min, the supernatant was removed and the samples placed in a speed-vac dryer overnight. The resulting dried cell pellet was ground to a fine powder in the Eppendorf tubes using liquid nitrogen and pestle pellet grinders, before being resuspended in 1 mL of protein extraction buffer (0.175 M Tris-HCl pH 8.8, 5% SDS, 15% glycerol and 0.3 M DTT). Samples were then vortexed for 30 s and sonicated for 30 s to maximise protein extraction. The cell homogenate was then centrifuged at 4500 g for 10 min at RT, with the supernatant used for protein determination using the BCA protein assay kit (as per section 2.3.9). Dialysis of these samples was not necessary due to low levels of sucrose present.
2.3.18 Nitric oxide levels in BY-2 cells grown in various media

BY-2 cells were grown as 2 mL cultures in 12 well plates, with media described in section 2.3.1. Subculturing was staggered (from appropriately aged flask cultures) to allow measurement of different stages of growth on the same day. Two treatment groups were assembled; cells treated with 10 µL of SNAP (S-nitroso-N-acetylpenicillamine) (1 mM, dissolved in DMSO) and a control group treated with 10 µL of DMSO. After 24 h of incubation (with SNAP or DMSO), the cells were then washed with the appropriate medium (e.g. complete medium for complete medium grown cells) and were ready to harvest. Once harvested, the cells were converted into protoplasts (as per section 2.1.2) and stained for 30 min with DAF-FM (4-amino-5-methylamino-2’,7’-difluorofluorescein) diacetate at a final concentration of 5 µM. Excess DAF-FM probe was removed by washing the protoplasts with protoplast buffer (appendix 2.1). Finally, the protoplasts were analysed via flow cytometry using a 488 nm argon laser, capturing parameters of FS, SS and fluorescence at 525 nm. As there were various sizes of protoplasts in each sample, all events were recorded and only very small cellular debris was gated out based on the FS vs. SS plot.

DAF-FM diacetate is a cell permeant reagent used to detect and quantify low levels of nitric oxide (NO). Essentially non-fluorescent, DAF-FM forms the fluorescent benzotriazole compound upon interaction with NO. SNAP was used as a positive control, as it spontaneously releases NO under physiological conditions. This assay was based on guidelines provided by the manufacturer of the DAF-FM diacetate probe (Life Technologies, Carlsbad, CA, USA).

2.3.19 Intracellular calcium levels in BY-2 protoplasts

After culturing BY-2 cells in 12 well plates with complete medium, sorbitol substituted medium and KCl substituted medium, samples were taken at different stages of growth and the cells converted into protoplasts (see section 2.1.2). The protoplasts were incubated with indo-1 AM (2-[4-[Bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]phenyl]-1H-indole-6-carboxylic acid penta acetoxymethyl ester) (1 µM) for 30 min, before washing with BY-2 protoplast test buffer (appendix 2.1) and 30 min of further incubation (both incubation steps were at RT and protected from light). Flow cytometry was then employed (section 2.1.5) to measure the fluorescence at 400 nm and 475 nm, when the indo-1 stained cells were excited with a 347 nm UV laser. The ratio of fluorescence (400 nm emission:475 nm emission) was observed over time to establish a
Chapter 2: Methods

baseline intracellular calcium ratio level. Higher ratios indicate higher levels of intracellular calcium and to observe this, the samples were then spiked with the Ca$^{2+}$ ionophore ionomycin (2 µM). The samples were observed for between 300 and 500 sec, and recovery times after ionomycin addition were noted.

To estimate intracellular Ca$^{2+}$ concentrations based on the indo-1 fluorescence emission ratio, the following equation was used:

$$[\text{Ca}^{2+}] = K_d \cdot Q \cdot (R - R_{\text{min}})/(R_{\text{max}} - R)$$

Where:  
- $K_d$ (dissociation constant of indo-1) = 250 nM
- $Q$ ($F_{\lambda2\text{min}}/F_{\lambda2\text{max}}$) = 5.71
- $R_{\text{min}}$ ($F_{\lambda1\text{min}}/F_{\lambda2\text{min}}$) = 0.13
- $R_{\text{max}}$ ($F_{\lambda1\text{max}}/F_{\lambda2\text{max}}$) = 10.15
- $R$ ($F_{\lambda1}/F_{\lambda2}$) = measured indo-1 ratio

Values for $F_{\lambda1\text{min}}, F_{\lambda1\text{max}}, F_{\lambda2\text{min}}$ and $F_{\lambda2\text{max}}$ were determined by converting freshly subcultured BY-2 cells (grown in complete medium) into protoplasts (as per section 2.1.2) and replacing the BY-2 protoplast test buffer with either Zero Free Calcium Buffer or 39 µM Free Calcium Buffer (Invitrogen, Carlsbad, CA, USA). The protoplasts were then incubated with indo-1 AM (1 µM) for 30 min, before washing with the appropriate buffer and addition of ionomycin (2 µM). The protoplasts in each buffer were then analysed using flow cytometry (347 nm excitation) to determine their peak fluorescence intensity emission at 400 nm and 475 nm.

This assay was adapted from guidelines provided by the manufacturer of the indo-1 AM probe (Life Technologies, Carlsbad, CA, USA). Indo-1 is not cell permeable, but by esterification of its carboxylic acid groups to create indo-1 AM, the dye becomes neutral which allows it to cross the cell membrane. In the esterified form indo-1 will not bind Ca$^{2+}$, therefore any calcium in the buffer solution does not alter the fluorescent properties of the dye. Once loaded into the cell, ubiquitous esterases cleave the AM groups, allowing binding of intracellular Ca$^{2+}$ and an accompanying shift in
fluorescence emission is observed (unbound indo-1 fluoresces at 475 nm, whereas bound indo-1 fluoresces at 400 nm).

2.4 Chapter 6 methods

2.4.1 Methylation-sensitive restriction fingerprinting of BY-2 genomic DNA

2.4.1.1 DNA extraction from BY-2 cells

Genomic DNA was extracted from BY-2 cells using a phenol/chloroform extraction and purification method. BY-2 cells (1 g with as much medium removed as possible) were ground to a fine powder with an ice cold mortar/pestle and addition of liquid nitrogen. The frozen powder was then placed in a 15 mL tube and 4.2 mL of pre-warmed (55°C) CTAB extraction buffer (appendix 5.2) was added. After incubation for 1 h at 55°C, samples were allowed to cool at RT for 10 min. Then 3.3 mL of a 25:24:1 ratio mixture of phenol:chloroform:isoamyl alcohol (P:C:I) was added and mixed by vortexing. After centrifugation for 10 min at 13,000 g (RT), the upper aqueous layer was transferred to a new tube. The P:C:I extraction was repeated twice more (total of 3 extractions). To the upper aqueous layer was added a 2/3 volume of -20°C isopropanol, and mixed by inversion. DNA was then allowed to precipitate by placing tubes at -20°C for >30 min (usually overnight). A DNA pellet was then obtained by centrifugation at 13,000 g for 20 min (4°C). The pellet was redissolved in 600 µL of TE buffer (appendix 5.3), 6 µL of RNase solution (appendix 5.4) added and the sample incubated at 37°C for 30 min. Then 6 mL of a mixture of chloroform and isoamyl alcohol (C:I), in a ratio of 24:1 respectively, was added and mixed by inversion. The upper aqueous layer was placed in a new tube after centrifugation for 10 min at 13,000 g (RT). The C:I extraction was then repeated and a 1/2 volume of ammonium acetate (7.5 M) added to the aqueous layer, before mixing by inversion and addition of 2 volumes of 100% ethanol. After further mixing, the sample was chilled to -20°C for >30 min (usually overnight) and centrifuged at 13,000 g for 20 min (4°C). The supernatant was then removed and the remaining DNA pellet was rinsed twice with 70% ethanol and allowed to dry. After resuspending in 160 µL of TE buffer, the sample was incubated at 65°C for 1 h, with periodic mixing. A final centrifugation at 16,000 g for 10 min (4°C) and the aqueous portion was transferred into a clean tube, with the
pellet discarded, and stored at 4°C (long term storage at -20°C).

2.4.1.2 DNA quantitation and quality

DNA was diluted 20 times, before measuring absorbances at 260 nm and 280 nm, using a Varian Series 634 spectrophotometer (Varian Techtron Pty Ltd, Melbourne, Australia). Double stranded DNA was quantified using the formula; dsDNA µg/mL = Abs260 x 50 x 20 (dilution factor). The ratio of Abs260/Ab280 gave an indication of DNA purity, with the optimum ratio being between 1.8 and 2.0 (all samples were within this ratio range).

The quality of all DNA samples was also tested by amplifying a 555 bp fragment of the *N. tabacum* actin (Tob103) gene (GenBank accession number U60495). PCR conditions matched those used for DNA amplification used in section 2.4.1.4, with the exception of the primers themselves. The actin primers were, Tob103f (344-363) 5’-TAC AAC GAG CTT CGT GTT GC-3’ and Tob103r (879-898) 5’-CTT GTC CAT CTG GCA GTT CA-3’ . This fragment was also used as a positive control during subsequent PCR amplifications.

2.4.1.3 Restriction enzyme digestion

The DNA was then digested with restriction enzymes; either a combination of the *Mse* I and *BstU I* endonucleases or *Mse* I only (New England BioLabs, Ipswich, MA, USA). For each sample, duplicate tubes were set up containing 1 µg of DNA and 1 µL of 10X NE buffer 2 (New England BioLabs). In one of each pair, 10 U of *BstU I* was added, with the volume in both tubes then made up to 10 µL with distilled water. After incubating for 2 h at 60°C, the samples were cooled on ice and 10 U of *Mse* I added to both tubes. BSA was also added to both samples to give a final concentration of 1 µg/µL, before incubation at 37°C for 16 h.

2.4.1.4 PCR conditions

The DNA fragments that resulted from restriction enzyme digestion then underwent PCR amplification using three different primer pair combinations (Mills and Ramsahoye, 2002).

Set 1. Msrff1 5’-AGC GGC CGC G-3’ and Msrfr1 5’-ACC CCA GCC G-3’
Set 2. Msrff1 5’-AGC GGC CGC G-3’ and Msrfr2 5’-TGG TCG GCG C-3’
Set 3. Msrff1 5’-AGC GGC CGC G-3’ and Msrfr3 5’-GCA CCC GAC G-3’
Chapter 2: Methods

The 50 µL PCR mix contained 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), MgCl$_2$ (Invitrogen) (2 mM), dNTPs (0.2 mM), forward primer (0.4 µM), reverse primer (0.4 µM), Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) (1 unit) and DNA template (100 ng). The PCR cycle consisted of an initial 5 min denaturation at 94°C, followed by 35 cycles of 2 min denaturation at 94°C, 1 min annealing at 40°C and 2 min extension at 72°C, before a final extension for 10 min at 72°C. The PCR products were then held at 4°C. An Applied Biosystems GeneAmp PCR System 9700 thermocycler was used for all PCR amplifications.

2.4.1.5 Gel electrophoresis and visualisation

PCR products underwent electrophoresis on TBE polyacrylamide (6%) gels in 1X TBE buffer. Blue 5X DNA loading buffer (Bioline USA Inc, Randolph, MA, USA) (5 µL) was added to every 20 µL PCR product before loading. Fragment size was compared against a 1 kb+ DNA ladder (Invitrogen Corporation, Carlsbad, CA, USA). Gels were run at 200 V for 30 min, or until dye front was 10 mm from gel end. They were then stained with ethidium bromide in 1X TBE (final concentration of 0.5 µg/mL ethidium bromide) for 30 min and destained in tap water for a further 10 minutes. All gels were visualised under UV light, with images captured using a Nikon COOLPIX 4300 digital camera, recording 4 megapixel (2272 x 1704 pixels) JPG image files in the ‘fine’ detail mode.

2.4.2 Total 5-methylcytosine content of BY-2 cells during growth and starvation

2.4.2.1 DNA extraction from BY-2 cells

DNA was extracted as per section 2.4.1.1 for this experiment.

2.4.2.2 Preparation for HPLC analysis of 5-methyl-cytosine

DNA samples (10 µg) were diluted with distilled water to make up to a volume of 100 µL, before splitting each sample into two 50 µL lots and placing into 0.5 mL PCR tubes. Two PCR tubes were filled with 50 µL of distilled water as controls. All tubes were incubated at 98°C for 3 minutes in a PCR thermocycler. Tubes were then cooled on ice for 5 min. To consolidate condensation, tubes were briefly centrifuged, before adding 5 µL of ammonium acetate (100 mM, pH 5.3). Then 5 µL of nuclease P1 (1
mg/mL stored at 4°C) was added below the surface of each sample. After incubation at 45°C for 2 h in a PCR thermocycler, the tubes were briefly centrifuged and 6 µL of ammonium bicarbonate (1 M, freshly prepared) was added. A 10 µL volume of phosphodiesterase I (snake venom phosphodiesterase) (0.002 U/mL, stored at -20°C) was then added and the samples incubated at 37°C for 90 min in a PCR thermocycler. After adding 4 µL of alkaline phosphatase (0.1 U/mL, stored at -20°C), the samples were incubated at 37°C for 2 h in a PCR thermocycler. Finally, 40 µL of distilled water was added to each sample and the samples were stored at -20°C until HPLC analysis.

2.4.2.3 HPLC analysis of 5-methyl-cytosine

Samples were thawed, briefly centrifuged to consolidate liquid and approximately 120 µL added to HPLC vials. Along with water controls and samples, 120 µL of 2’-deoxycytidine (20 µM) and 5-methyl-2’-deoxycytidine (800 nM) were included as standards in each HPLC run. A 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a Primesphere column (C18-HC, 5 µ, 110 Å, 250 x 4.6 mm) (Phenomenex, Torrance, CA, USA) was used for sample analysis. The column was at a temperature of 30°C and the samples and injector were kept at 4°C. The following solvent gradient table was used during the run time of 30 min, with a flow rate of 0.6 mL/min:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Distilled water</th>
<th>Ammonium acetate (100 mM, pH 6.0)</th>
<th>Acetonitrile (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>46%</td>
<td>50%</td>
<td>4%</td>
</tr>
<tr>
<td>8.0</td>
<td>46%</td>
<td>50%</td>
<td>4%</td>
</tr>
<tr>
<td>12.0</td>
<td>40%</td>
<td>50%</td>
<td>10%</td>
</tr>
<tr>
<td>20.0</td>
<td>10%</td>
<td>50%</td>
<td>40%</td>
</tr>
<tr>
<td>20.1</td>
<td>35%</td>
<td>0%</td>
<td>65%</td>
</tr>
<tr>
<td>23.0</td>
<td>46%</td>
<td>50%</td>
<td>4%</td>
</tr>
</tbody>
</table>

The resulting signal was measured using an ultra violet (UV) diode array detector (DAD). Unmethylated cytosine had a retention time of ~6.7 min and methylated cytosine had a retention time of ~10.6 min. Cytosine methylation percentage was then established using the following formula: Cytosine methylation (%) = (methylated peak area / (unmethylated peak area + methylated peak area)) x 100.
2.4.3 Anti-histone H3 mono-methyl K4 and anti-histone H1 antibody analysis

BY-2 protoplasts were prepared as in section 2.1.2 from 12 well plate cultures and their concentration adjusted to approximately 1 x 10^5 protoplasts/mL. The protoplasts were then incubated in PBS containing 0.1% Triton X-100 (to release the nuclei) and either mouse anti-histone H1 (MorphoSys US Inc (Biogenesis), Kingston, NH, USA) or rabbit anti-histone H3 mono-methyl K4 (H3K4me) antibodies (Abcam Inc, Cambridge, MA, USA), diluted 1:1000. After a 20 min incubation period at RT, secondary reporter antibodies bound to a FITC fluorophore were added and incubated for a further 20 min at RT. Goat anti-mouse IgG (Fc): FITC antibodies (MorphoSys US Inc (Serotec), Kingston, NH, USA) were added to tubes containing anti-histone H1 and goat anti-rabbit IgG (H&L): FITC antibodies (Abcam Inc, Cambridge, MA, USA) were added to tubes containing anti-histone H3K4me. Nuclei were then washed in protoplast test buffer (appendix 2.1), before analysis via flow cytometry. Cellular debris was electronically gated out/excluded, based on its low scattering ability and low level of fluorescence when observed on a FS versus PMT1 (FITC) plot. Replicate nuclei samples were also labelled with PI (18 µM) as described in section 2.2.3 to allow direct comparison of either histone H1 or H3K4me staining with PI staining.
3. Analysis of BY-2 nuclei staining using flow cytometry

3.1 Introduction

The use of DNA specific stains in the analysis of the cell cycle and PCD has made it possible, to better understand the role chromatin plays in cell proliferation and cell death. The most commonly utilised stain is propidium iodide (PI), which binds to DNA by intercalating between base pairs, with a stoichiometry of one molecule every 4 or 5 bases. It does so with no sequence specificity and also binds to RNA, therefore requiring the use of ribonuclease to measure DNA content selectively (Wilson et al., 1986; Shapiro, 2003). Once the dye binds to DNA, the hydrophobic environment causes a 30-40 nm shift in excitation spectrum (towards the red) and an increase in fluorescence quantum efficiency (approximately 20-30 times greater emission than the unbound dye). Emission is also shifted approximately 15 nm toward blue upon binding (figure 3.1). DNA binding also concentrates PI molecules, further increasing the fluorescence intensity. PI does not permeate through viable cell membranes and its binding affinity is affected by the ionic strength of the surrounding environment/solution (Shapiro, 1988). The fluorescence intensity of PI is sensitive to the degree of supercoiling within the DNA structure (Taylor et al., 1991; Shapiro, 2003).

Contrary to PI, some fluorescent Hoechst dyes such as Hoechst 33342 (H342) permeate unfixed cells and bind DNA preferentially, lodging in the minor groove, with greater affinity for AT-rich sites. AT-rich dsDNA strands show an approximate two-fold enhancement of fluorescence over GC-rich DNA, and the blue fluorescence of these dyes is sensitive to chromatin state and DNA conformation (Shapiro, 1988; Searle and Embrey, 1990; Brown and Fox, 1996). Hoechst dyes have a strong affinity for DNA, replacing many intercalating dyes (including PI) and although able to stain living cells, some cell types do not stain well due to export of the dye by membrane pumps. Dye concentrations must be fine tuned for different cell types and phosphate content in the medium/buffer. A low dye to phosphate ratio results in high affinity binding of Hoechst dyes and greater blue fluorescence. Conversely, high dye concentrations result in low affinity binding to secondary sites, fluorescence quenching, a shift in fluorescence emission (towards green) and can lead to DNA precipitation (figure 3.1).
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

(Shapiro, 1988).

![Figure 3.1](image)

Figure 3.1. The structure of PI (a) and excitation/emission spectrum of PI bound to dsDNA (c), along with the structure of the Hoechst dyes (b) and excitation/emission spectrum of H342 bound to dsDNA (d). Adapted from Invitrogen product information leaflet.

PI and the Hoechst dyes can be used to quantify DNA content in plant cells and their use as such is well established (Conia et al., 1987; Bharathan et al., 1994; Dolezel et al., 1998; Dolezel et al., 2007b). Previously, DNA content was estimated using methods such as Feulgen microspectrophotometry but flow cytometry has superseded such methods, providing fast and accurate results. In the case of analysing the DNA content of plant cells, first the nuclei must be made available to the dye. This can be achieved several ways, with the two simplest methods being; chopping to release the nuclei (useful with fresh whole tissue) (Galbraith et al., 1983) and enzymatic degradation of the cell wall before lysis of the cell membrane (often used with cell culture samples) (O’Brien and Lindsay, 1993; O’Brien et al., 1998b).

The 2C nuclear DNA (nDNA) content in the *Nicotiana* genus ranges from 2.86 pg in *N. trigonophylla* (*2n* = 2x = 24 chromosomes) to 14.57 pg in *N. rustica* (*2n* = 4x = 48 chromosomes). It is reported that *N. tabacum* cv. BY-2 has approximately 12.3 Gbp in its genome (Yokota et al., 2005), and is tetraploid (*2n* = 4x = 48 chromosomes) (Bennett and Smith, 1976; Kwade et al., 2005). Given that 1 pg of DNA is equivalent to 978 Mbp (Dolezel et al., 2003), the 2C nDNA content of a BY-2 cell should be...
approximately 12.6 pg. By comparison, a plant that is often used as a standard in DNA content estimation via flow cytometry is *Pisum sativum* (Dolezel and Bartos, 2005), with a 2C nDNA amount of 9.80 pg \((2n = 2x = 14 \text{ chromosomes})\) (Bennett and Smith, 1976).

Plant DNA content measurements assayed using flow cytometry are for the most part precise, but several examples exist where inaccuracies have arisen. Sunflower (*Helianthus annuus*) leaves have been shown to contain unknown compounds that interfere with PI intercalation and/or fluorescence (Price *et al*., 2000) and seasonal differences in fluorescence intensity have been noted in kiwifruit (*Actinidia deliciosa*) nuclei stained with PI (Hopping, 1994). Previous flow cytometric studies searching for evidence of apoptosis in tobacco cells have also revealed fluctuations in PI fluorescence over the course of batch culture growth (O'Brien *et al*., 1998a).

Aims of this chapter:

To investigate the differences in the PI staining patterns observed during the course of growth in a BY-2 batch culture and whether these differences represent a reproducible phenomenon.

### 3.2 Results

#### 3.2.1 The effect of propidium iodide loading on the DNA peak positions of BY-2 nuclei

Staining saturation of the BY-2 nuclei was achieved with PI concentrations \(\geq 18 \mu M\). Slight fluctuations in the mean PI fluorescence intensity of the G\(_0\)/G\(_1\) and G\(_2\)/M populations were seen from the Coulter Epics Elite flow cytometer over time (figure 3.2). Measurements from 50 min onward were not recorded, due to the loss of PI signal. This was attributed to the eventual degradation of the nuclear membrane and dissipation of DNA into the buffer solution, as seen by bright field microscopy (data not shown).
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

(a) 

(b) 

(c) 

(d) 

(e) 

(f)
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

Figure 3.2. PI staining profile of BY-2 nuclei, with the subsequent measurement time points overlaid. Nuclei were isolated from protoplasts, which in turn were prepared from two day old BY-2 cells grown in flasks containing 100 mL of culture. Approximately $1 \times 10^5$ protoplasts/mL were present in each sample, with 50,000 events (ungated) recorded during flow cytometric analysis. The red profile represents readings taken just after PI addition (0 min) and other coloured lines represent profiles from 10-40 min. Profiles with 3 µM of PI are shown in (a), 6 µM in (b), 9 µM in (c), 12 µM in (d), 15 µM in (e), 18 µM in (f), 21 µM in (g) and 24 µM in (h). This experiment was run in duplicate, with representative results presented here.

3.2.2 The effect of propidium iodide loading on the DNA peak positions of *P. sativum* nuclei

*P. sativum* nuclei required less PI to saturate the DNA, as there was little difference in fluorescence intensity between samples containing between 15 µM and 24 µM PI. This was quantified using the Kolmogorov-Smirnov test to determine if any two of the data sets differed significantly. The most difference was found between samples stained with 15 µM and 18 µM of PI, but the reported maximum difference between the cumulative distributions ($D$) was 0.14, with a corresponding *p*-value of 0.97, indicating that they were very similar data sets (figure 3.3).
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

3.2.3 G₀/G₁ peak position changes during growth of BY-2 cells in complete medium

Shifts in the fluorescence of PI stained nuclei were observed during the normal growth and proliferation of BY-2 cells in complete medium. Maximal PI fluorescence intensity was attained by day 4 of growth, after which PI fluorescence decreased in intensity and by day 8, nuclear PI fluorescence had decreased to similar levels as seen on day 2 (figure 3.4).

Figure 3.3. Overlaid histograms of *P. sativum* nuclei stained with PI at various concentrations. Apical shoots were finely chopped on ice in Galbraith’s buffer (appendix 4.1), strained, incubated with PI at differing concentrations for 5 min and analysed using flow cytometry. Approximately 1 x 10⁵ nuclei/mL were present in each sample and 50,000 events (ungated) were recorded in each flow cytometry sample. The CV values ranged from 2.1-2.5%.
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

Figure 3.4. Overlaid DNA histograms representative of PI fluorescence over 8 days of growth of BY-2 cells in complete medium. BY-2 cells were grown as 2 mL cultures in 12 well plates and harvested every two days for production of protoplasts. Nuclei were isolated from the protoplasts during incubation with PI (18 µM) and Triton X-100 (0.1%) for 5 min, before flow cytometric analysis. Approximately 1 x 10^5 protoplasts/mL were present in each sample and 50,000 events (ungated) were recorded in each flow cytometry sample. The fluorescence intensity of both the G_0/G_1 and G_2/M phase peaks increased as the culture proliferated, reaching maximal intensity by day 4. From this stage, the fluorescence intensity of the DNA peaks then diminished, reaching minimal intensity by day 8. This data is representative of a preliminary experiment that is repeated and expanded on later in this section (see figure 3.6).
Shifts in the BY-2 DNA histogram positions between day 0 and day 4 were compared to a *P. sativum* DNA histogram. These three samples were run separately in sequence, using identical settings on the flow cytometer and illustrate the interspecies difference and BY-2 intraspecies shift in PI fluorescence (figure 3.5).

![Figure 3.5](image)

**Figure 3.5.** Overlaid DNA histograms comparing three samples stained with PI; *P. sativum* nuclei, BY-2 nuclei at day 0 and BY-2 nuclei at day 4. BY-2 cells were grown as 2 mL cultures in 12 well plates and harvested for production of protoplasts. Their nuclei were isolated from the protoplasts during incubation with PI (18 µM) and Triton X-100 (0.1%) for 5 min, before flow cytometric analysis. Apical shoots of *P. sativum* were finely chopped on ice in Galbraith’s buffer (appendix 4.1), strained, incubated with PI (18 µM) for 5 min and analysed using flow cytometry. Approximately $1 \times 10^5$ nuclei/mL were present in each sample. Note that the PMT4 voltages (measuring PI fluorescence) used to obtain these data were higher than used in figure 3.4, in order to accommodate the less fluorescent *P. sativum* signal on the same x-axis scale.
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

A more complete picture of the shifts in PI fluorescence was seen when samples were taken each day for 10 days of growth in complete medium. The x-axis mean for the G₀/G₁ peak increased from day 0 to day 4, and reached a maximum at day 4 of 210 channels above the day 0 value. From day 4 onward, the G₀/G₁ peak fluorescence dropped, and by day 8, it dropped below the value at day 0 by 15 channels. These perceived shifts in PI fluorescence correlated closely with cell proliferation (figure 3.6).

Figure 3.6. BY-2 G₀/G₁ DNA peaks measured by PI staining and the corresponding cell proliferation curve over ten days, when grown in complete medium. BY-2 cells were grown as 2 mL cultures in 12 well plates and harvested every day for production of protoplasts. Nuclei were isolated from the protoplasts during incubation with PI (18 µM) and Triton X-100 (0.1%) for 5 min, before flow cytometric analysis. Approximately 1 x 10⁵ protoplasts/mL were present in each sample. Each bar represents the mean of six sample replicates of the G₀/G₁ peak x-axis mean channel number. Cell counts were performed on parallel samples using a haemocytometer grid. Error bars are shown as ± 1 standard error.

When the same samples were stained with H342, the BY-2 nuclei showed little variation during the 10 day course of culturing, with x-axis means ranging between channels 113 and 140 (figure 3.7).
Figure 3.7. BY-2 G₀/G₁ DNA peaks measured by H342 staining and the corresponding cell proliferation curve over ten days. BY-2 cells were grown as 2 mL cultures in 12 well plates and harvested every day for production of protoplasts. Nuclei were isolated from the protoplasts during incubation with H342 (16 µM) and Triton X-100 (0.1%) for 5 min, before flow cytometric analysis. Approximately 1 x 10⁵ protoplasts/mL were present in each sample. Each bar represents the mean of six sample replicates of the G₀/G₁ peak x-axis mean channel number. Cell counts were performed on parallel samples using a haemocytometer grid. Error bars are shown as ± 1 standard error.

3.2.4 Fluorescence microscopy of PI and Hoechst 33342 stained BY-2 nuclei

The nuclei analysed by flow cytometry were also observed using epifluorescence microscopy. Nuclei stained with PI became swollen upon accepting the dye, with the greatest changes seen in nuclei taken from cultures at day 4 of growth. H342 staining did not seem to cause any appreciable nuclear swelling at any stage post subculturing (figure 3.8).
Figure 3.8. BY-2 cell nuclei were isolated from freshly prepared protoplasts and stained with either PI (18 µM) or H342 (16 µM) in the presence of Triton X-100 (0.1%). Representative images were captured on an epifluorescence microscope at day 0 (a) & (e), day 2 (b) & (f), day 4 (c) & (g) and day 8 (d) & (h) post subculturing into 12 well plates (2 mL cultures). Images on the top row (a)-(d) were taken using the interference green (G-2A) filter to visualise PI fluorescence and the images on the bottom row (e)-(h) were taken using the ultra violet (UV-2A) filter to visualise H342 fluorescence. Swelling of the nuclei was observed upon staining with PI and became increasingly evident up to day 4, after which the swelling then became less pronounced (a)-(d). Corresponding duplicate samples were also stained with H342 and representative images showed little swelling or variation in size over the course of culturing (e)-(h). Scale = 10 µm.

When nuclei were stained simultaneously with both PI and H342, the same pattern of nuclear swelling was observed. Nuclei harvested from four day old cultures exhibited the most pronounced swelling, with nuclei from older and younger cultures remaining smaller after addition of the PI and H342 dyes (figure 3.9).
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

3.2.5 Mixing of PI stained *P. sativum* nuclei with BY-2 nuclei

To highlight interspecies differences in nuclear staining with PI and exclude the possibility that buffer conditions were influencing them, mixing of *P. sativum* and BY-2 nuclei was performed. *P. sativum* shoot apices were chopped, strained and stained with PI (18 µM), before being mixed with PI stained (18 µM) BY-2 nuclei (2 days post subculturing). The mixed population sample was then analysed using flow cytometry, with the voltage applied to PMT4 (PI fluorescence) increased to accommodate both profiles on the same linear x-axis (figure 3.10).
Chapter 3: Analysis of BY-2 nuclei staining using flow cytometry

Figure 3.10. *P. sativum* nuclei mixed with BY-2 nuclei (2 days old) and stained with PI (a). The *P. sativum* nuclei were prepared by chopping apical shoots in Galbraith’s buffer (appendix 4.1) and BY-2 nuclei were isolated from freshly prepared protoplasts using Triton X-100 (0.1%), before mixing, staining with PI (18 µM) and analysing using flow cytometry. The two nuclei populations were discernable by gating a two-dimensional plot of PI fluorescence (log scale) versus side scatter. The gate labelled 9.80 (% of events) represents the *P. sativum* nuclei and the gate labelled 29.35 (% of events) represents the BY-2 nuclei at day 2. A gate encompassing both species is labelled 39.60 (% of events) (b). Based on these gates, the resulting overlaid histograms allowed distinction of the two species based on the PI fluorescence of their nuclei (c).
3.2.6 Mixing of PI stained BY-2 nuclei from different time points

When BY-2 nuclei harvested at different time points were stained with PI, mixed and analysed by flow cytometry, the different populations could be discriminated by gating methods. This experiment was undertaken to provide further evidence that temporal differences in PI staining of BY-2 nuclei was not due to variation between samples. Nuclei from day 0 were not easily discernable from day 4 nuclei, and gating was necessary to distinguish between the two populations when mixed, due to the overlap of the day 0 G2/M peak with the day 4 G0/G1 peak. The PI fluorescence of nuclei from day 4 and day 8 were easily recognised after mixing, due to less overlap between each samples fluorescent signature (figure 3.11).

![Graphs showing PI fluorescence and side scatter plots for different time points.](image-url)
Figure 3.11. BY-2 nuclei from different time points were isolated from freshly prepared protoplasts using Triton X-100 (0.1%), mixed together, stained with PI (18 µM) for 5 min and analysed using flow cytometric analysis. Nuclei from day 0 were mixed with nuclei from day 4 (a), with the resulting DNA peaks better resolved through gating. The gate labelled 6.07 (% of events) represents day 0 nuclei, whereas the gate labelled 3.11 (% of events) represents day 4 nuclei, and a gate incorporating nuclei from both time points is labelled 9.26 (% of events) (b). Separate peaks were observed with more clarity by using an overlay histogram incorporating each gated peak (c). The same approach was used for combined nuclei from day 4 and day 8 sampling points (d). To better distinguish between the populations, day 4 nuclei are present in the gate labelled 3.76 (% of events), day 8 nuclei are present in the gate labelled 3.83 (% of events) and a gate containing nuclei from both time points is labelled 7.77 (% of events) (e). These gates were then used to generate separate histograms that were overlaid (f). Each sample was also analysed separately (without mixing), for peak identification purposes (data not shown).
3.3 Discussion

The aim of these experiments was to investigate the apparent DNA content changes observed during the growth of BY-2 cultures as measured with PI staining, employing a range of cytological techniques. The apparent content of BY-2 plastid DNA (ptDNA) has been previously reported to change over the course of culturing (Yasuda et al., 1988), and perceived changes to tobacco nuclear DNA (nDNA) content have also been noted (O'Brien et al., 1998a; O'Brien et al., 1998b). The results presented in these previous studies have been expanded upon in this chapter, with the utilisation of microscopy and flow cytometry.

Because of the variable sensitivity of flow cytometric instruments and variable staining of cell suspensions, measures were put in place to ensure reproducible and valid results. The Epics Elite flow cytometer used in experimentation was aligned against standardised fluorescent beads on a daily basis and the saturation point for PI staining in BY-2 nuclei was identified to eliminate staining variation. Figure 3.2 showed that above concentrations of 18 µM, PI was seen to saturate BY-2 nDNA and therefore this concentration was chosen for further experiments. Incubation time of the BY-2 nuclei with PI was not an important factor, due to the very quick infiltration and binding of the PI molecule, although above 50 min, loss of fluorescence occurred, probably due to eventual nuclear membrane destruction from the Triton X-100. As the nDNA content of the pea (P. sativum) cell is known and the nuclei widely utilised as a DNA content standard in plant flow cytometry (Baranyi and Greilhuber, 1995; Dolezel et al., 2007b), they were also used as an external biological standard in the experiments described here. The P. sativum nuclei required less than 15 µM of PI to saturate the fluorescence intensity signal, with staining stability reached quickly after PI addition (figure 3.3).

It was found that during the growth of BY-2 cells in a batch culture, DNA content (when assayed with flow cytometric analysis of PI stained nuclei) seemed to increase over the first four days before returning to a level slightly above that of a freshly subcultured batch. At their peak (day four), PI fluorescence intensities were almost 2.5 times greater than those measured in freshly subcultured samples (figure 3.6). A possible explanation for this change is that during growth of the culture, changes in the structure of the chromatin influence the accessibility of DNA to the PI fluorochrome. Two alternative explanations are that during this phase, substances produced by the BY-2 cells somehow enhance fluorescence intensity, or that substances suppressing fluorescence are not produced. This has been reported in other plant species (Price et al., 2000). The former explanation may be more applicable in this instance, due to the
physical swelling seen when nuclei are exposed to PI. Times of peak fluorescence intensity (day four) correlated with maximal nuclear swelling, hinting toward structural changes in the DNA conformation, i.e. chromatin relaxation allowing improved PI intercalation and as a consequence, an increase in nuclear swelling (figure 3.8). A further explanation may involve changes in nuclear membrane permeability to PI, although this is unlikely as the surrounding solution was saturated with PI and contained the detergent Triton X-100. It could be argued that the nuclear swelling observed upon exposure to PI was an artefact of the method used (notably addition of Triton X-100), although nuclei treated using the same method and stained with H342 did not display variation in nuclear size (figure 3.8).

Previous studies have noted that the DNA content (measured using DAPI fluorescence) within plastid nucleoids of BY-2 cells increased when older cells were transferred to fresh medium (Yasuda et al., 1988; Nagata et al., 2004). The copy number of ptDNA was seen to increase by 11-fold during the first day of growth in fresh medium, before a gradual decrease back to pre-subculture levels over the next 7 days. After the initial burst of ptDNA synthesis, plastids were distributed amongst daughter cells and thereafter, formation of starch granules was observed in the plastids (Yasuda et al., 1988). The authors supposed the proplastids produce factors essential for growth of heterotrophic plant cells and concluded that the introduction of fresh culture medium offered a good experimental system to study ptDNA synthesis control mechanisms (Yasuda et al., 1988).

Although ptDNA was thought to be excluded in the experiments discussed in this chapter (DNA fragments smaller than G0/G1 nuclei were gated out), this introduces the possibility that these high copy number plastid nucleoids can bind to cell nuclei during the harvest process, thereby increasing the perceived DNA fluorescence intensity of the BY-2 nuclei. But this is unlikely because no satellite nucleoid fluorescence was noted when microscopically observing nuclei (figure 3.8); although to test this, DNA specific protoplast permeant dyes could have been employed. Another argument against this speculation is the differing timeline of fluorescence changes, with ptDNA reaching a peak after day 1 (Yasuda et al., 1988) and nDNA peaking by day 4 (figure 3.6).

It was interesting to note that neither significant changes in fluorescence intensity or swelling were seen in BY-2 nuclei exposed to H342 at any stage in culture growth (figures 3.7 and 3.8). If DNA conformational changes were indeed occurring during culturing, binding of the H342 dye did not appear to be effected. In this model system, H342 was more robust and reliable than PI when used as a tool to estimate DNA content.
using flow cytometry. The fluorescence stability of H342 also established that DNA content changes were not the reason for fluctuations in the PI fluorescence seen in BY-2 nuclei.

Mixing experiments were employed to further check the validity of the PI fluorescence changes observed during BY-2 culturing. Two day old BY-2 nuclei were mixed with *P. sativum* nuclei as an initial control experiment, highlighting the staining profile of nuclei with different genome sizes (figure 3.10). Subsequent mixtures of BY-2 nuclei at day zero with day four, and day four with day eight showed that the mixed nuclei populations were truly different in their PI fluorescence profile and able to be effectively separated based on PI fluorescence intensity (figure 3.11).

An apparent lack of a large S phase population in the BY-2 DNA histograms was noted during this study. Rapid progression from the G1 phase through to the G2 phase with a short S-phase may account for this observation. However, a defined and significant S phase was noted in the *P. sativum* nuclei samples, although these were obtained through a different method of harvesting to the BY-2 nuclei, which perhaps had an impact on appearance of the DNA peaks. Galbraith’s buffer (appendix 4.1) was used in *P. sativum* nuclei preparation, while PBS/Triton X-100 solution (appendix 4.2) was used in BY-2 nuclei preparation. Unfortunately, these two solutions were incompatible with the two plant cell types i.e. BY-2 protoplasts incubated in Galbraith’s buffer or *P. sativum* chopped tissue incubated in PBS/Triton X-100 solution resulted in poorly defined DNA histograms with higher coefficient of variation (CV) values. This was seen in a mixing experiment, when *P. sativum* and BY-2 nuclei were co-incubated in the PBS/Triton X-100 solution, with the *P. sativum* DNA peak having a higher CV and less S phase nuclei than noted using Galbraith’s buffer (figure 3.10).

In summary, while these results are compatible with previous observations (O’Brien *et al.*, 1998b), they also help resolve the question of whether they are caused by actual changes in nDNA content or changes in the fluorescence intensity of PI. PI fluorescence shifts were validated by mixing experiments and the stability of H342 fluorescence over time suggests that total nDNA content does not change. It is evident that the PI fluorescence shifts are not artefacts caused by quenching of fluorescence, but rather may be explained by changes in chromatin conformation as suggested by the effects of PI on nuclear morphology. The time dependent nature and reproducibility of PI fluorescence changes warrants further investigation, especially with regard to chromatin modification.
4. Carbon source availability and its effect on BY-2 cell growth and chromatin state

4.1 Introduction

In plants, sugars are the main respiratory source for energy production, form part of the structure of DNA and RNA, are used in construction of cell walls and are vital to the function of many proteins and lipids. Most of a plant's sugar requirement is provided by photosynthesis, but changes in environmental conditions can limit the production of sugars and reduce carbohydrates to tissues considered carbon heterotrophic (non-photosynthetic organs). Several coping mechanisms have evolved in plants, the most common being starch storage in tissues such as stems and tubers.

Changes to cell physiology and patterns of gene expression have been studied in several plant species and provide insight into the coping strategies involved to maintain survival. An arrest in cell growth, decrease in respiration, degradation of lipids and proteins, accumulation of phosphorylcholine and amino acids, and a decline in glycolytic enzyme activity are the result of sugar starvation (Yu, 1999). Catabolism of fatty acids, amino acids and proteins is achieved by upregulation of genes encoding the appropriate enzymes in an attempt to substitute carbohydrate metabolism (Dieuaide et al., 1992). With sustained sugar starvation, irreversible damage occurs and results in cell death. This type of cell death has been suggested by some as senescence (Wingler et al., 2009), but the involvement of autophagic cell death (ACD) remains an alternative possibility (Debnath et al., 2005).

The results of the previous chapter elucidated the possibility that chromatin conformation (as indicated by PI staining properties) changed during growth of BY-2 cell cultures. A likely cause of this change is nutrient depletion during culture. Nutrient withdrawal and subsequent autophagy have profound effects on cellular processes and physiology, and are reflected in the morphology of the cell. Morphological indicators in plant cells, such as vacuolisation or the appearance of autolysosomes, give interesting insights into the method in which these cells select, package and process cytoplasm/organelles for autophagic degradation. Currently, two distinct autophagic pathways have been observed in plant cells, one in which cytoplasmic inclusions accumulate as autophagic bodies in the vacuole and one where the inclusions accumulate within autolysosome organelles, which exist outside of the central vacuole.
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

(Inoue et al., 2006). These differences in autophagosome packaging and targeting seem to be species dependent, with Arabidopsis and barley (Hordeum vulgare) cells examples of vacuole targeted autophagosomes (Rose et al., 2006) and tobacco cells an example of autolysosomal packaging (Moriyasu and Ohsumi, 1996).

Organelle specific probes have been used successfully in the identification and tracking of autophagosomes from formation to degradation, providing a visual demonstration of the autophagic process. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a mammalian homologue of Atg8p in yeast and appears to be the most reliable marker of autophagosomes. It is a soluble protein with a mass of approximately 17 kDa and is widely expressed in mammalian tissue and cultured cells. During formation of autophagosomes, the cytoplasmic form (LC3-I) is conjugated to phosphatidylethanolamine, creating LC3-phosphatidylethanolamine conjugate (LC3-II), which is then recruited into the autophagosome membrane. Antibodies targeted to endogenous LC3-II or GFP-Atg8 fusion proteins can therefore provide a snapshot of autophagosome presence in the cell before degradation in the lysosome (Kabeka et al., 2000).

Another marker that has provided positive identification of autophagosomes is monodansylcadaverine (MDC). While the specificity of MDC for staining autophagosomes has been reported (Niemann et al., 2000; Contento et al., 2005), it has also shown less specific staining by including other acidic vesicles such as lysosomes (Bampton et al., 2005). It seems that in some experimental systems MDC provides similar staining patterns to dyes such as LysoTracker Red and lysosome specific LAMP-1 or LAMP-2 targeted probes. The mechanism by which this lysosomotropic agent selectively enters acidic vesicles such as lysosomes and autophagosomes is not fully understood.

Evidence of autophagosome formation in BY-2 cells has been viewed directly with the application of a cysteine protease inhibitor ((2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane or E-64c) in sucrose starved cells. Accumulation of membrane bound spherical bodies surrounding the nuclear region that were positive for quinacrine staining suggested to the authors that they were acidic vesicles that contributed to the protein degradation noted during sucrose deprivation (figure 4.1). This result was interpreted as a direct observation of autophagosomes and therefore the process of autophagy in tobacco BY-2 cells (Moriyasu and Ohsumi, 1996).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

Figure 4.1. Morphological changes of cultured BY-2 cells during sucrose starvation. Cells were treated with 10 µM E-64c for day 0 (a), 1 day (d) and 2 days (e) of sucrose starvation or with 1% methanol (control) for 1 day (b) and 2 days (c) of sucrose starvation. Scale = 10 µm. Modified from Moriyasu and Ohsumi, 1996.

While there is an immense amount of histological information concerning the study of autophagic processes in mammalian cells, very few studies follow the equivalent processes in plant cells. The experiments presented in this chapter were designed to investigate autophagy and accompanying autophagic cell death (ACD) in a carbon deprived model plant system (tobacco BY-2 cells), using a suite of cytological techniques. Also of specific interest, were any correlations between carbon starvation and the chromatin changes observed in the previous chapter.

When inducing cell culture starvation and observing the processes of autophagy, it was important to simultaneously monitor the micro-environment in which the cells inhabit. Tracking the growth and population dynamics, as well as factors such as sugar consumption, pH, hormone levels and protein content in the cell culture of interest were basic, yet informative indicators of cellular processes during starvation.

As an albino cell line, BY-2 cells lack chlorophyll and therefore require a carbon source, of which sucrose is the most readily utilised (Nagata et al., 2004). Sugar alcohols such as mannitol and sorbitol are absorbed by tobacco callus cells, and are often used as metabolically inert osmotica. Mannitol has been reported to be metabolised by tobacco callus cells, albeit to a lesser degree than sucrose (Thompson et al., 1986), and was therefore not recommended as an ideal osmoticum for long term study of carbon source deprivation. Reports of sorbitol metabolism in tobacco callus
cells were not available, but its utilisation in other plant species suggests a minimal role as a carbon source in non-embryogenic callus tissue (Verma and Dougall, 1977; Swedlund and Locy, 1993). For this reason, sorbitol was a suitable inert osmoticum candidate and used for the ensuing study of carbon starvation and autophagy in BY-2 cells.

Aims of this chapter:

To measure changes in PI staining of the model plant cell line (BY-2) following deprivation of carbon (and carbon plus nitrogen); and to compare these changes with other physiological variations occurring after starvation from the carbon (and nitrogen) sources, observing alterations in cell growth and medium composition, with an attempt to describe any possible autophagic condition.

4.2 Results

4.2.1 DNA peak position changes during growth of BY-2 cells in various media

The mean $G_0/G_1$ DNA peaks of six replicate samples (taken each day) were plotted over the course of culturing in different growth media. They consistently showed an increase in PI fluorescence intensity during the initial phase of growth, before a decrease in fluorescence intensity during later stages. BY-2 cells grown in complete medium (data from a parallel experiment in chapter 3 (figure 3.6) were included for comparison) and medium lacking sucrose, the highest intensities were seen around day 4, whereas in the other media presented here, the highest intensities were delayed by 2 days (figure 4.2).
Figure 4.2. Mean G_0/G_1 DNA peaks of PI stained BY-2 nuclei, grown in various medium types over the course of ten days. BY-2 cells were grown as 2 mL cultures in 12 well plates and harvested every day for production of protoplasts. Nuclei were isolated from the protoplasts during incubation with PI (18 µM) and Triton X-100 (0.1%) for 5 min, before flow cytometric analysis. Approximately 1 x 10^5 protoplasts/mL were present in each sample. The PI fluorescence reached maximum intensity by day 4 in nuclei obtained from cells grown in complete medium and medium lacking sucrose, after which the PI fluorescence decreased. In nuclei isolated from cells grown in sorbitol substituted medium, as well as KCl and sorbitol substituted medium, fluorescence maxima were observed by day 6 before a decrease in fluorescence occurred. Each data point represents the average of six replicate samples. Error bars of ± 1 standard error are included (indiscernible on this scale).

The trend in PI fluorescence variation seemed to correlate with cell proliferation (cell counts taken concurrently and also presented in figure 4.8); therefore the two measurements were compared directly. With the absence of sucrose, cell numbers did not peak until day 7 and although a maximum PI fluorescence value was reached on day 4, the drop in fluorescence intensity was more gradual between days 5 and 7 (figure 4.3), when compared to cells grown in complete medium (figure 4.2).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

Figure 4.3. BY-2 G₀/G₁ DNA peaks measured using PI staining and the corresponding cell proliferation curve, when grown in medium lacking sucrose over ten days. BY-2 cells grown in 12 well plates were converted to protoplasts, which were then incubated for 5 min with PI (18 μM) and Triton X-100 (0.1%), before analysis using flow cytometry. Each sample comprised of approximately 1 x 10⁵ protoplasts/mL and 50,000 events were captured during the flow cytometry run. Cell counts were performed using a haemocytometer. Proliferation of the cells in this medium was slow, and the culture reached a maximum density by day 7, whereas PI fluorescence intensity of the cell nuclei was maximal on day 4. Each data point represents the average of six replicate sample measurements. PI staining data replicated from figure 4.2 for comparative purposes. Error bars of ± 1 standard error are included.

When the BY-2 cells were grown in sorbitol substituted medium, the increase in PI fluorescence intensity reached a maximum at day 6, correlating well with the cell concentration maximum also attained at day 6 (cell counts were taken before PI staining of samples, with the data also presented in figure 4.8 to compare treatment relationships) (figure 4.4).
Figure 4.4. BY-2 G₀/G₁ DNA peaks measured using PI staining and the corresponding cell proliferation curve over ten days, when grown in sorbitol substituted medium. Growth of cells occurred in 12 well plates (2 mL culture volume) and protoplasts (approximately 1 x 10⁵ protoplasts/mL) were freshly made before incubation with PI (18 µM) and Triton X-100 (0.1%) for 5 min. The released nuclei were then analysed using flow cytometry, with 50,000 events recorded in each sample. Cells were counted using a haemocytometer. Both cell density and PI fluorescence intensity of the nuclei were maximal on day 6. Each data point represents the average of six replicate sample measurements. PI staining data replicated from figure 4.2 for comparative purposes. The error bars represent ± 1 standard error.

BY-2 cells deprived of both nitrates and an exploitable carbon source had a maximum PI fluorescence intensity at day 6, which also correlated with the maximum density of this culture (data included from figure 4.8 for comparison) (figure 4.5).
Figure 4.5. G₀/G₁ DNA peaks measured using PI staining and the corresponding cell proliferation curve over ten days, when BY-2 cells were grown in KCl and sorbitol substituted medium. BY-2 cells grown in 12 well plates were converted to protoplasts, which were then incubated for 5 min with PI (18 µM) and Triton X-100 (0.1%), before analysis using flow cytometry. Each sample comprised of approximately 1 x 10⁵ protoplasts/mL and 50,000 events were captured during the flow cytometry run. A haemocytometer was used to provide cell counts. By day 6 both the cell density and PI fluorescence intensity of the nuclei reached their highest levels, before a decline in both parameters. Each data point represents the average of six replicate sample measurements. PI staining data replicated from figure 4.2 for comparative purposes. Error bars represent ± 1 standard error.

4.2.2 DNA peak position during growth of BY-2 cells in different media using Hoechst 33342 fluorescence

When duplicate samples from section 4.2.1 were stained with Hoechst 33342 (H342), BY-2 nuclei did not exhibit the same fluctuations of fluorescence intensity over time as seen in the PI stained nuclei, remaining stable throughout the course of culturing. Data from chapter 3 (complete medium) was obtained during the same experiment and included here for comparison (figure 4.6).
Figure 4.6. $G_0/G_1$ DNA peaks of BY-2 nuclei grown in various media, stained with H342 and measured over ten days. Growth of cells occurred in 12 well plates (2 mL culture volume) and protoplasts (approximately $1 \times 10^5$ protoplasts/mL) were freshly made before incubation with H342 (16 µM) and Triton X-100 (0.1%) for 5 min. The released nuclei were then analysed using flow cytometry, with 50,000 events recorded in each sample. The position of the $G_0/G_1$ DNA peak did not vary in the same manner as observed in PI stained nuclei and remained constant over time and between treatment types. Each data point represents the average of six sample replicates, where the mean channel number for the $G_0/G_1$ DNA peak was measured in each replicate. Error bars of ±1 standard error are included (indiscernible on this scale).

4.2.3 The effect of 2,4-dichlorophenoxyacetic acid supplementation on DNA peak position

The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is required for growth of BY-2 cells and is quickly consumed during culturing (see section 4.2.9). To test whether 2,4-D deprivation was responsible for the chromatin changes (PI staining), cultures were supplemented with 2,4-D during the course of BY-2 growth and PI fluorescence intensity fluctuations were measured. Supplementation with 2,4-D caused a decrease in the maximal PI fluorescence intensities attained (compared to figure 4.2), with none of the samples having mean $G_0/G_1$ peak positions above 300 channels. Most fluorescence maxima were reached by day 5, except for the cell nuclei that grew in KCl and sorbitol substituted medium (figure 4.7).
Figure 4.7. Changes in the mean G₀/G₁ peak position of BY-2 nuclei grown in various media supplemented with a 2,4-D. Cells were grown in 12 well plates (2 mL of culture in each well) and supplemented with 700 nM of 2,4-D each day. After daily harvesting, cells were converted to protoplasts (sample concentration of approximately 1 x 10⁵ protoplasts/mL) and incubated for 5 min with PI (18 µM) and Triton X-100 (0.1%). The released nuclei were then analysed using flow cytometry, where 50,000 events were recorded. Maximal PI fluorescence was attained on day 5 for all of the medium treatments, except in the KCl and sorbitol treated nuclei, where the maximal fluorescence occurred on day 6. Each data point represents the average of two duplicate samples, where the mean channel number for the G₀/G₁ DNA peak was measured in each duplicate. Error bars represent ± 1 standard error.

4.2.4 Growth curves of BY-2 cells grown in 12 well plates

BY-2 cells grown in complete medium quickly established a high rate of cell proliferation, doubling in number within the first 12 h post subculturing and reaching a maximum density of 1.53 x 10⁶ cells/mL on day 5 (data reproduced from chapter 3 for comparative purposes). Sucrose appeared to be a very important medium component, with its absence causing severe limitations on cell proliferation, as none of the cultures grown without sucrose attained a density greater than 6 x 10⁵ cells/mL (figure 4.8). Cell counts were obtained from the samples used in section 4.2.1 (before PI staining).
Figure 4.8. Cell counts were taken from BY-2 cells grown in various media preparations and plotted over the course of ten days. The cells were grown as 2 mL cultures in 12 well plates and counted with the aid of a haemocytometer. Cells grown in media lacking sucrose divided very slowly and took longer to reach maximum counts when compared to cells grown in complete medium. Each data point represents the mean of six counts. Error bars represent ± 1 standard error.

Once cell counts were calculated, optical density measurements were taken for the same samples. The two cell estimation methods did not correlate exactly, with cell counts peaking between day 5 and 7 (figure 4.8) and cell densities peaking between day 6 and 10 (figure 4.9). This discrepancy may be explained by a build up of cellular debris influencing the later optical density measurements, but the overall difference in cell growth between the complete medium and medium lacking sucrose was apparent.
Figure 4.9. Optical density measurements were taken from BY-2 cell samples grown in different media, over a ten day growth period. The cells were grown as 2 mL cultures in 12 well plates and optical density was measured at 620 nm in a 96 well plate. Cell densities in the sucrose starved cultures attained just over half of the maximum density measured in the complete medium grown culture. Six replicate samples were averaged for each time point and the included error bars designate ± 1 standard error.

4.2.5 Cell viability assessment during growth of BY-2 cells in various media

Sucrose starvation and its impact on the rate of BY-2 cell attrition was assessed over time and comparisons made between the medium types tested. Evaluation of live and dead cells utilised a combination of the vital stain fluorescein diacetate (FDA), which is taken up by living cells and metabolised to a fluorescent, cell non-permeable acid; and PI, which is actively pumped out of viable cells and reaches the nucleus of non-viable cells due to a compromised cell wall/membrane (figure 4.10).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth

4.2.5.1 Live and dead cell proportions when grown in complete medium

The percentage of live cells remained stable at approximately 90% for the first 4 days, with viability steadily declining from day 5, until no FDA positive cells were present at day 13 (figure 4.11).
Figure 4.11. Proportion of live versus dead BY-2 cells as measured by co-staining with FDA and PI. Cells were grown as 2 mL cultures in 12 well plates, with samples taken daily, co-stained with FDA (2.4 μM) and PI (3.0 μM) for 10 min, before counts were taken under a haemocytometer grid. Maximum viability was seen on day 4, with no viable cells observed after day 13. Cells were sampled over the course of twenty days when grown in complete medium, with the mean of five replicate counts presented for each time point. Error bars correspond to ± 1 standard error.

4.2.5.2 Live and dead cell proportions when grown in sucrose depleted medium

The decline in viability of BY-2 cells grown in medium lacking sucrose and in sorbitol substituted medium was more gradual than that seen when cells were grown in complete medium. FDA positive cells were still observed in cultures up until day 16 and 50% viability was reached at day 10, compared to day 8 in the complete medium culture (figures 4.12 and 4.13).
Figure 4.12. Proportion of live versus dead BY-2 cells as measured by co-staining with FDA and PI. Cells were grown as 2 mL cultures in 12 well plates, with samples taken daily, co-stained with FDA (2.4 μM) and PI (3.0 μM) for 10 min, before counts were taken under a haemocytometer grid. Initial viability did not improve after subculturing and by day 16, the culture was not viable. Cells were observed over the course of twenty days when grown in medium lacking sucrose. Each sampling point is the mean of five replicate counts and the error bars represent ± 1 standard error.
Figure 4.13. Proportion of live versus dead BY-2 cells as measured by co-staining with FDA and PI. Cells were grown as 2 mL cultures in 12 well plates, with samples taken daily, co-stained with FDA (2.4 μM) and PI (3.0 μM) for 10 min, before counts were taken under a haemocytometer grid. The proportion of cells within the culture that were viable improved until day 4, after which viability declined until no viable cells were present by day 16. Cells were observed over the course of twenty days when grown in sorbitol substituted medium. For each sampling point, a mean of five replicate counts was taken and the error bars represent ± 1 standard error.

4.2.5.3 Live and dead cell proportions when grown in sucrose and nitrate depleted medium

A sharp decline in viability was noted from day 5 and few cells remained viable by days 11 and 12, when cultured in medium lacking both carbon and nitrogen (sorbitol and KCl substituted). Between days 5 and 9, a population of cells were identified as both FDA and PI negative. Although these cells seemed intact and resembled FDA positive cells (structurally) when viewed using phase contrast microscopy, they did not fluoresce with either stain and were categorised as having an ‘unknown’ viability capacity. It is possible that these cells were in an intermediate phase, where FDA was no longer being hydrolysed, but the cell membrane was still functional, thereby excluding PI (figure 4.14).
Figure 4.14. Proportion of live versus dead BY-2 cells as measured by co-staining with FDA and PI. Cells were grown as 2 mL cultures in 12 well plates, with samples taken daily, co-stained with FDA (2.4 μM) and PI (3.0 μM) for 10 min, before counts were taken under a haemocytometer grid. Culture viability reached its peak on day 4, but quickly declined on days 5 and 6. Some cells were negative for both stains, and therefore these cells were assigned a status of unknown vitality. No viable cells were detected by day 13. Cells were observed over twenty days when grown in medium substituted with sorbitol and KCl, and each time point consists of the mean of five replicate counts. Error bars represent ± 1 standard error.

4.2.6 Changes in pH during the growth of BY-2 cells

The pH of cultures medium is an important factor for cell growth and the effect of sucrose deprivation on culture pH was determined. All media formulations were adjusted to pH 5.8 before autoclaving, as without adjustment the media were too acidic to support BY-2 cell growth. After autoclaving, the pH decreased in all medium types, which was especially evident in the complete medium, which dropped from pH 5.8 to pH 5.2. This then increased and stabilised to around pH 5.4 after 5 days of growth. In growth media that lacked sucrose, cultures became more basic, with a range of values between pH 5.8 and pH 6.1. The inclusion of sucrose created a more acidic environment, but seemed to buffer the pH better during growth (figure 4.15).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

Figure 4.15. The pH levels plotted during the course of BY-2 cell growth in various medium types. Cultures were grown in 12 well plates (2 mL culture volume), with their pH tested on a daily basis. The complete medium (containing sucrose) decreased by 0.6 pH units after autoclaving, whereas the media lacking sucrose had a decrease of approximately 0.2 pH units post autoclaving. All cultures exhibited an increase in pH after subculturing, with the complete medium culture remaining more acidic throughout the ten days of sampling. Each point represents the average pH value of six supernatant sample replicates and the included error bars represent ± 1 standard error.

4.2.7 Sucrose concentration in BY-2 12 well cultures grown in various media

To get a better understanding of sucrose utilisation and determine the point at which sucrose is exhausted in a batch culture grown in complete medium, sucrose concentration analysis was undertaken. Levels in cell-free medium samples were rapidly depleted, with concentrations ranging from 3.7 to 7.5 mM after 6 days of culturing in complete medium. It is likely that there was no sucrose present after day 6 and these residual levels are a result of the assays detection limit. As expected, sucrose was not reported at high levels in media lacking sucrose addition (figure 4.16).
Figure 4.16. Sucrose concentrations in different BY-2 media were measured over the course of ten days of growth. Cells were grown as 2 mL cultures in 12 well plates. Samples were centrifuged and the supernatant treated with invertase, to convert sucrose to glucose. A PGO enzyme preparation (Sigma-Aldrich, Saint Louis, MO, USA) was used to quantify glucose as per the manufacturer’s instructions. The sucrose concentration in the complete medium grown culture decreased for the first six days, before approaching measured levels in the cultures lacking sucrose. Each data point represents the average of duplicated supernatant sample measurements. Included error bars represent ± 1 standard error.

4.2.8 Protein content in the different BY-2 media from 12 well BY-2 cultures

Total protein content within the cell-free medium showed disproportionally high levels to be present in medium containing sucrose, when assayed using the BCA protein quantification kit. Although the kit stated that its reagents were not affected by sucrose (below 40%), there seemed to be large differences in protein content of sucrose-containing and sucrose-free media. Dialysis of each medium sample allowed a better comparison of total protein levels, but the sucrose-containing medium (complete medium) still displayed elevated protein content. Regardless, the overall trend in all of the media analysed was a decrease in the total protein present from day 1, with a sharp increase on day 10 (figure 4.17). This was perhaps due to increased amounts of cell death and subsequent cell lysis releasing cytoplasmic proteins into the medium.
Figure 4.17. Total protein content in different BY-2 media following dialysis, over the course of a ten day batch culture. Cultures were grown in 12 well plates, with a volume of 2 mL per well. Supernatant samples were dialysed over 15 h using 6-8 kDa cut-off dialysis tubing, and the resulting dialysed liquid was tested for total protein content using a BCA protein assay (Pierce, Rockford, IL, USA) as per the manufacturer’s instructions. In cultures lacking sucrose, levels of protein in the dialysed supernatant were consistently lower than seen in the complete medium grown culture, and trended downward from day 1. All of the final samples on day 10 were markedly elevated, perhaps as a result of cell death and cell lysis. Each data point represents the average of duplicate samples and error bars of ± 1 standard error are reported.

4.2.9 Depletion of 2,4-D in different BY-2 media from 12 well BY-2 cultures

The role of 2,4-D during sucrose starvation was investigated and found to drop dramatically within the first 24 h of growth in the media analysed. Initially added to achieve a final concentration of 900 nM in all the medium formulations, the 2,4-D levels beyond day 5 were undetectable in the complete medium. In the sorbitol substituted medium culture, 2,4-D levels ranged between 168 nM and 250 nM from day 1 to day 10, perhaps suggesting a stall in cell growth and proliferation (figure 4.18).
Figure 4.18. Depletion of 2,4-D levels in the medium of BY-2 cells grown in the presence of sucrose (complete medium) and absence of sucrose (sorbitol substituted medium) over the course of ten days. Samples from shaker cultures (100 mL culture volume) were centrifuged and passed through a 0.2 µm filter. The 2,4-D content was then quantified using HPLC. Beyond day 5, 2,4-D levels were undetectable in the complete medium culture, but remained at low levels in the sorbitol substituted culture. Each data point represents a single sampling.

4.2.10 BY-2 cell morphology

When cultured in complete medium, BY-2 cells grew in a chain formation, ranging from 2 to >30 cells in length. Individual cells were also seen, but were less frequent in young cultures. Cells forming the chains interior were generally cylindrical in shape, whereas the cells on both ends tapered and were more of a conical shape. Most nuclei were in a central position and transvacuolar strands were an abundant and dominant feature of the intracellular space. With further culture growth, more cells broke away from the chain formation and discrete cells became more common. The isolation of single cells meant that their shape became more spherical and as they aged, transvacuolar strands became less common. Most cells appeared non-viable after 12 days of culture, with the cell contents appearing shrivelled and the cell membrane disassociated from the cell wall (figure 4.19).
Figure 4.19. Morphology of BY-2 cells grown in complete medium using phase contrast microscopy, over a time course of twelve days. Cells at day 0 (a) had distinct transvacuolar strands and appeared approximately rectangular when viewed from above. At day 4 (b), the cells had not changed substantially, but at day 8 (c) many appeared more rounded and separate from their normal chain formation. By day 12 (d), many cells had become non-viable. Scale = 20 µm.

Sucrose deprivation had pronounced effects on the morphology of the BY-2 cells. The overall size and shape was altered, with cells shrinking and becoming more spherical in form. Transvacuolar strands were lost and most of the nuclei became marginalised by day 4. Cell chains appeared to form clumps more readily and the clumps often consisted of several hundred cells. Although some cells were dead by day 12, there were seemingly more viable cells present than seen at day 12 in the complete medium culture (figure 4.20).
Figure 4.20. Morphology of BY-2 cells grown in medium lacking sucrose (sorbitol substituted) using phase contrast microscopy, over a time course of twelve days. Cells at day 0 (a) had distinct transvacuolar strands and appeared approximately rectangular when viewed from above. After day 4 (b), the cells became more spherical and lost the majority of their transvacuolar strands, also seen at day 8 (c). By day 12 (d), some cells had become non-viable. Scale = 20 µm.

4.2.11 Formation of spherical bodies in response to protease inhibition

To induce the formation of suspected autophagosomes (by arresting the assimilation of recycled proteins into the vacuole), BY-2 cells growing in complete medium were treated with a cocktail of protease inhibitors that resulted in the formation of spherical bodies after only 24 h. Although similar to the vesicles seen during nitrate starvation (see section 5.2.10), as they often formed around the nucleus, these vesicles differed in several aspects; they appeared to be smaller and were not always limited to the perinuclear region (figure 4.21).
BY-2 cells grown in complete medium and treated with a cocktail of protease inhibitors over the course of fifteen days. After the first day of treatment (a), the cells had already developed spherical bodies, which were retained through day 4 (b) and day 8 (c). By day 15 (d), most cells had become non-viable (PI positive and FDA negative staining). Scale = 20 µm.

BY-2 cells treated with the cocktail of protease inhibitors and starved of sucrose (sorbitol substituted medium) also presented spherical bodies in a similar manner to those seen in cells grown in complete medium, with one major difference. During the later stages of culture development, some cells appeared to be transparent and devoid of organelles. These ‘empty’ cells were smaller than normal healthy cells, likely comprising of only the cell wall and cell membrane (figure 4.22).
Figure 4.22. BY-2 cells grown in sucrose deprived medium (sorbitol substituted) and treated with a cocktail of protease inhibitors over the course of fifteen days. Protease inhibition resulted in formation of spherical bodies in the perinuclear region after only one day (a), with their retention through day 4 (b) and day 8 (c). Many cells appeared devoid of organelles or had become non-viable by day 15 (d). Scale = 20 µm.

4.2.12 Staining of sucrose deprived BY-2 cells with monodansylcadaverine

During withdrawal of sucrose, the BY-2 cells were converted into protoplasts and stained with MDC to test for the appearance of acidic vesicles. In cells starved of sucrose (sorbitol substituted), MDC positive vesicles were seen 3 days after transferral to the new medium, reaching a maximum density on day 4 (figure 4.23). Very few MDC positive vesicles were observed in BY-2 cells grown in complete medium at day 4, but such vesicles reached a similar maximum density to that observed in 4 day old sorbitol substituted cells, after 8 days of culturing (data not shown).
Figure 4.23. MDC fluorescence emitted by a protoplast that was isolated from a BY-2 cell grown in sorbitol substituted medium (starved of sucrose). Cells were grown in 100 mL shaker cultures, converted into protoplasts at different sampling points, incubated for 10 min with MDC (40 µM), washed and viewed under a fluorescent microscope. The image above was taken four days after subculturing into the new medium. MDC fluorescence highlighted the presence of acidic vesicles. Scale = 20 µm.

4.2.13 BY-2 cell area measured in two dimensions

When measurements of length and width were taken from BY-2 cells grown in each of the medium types, trends in the two dimensional area and therefore the inferred volume of cells could be followed over time. Changes could be classified into three general trends; cells grown in complete medium retained a relatively stable size, cells deprived of sucrose (medium lacking sucrose and sorbitol substituted medium) became progressively smaller after day 3 and cells deprived of nitrates and sucrose (KCl and sorbitol substituted) became progressively larger after day 5 (figure 4.24).
Figure 4.24. Cell areas based on two dimensional (length x width) measurements in BY-2 cultures grown in different media. Cultures grown in 12 well plates (2 mL per well) were sampled to view under a microscope and representative images taken to allow dimensional measurements. Cells deprived of sucrose (minus sucrose or sorbitol substituted media) became progressively smaller, whereas cells deprived of both sucrose and a nitrogen source developed a larger two dimensional area. When grown in complete medium, BY-2 cell area was more stable throughout culturing. Each time point represents the average measurement of 12 cells chosen at random from a slide preparation. Error bars are included and represent ± 1 standard error.

4.2.14 Total protein content estimation within BY-2 cells during growth in various media

The total protein content within BY-2 cells (excluding the surrounding medium) was analysed over the course of ten days and expressed as protein weight per litre of culture (figure 4.25).
Figure 4.25. Total protein content of BY-2 cultures grown in different media over the course of ten days. During culturing in 12 well plates, the samples taken were centrifuged, the supernatant discarded and the cell mass dried. The cell pellet was then frozen in liquid nitrogen, ground to a powder and resuspended in a protein extraction buffer. After vortex mixing and sonication, the cell homogenate was centrifuged. The resulting supernatant was used for protein determination using a BCA protein assay kit (Pierce, Rockford, IL, USA) as per the manufacturer’s instructions. With a complete medium, total protein content in the BY-2 culture increased until day 5, after which a decrease in content was observed. Cultures lacking sucrose had lower total protein content measurements than the complete medium grown cultures. Protein content was measured from cell dry mass (excluding medium) and expressed as grams of protein per litre of culture. Error bars are representative of ± 1 standard error, from duplicated samples.

On a per cell basis (cell count data not shown), cells grown in complete medium, medium lacking sucrose and sorbitol substituted medium retained similar levels of proteins, ranging from approximately 690 pg/cell at day 0, to approximately 400 pg/cell at day 10. All cultures had less protein per cell by day 10 than when they were freshly subcultured (day 0), with the culture grown in KCl and sorbitol substituted medium losing the most protein overall (figure 4.26).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

Figure 4.26. Protein content expressed on a per cell basis, for BY-2 cells grown in various media over the course of a ten day batch culture. Previous total cell protein content measurements (see figure 4.25) were divided by cell counts taken earlier from the same samples (data not shown) to obtain a measurement of protein per cell. Cells grown in KCl and sorbitol substituted medium retained less protein than those grown in complete medium or medium lacking sucrose (minus sucrose and sorbitol substituted media). When compared to protein levels present following subculturing, all cultures consisted of cells with depleted protein content at day 10.

4.2.15 Nitric oxide levels in BY-2 cells grown in various media

Nitric oxide (NO) is now regarded as a key signalling molecule in plants, where it orchestrates a plethora of cellular activities associated with growth and development. To determine if NO plays a role in autophagic processes and/or chromatin remodelling, levels of cellular NO were measured using the indicator 4-amino-5-methylamino-2’,7’-difluorofluorescein (DAF-FM), with positive controls elicited by applying the NO-releasing agent S-nitroso-N-acetylpenicillamine (SNAP) 24 h before sampling. Interpretation of the histograms displaying DAF-FM fluorescence proved difficult (figure 4.27) and trends were easier to visualise when plotted over time (figure 4.28).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

(a) (b) (c) (d) (e) (f)

Complete medium

Sorbitol medium

DAF-FM fluorescence

DAF-FM fluorescence

DAF-FM fluorescence

DAF-FM fluorescence

DAF-FM fluorescence

DAF-FM fluorescence

Count

Count

Count

Count

Count

Count

No sucrose medium

Sorbitol medium

DAF-FM fluorescence

DAF-FM fluorescence

DAF-FM fluorescence
BY-2 protoplasts obtained from cells grown in complete medium displayed a peak mean DAF-FM fluorescence value that ranged between channel 23 and channel 34 during the first 6 days, after which the mean DAF-FM fluorescence intensity increased to channel 54 by day 9. In comparison, the cells sampled from the other media types displayed lower x-peak mean channel values between day 5 and day 10 (figure 4.28).
Figure 4.28. Relative NO levels inferred from the fluorescence of DAF-FM stained BY-2 protoplasts obtained from cells grown in various media. Cultures grown in 12 well plates (2 mL volume) were treated with DMSO (10 µL) 24 h before sampling. On the day of sampling, the cells were converted to protoplasts (approximately 1 x 10^5 protoplasts/mL), stained with DAF-FM (5 µM), washed and analysed using flow cytometry. In complete medium grown cells, DAF-FM fluorescence increased markedly after day 5, whereas no large increases were observed in cells grown in the other media types during this time. Each data point represents an average of duplicate samples, measuring the DAF-FM fluorescence peak mean channel value. The included error bars represent ±1 standard error from duplicated samples.

When replicates of the cultures presented in figure 4.28 were treated with SNAP (24 h before staining with DAF-FM), much higher fluorescence intensities were observed. The ability of SNAP to release NO or the response of DAF-FM to NO in cells older than 2 days post subculturing seemed to be suppressed. There was some increased fluorescence observed in complete medium cultured BY-2 protoplasts after day 4, reaching maximal fluorescence intensity by day 8, after which the signal intensity dropped off (figure 4.29).
Figure 4.29. DAF-FM fluorescence inferring relative NO levels in BY-2 protoplasts treated with SNAP, during the course of ten days. Cultures grown in 12 well plates (2 mL volume) were treated with SNAP (25 nM) 24 h before sampling. On the day of sampling, the cells were converted to protoplasts (approximately $1 \times 10^5$ protoplasts/mL), stained with DAF-FM (5 μM), washed and analysed using flow cytometry. A substantial decrease in DAF-FM fluorescence following subculturing was observed until day 2 in all of the cultures. From day 4, elevated DAF-FM fluorescence was seen in samples taken from the complete medium culture, whereas samples from the other medium treatments displayed comparatively lower fluorescence. Each data point obtained from the various medium treatments was the mean of duplicate samples measuring the DAF-FM peak mean channel number. Error bars are included and represent ± 1 standard error from duplicated samples.

### 4.2.16 Intracellular calcium levels in BY-2 protoplasts

#### 4.2.16.1 Intracellular calcium levels in freshly subcultured BY-2 protoplasts

One possible marker of the cellular stress associated with sucrose starvation (and therefore a potential autophagic marker) is calcium release from the endoplasmic reticulum into the cytosol. Intracellular calcium levels were measured using the cell permeant dye 2-[4-[Bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxyl]ethoxy]phenyl]-1H-indole-6-carboxylic acid penta acetoxyethyl ester (indo-1 AM). After establishing a baseline indo-1 fluorescence ratio (400:475 nm fluorescence emission ratio), a calcium flux was elicited by spiking the sample with ionomycin. The ability of the protoplasts to recover was then observed. Freshly subcultured BY-2 protoplasts had a mean baseline
intracellular [Ca$^{2+}$] of 395 nM. After ionomycin addition, there was a recovery period of 40 s, which resulted in a final mean intracellular [Ca$^{2+}$] of 447 nM (figure 4.30).

Figure 4.30. Response of the indo-1 fluorophore to the addition of ionomycin, in BY-2 protoplasts from a day 0 culture (freshly subcultured). Protoplasts isolated from 12 well plate cultures were incubated with indo-1 AM (1 µM) for 30 min, before washing and analysing using flow cytometry. After addition of ionomycin (2 µM), intracellular [Ca$^{2+}$] were returned to near baseline levels of 395 nM, demonstrating the protoplasts ability to modulate intracellular calcium levels. The ratio displayed on the y-axis is calculated from indo-1 fluorescence emissions at 400 nm and 475 nm. The trace is divided into four chronologic sections; initial baseline, ionomycin uptake, recovery period and final baseline. The arrow represents the point at which ionomycin was added and intracellular calcium concentration is measured in nM.

### 4.2.16.2 Intracellular calcium levels in BY-2 cells grown in complete medium

While protoplasts derived from a 4 day old culture had a similar mean baseline indo-1 ratio to freshly subcultured cells (section 4.2.16.1), there did not seem to be a recovery period after ionomycin treatment. The day 4 sample did not attain baseline (or close to baseline) ratios within the 450 s sample run, suggesting that high levels of intracellular Ca$^{2+}$ remained in these protoplasts after ionomycin treatment. When 8 day old protoplasts were subjected to the same test procedure, the mean baseline intracellular [Ca$^{2+}$] was higher at 490 nM. After addition of ionomycin, there was a recovery period.
of 58 s, before a final mean intracellular \([\text{Ca}^{2+}]\) of 1297 nM was reached, suggesting that a high level of intracellular \(\text{Ca}^{2+}\) also remained in 8 day old protoplasts after ionomycin treatment (figure 4.31).

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Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

Figure 4.31. The response of the indo-1 fluorophore to the addition of ionomycin, in BY-2 protoplasts at day 4 (a) and day 8 (b), when grown in complete medium. Protoplasts were isolated from 12 well plate cultures and incubated with indo-1 AM (1 µM) for 30 min, before washing and analysing using flow cytometry. Baseline intracellular calcium levels became progressively higher from day 4 to day 8, and the ability of protoplasts to recover their baseline $[\text{Ca}^{2+}]$ after ionomycin (2 µM) challenge was non-existent or severely limited. The ratio displayed on the y-axis is calculated from indo-1 fluorescence emissions at 400 nm and 475 nm. The trace is divided into four chronologic sections; initial baseline, ionomycin uptake, recovery period (absent in the day 4 sample) and final baseline. The arrow represents the point at which ionomycin was added and intracellular calcium concentration is measured in nM. The flow cytometer became blocked between 400 s and 500 s while running the day 8 sample, causing apparent spikes in indo-1 fluorescence.

4.2.16.3 Intracellular calcium levels in BY-2 cells grown in sorbitol substituted medium

When grown in sorbitol substituted medium for 4 days, the BY-2 protoplasts had a lower mean baseline intracellular $[\text{Ca}^{2+}]$ of 365 nM, which was raised slightly upon addition of ionomycin to 492 nM, with no discernable recovery period. By day 8, the mean baseline intracellular $[\text{Ca}^{2+}]$ was very high (5045 nM) and did not respond significantly to addition of ionomycin, indicating the presence of constant high levels of intracellular $\text{Ca}^{2+}$ (figure 4.32).
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

![Graph showing carbon source availability and BY-2 cell growth](image)

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Figure 4.32. Response of the indo-1 fluorophore to the addition of ionomycin, in BY-2 protoplasts at day 4 (a) and day 8 (b), when grown in sorbitol substituted medium. Protoplasts were isolated from 12 well plate cultures and incubated with indo-1 AM (1 µM) for 30 min, before washing and analysing using flow cytometry. While the baseline $[Ca^{2+}]$ on day 4 was only slightly above that seen at day 0, by day 8 the baseline was elevated by almost thirteen times that of the day 0 measurement. Upon ionomycin (2 µM) challenge, no recovery was observed in the protoplasts produced at day 4 and a weak uptake response was measured in day 8 protoplasts. The ratio displayed on the y-axis is calculated from indo-1 fluorescence emissions at 400 nm and 475 nm. The trace is divided into three chronologic sections; initial baseline, ionomycin uptake and final baseline. The arrow represents the point at which ionomycin was added and intracellular calcium concentration is measured in nM.

### 4.3 Discussion

The previous chapter described fluctuations of PI staining in BY-2 cells cultured in complete medium. It was theorised that these changes were a result of rapid sucrose depletion, subsequent autophagy and potentially autophagic cell death (ACD). Would withdrawal of sucrose from the growth medium exacerbate or bring on early fluctuations in PI staining, thereby providing a link between this observation and sucrose starvation or autophagy? While sucrose starvation did not intensify the PI staining maxima, there was a delay in the return to baseline PI fluorescence intensities when compared to cells grown on complete medium (figure 4.2). This observation suggests that the changes in PI fluorescence are unlikely to be a direct result of sucrose...
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

starvation and the delay in fluorescence maxima is caused by slow growth and extended vitality in the sucrose starved cultures (figure 4.12 & 4.13). Interestingly, 2,4-D supplementation removed the delay in PI fluorescence returning to baseline levels in sucrose starved cells (figure 4.7). Further experiments would be necessary as the mechanism causing this change in timing is not known, but may involve increased cell proliferation as a result of the synthetic auxin supplementation.  

Examination of medium composition during the course of culture growth highlighted the highly active nature of BY-2 cells and provided insights into the utilisation and metabolism of nutrients. Coupled to this, the timeframe involved in culture growth from initiation through proliferation and stationary phase to death was investigated with regard to understanding the underlying processes in both nutrient rich and deprived conditions. Because it is important to understand BY-2 cell growth under both carbon limitation and ‘normal’ conditions (growth in complete medium), the experiments carried out attempted to provide a basis for subsequent chapters.  

Growth curves of the BY-2 cells cultivated in complete medium displayed an initial doubling time of 12 h, compared to a reported doubling time of between 13 h (Nagata and Kumagai, 1999) and 16 h (seen in continuous culture) (Nagata et al., 1992). It seems that the major limiting factor in BY-2 culture proliferation was the carbon source, sucrose, without which, the initial doubling time was extended to 89 h. None of the three sucrose starved cultures produced a density of more than $6 \times 10^5$ cells/mL (figure 4.8).  

A comparison of BY-2 culture health as assayed using live/dead differential stains, showed a distinction between cell growth and longevity in the different medium types. When grown in complete medium, BY-2 cultures retained $>90\%$ viability until day 4, and retained a vital population until day 13 post subculture (figure 4.11). Sucrose starvation seemed to extend the lifespan of the BY-2 cultures, with viable (FDA positive) cells still detectable after 15 days of culturing (figure 4.12 & 4.13). Longevity studies in other cellular model organisms placed in a nutrient poor environment have also produced similar results (Hansen et al., 2008; Vellai, 2009), although in the case of BY-2 cells deprived of both sucrose and nitrates, the result was a culture of shortened lifespan. In these cultures it was interesting to note that when stained with FDA and PI a double negative staining population was observed in older cells (figure 4.14). This was most likely attributed to cells in a transitional phase, no longer able to hydrolyse FDA, but still retaining an intact cell membrane and poses further questions surrounding the ‘point of no return’, where the cell is unable to be rescued from death.
When analysing the medium composition during BY-2 cell growth, pH values, sucrose levels, total protein content and 2,4-D concentrations were taken into account. A cell cultures micro-environment can have major impacts on individual cell metabolism and therefore the activation or inactivation of cellular pathways utilised to cope with the change. Examples include; pH changes effecting cell differentiation in carrot suspension cultures (Jay et al., 1994), morphological modifications linked to levels of 2,4-D in tobacco cells (Boot et al., 1993; Nagata et al., 2004) and sucrose impacting on expression of cell cycle genes (Richard et al., 2002). It was necessary to correlate alterations in the medium composition to the morphological and physiological changes discussed in further chapters.

During observation of pH levels in the different growth media, it was apparent that sucrose had a significant effect on the stability of pH levels during culture growth. Cultures lacking sucrose displayed a gradual increase in pH, reaching between pH 5.8 and 6.1, whereas the sucrose containing culture (complete medium) maintained a pH of between 5.2 and 5.5 (figure 4.15). Sucrose has been known to lower the pH of phosphate containing solutions (Chuy and Bell, 2006), but it may prevent the raised pH condition seen in cultures that lack it. Other explanations include the possibility of a proliferating cultures improved ability to modify the surrounding pH to optimum levels (i.e. in cultures provided with sucrose), production of substances in sucrose starved cells that increase the pH or consumption of acidic species in the medium by sucrose starved cells.

Further analysis of the surrounding medium during growth of BY-2 cells in the different medium formulations focussed on protein content. While it was clear that sucrose was interfering with the BCA protein quantification assay (despite the manufacturer stating that the kit was not affected by sucrose levels lower than 40%), dialysis of the samples removed most of it. Despite this, perceived total protein levels in the complete medium still remained high over the course of culture growth, perhaps due to the contribution of residual sucrose to the BCA assay. High cell turnover, with the death and degradation of cells may also account for the higher protein levels in the complete medium culture. Total protein content in the medium of sucrose deprived cultures decreased gradually over time, but a spike on day 10 (in all cultures) may have been due to increased cellular degradation as the batch cultures declined in viability (figure 4.17).

Uptake of 2,4-D from the medium was examined in two of the different medium formulations, with most 2,4-D utilised (or degraded) during the first 24 hours post
subculturing (between a 75% and 77% reduction in levels). Levels became undetectable by day 5 in the complete medium and levels remained low, but still detectable at approximately 200 nM in the sorbitol substituted culture for the duration of testing (until day 10) (figure 4.18). Low levels of auxins (of which 2,4-D is a synthetic form) have been observed to promote cell elongation and growth, whereas higher levels stimulate cell division (Hasezawa and Syono, 1983). The initial drop in 2,4-D levels after 24 h did not have predictable effects on cell morphology or cell division in BY-2 cells grown in the absence of sucrose or in complete medium.

Very distinct cell morphologies became apparent when the BY-2 cells were exposed to sucrose deprivation. Sucrose withdrawal resulted in cells with an approximately 27% smaller footprint (two dimensional area) than those grown in complete medium (figure 4.24). Other notable changes were the disappearance of transvacuolar strands and marginalization of the nuclei in the sucrose deprived cultures (figure 4.20). These observations offer clues as to how nutrient withdrawal effects BY-2 cell growth and function. Previous studies in BY-2 cells identified the presence of membrane bound vesicles thought to be autophagosomes when starved of sucrose and exposed to a cysteine protease inhibitor (Moriyasu and Ohsumi, 1996). Similar vesicles were seen in this study, when sucrose starved cells and cells grown in complete medium were treated with a cocktail of protease inhibitors. They formed in the perinuclear region, were smaller than starch vesicles formed during nitrogen deprivation (see section 5.2.11) and did not increase in volume as the cells aged (figure 4.21 & 4.22). Of particular note was the appearance of cells apparently devoid of organelles in 15 day old cells starved of sucrose (sorbitol substituted medium) and treated with protease inhibitors (figure 4.22). Similar cell corpses have been attributed to a form of cell death termed ‘vacuolar’ cell death, where lytic vacuoles progressively engulf the cytoplasm to a point where the vacuolar membrane (tonoplast) ruptures, releasing hydrolases that cause destruction of the protoplast (VanDoorn et al., 2011). An empty ‘shell’ is all that remains.

MDC staining identified the presence of acidic vesicles, which were observed at an earlier stage in cells grown in the sucrose starved media than complete medium grown cells. These MDC positive vesicles did not have a perinuclear distribution pattern like the vesicles observed with protease inhibitor treatment. It is possible that the decrease in protease activity caused an accumulation of these vesicles in the perinuclear area, while without treatment the vesicles were distributed more evenly throughout the cell before degradation in the vacuole (figure 4.23).

The total protein content in sucrose starved BY-2 cultures was approximately three
times lower than that seen in cultures grown with complete medium at peak protein concentrations (figure 4.25). But on a per cell basis, there appeared to be less protein in the cells grown in complete medium than the sucrose deprived cells, with protein content declining over the course of growth in all media tested (figure 4.26). This trend of declining cellular protein levels is compatible with the observations of others (Inoue and Moriyasu, 2006b) and is often associated with a decline in protein production and an increase in protein degradation seen during autophagy (Abeliovich and Klionsky, 2001; Reggiori and Klionsky, 2002).

The role NO plays during sucrose starvation in plants has not been previously described, but its use as a signalling molecule in plant stress responses and programmed cell death (PCD) associated with the hypersensitive response (HR) (Hong et al., 2008) were reasons to investigate the possible involvement of NO in mediating autophagy induced by sucrose withdrawal. There did appear to be increased NO production toward the later stages of BY-2 cell culturing in complete medium (figure 4.28) and this could have contributed to the increase in cell death seen as the culture aged and became nutrient limited (figure 4.11). A proposed connection between NO signalling and autophagy has been suggested (Greenberg, 2005), citing evidence from a study that showed PCD of plant cells in response to distant pathogen-induced HRs (Alvarez et al., 1998). Contrary to this observation, but another possible link between autophagy and NO-initiated PCD involves the Nicotiana benthamiana gene BECLIN 1 (a plant ortholog of mammalian ATG6/VPS30/beclin 1, which is required for the initiation of autophagosomes). BECLIN 1-deficient plants infected with tobacco mosaic virus (TMV) showed a HR not only at the site of infection, but in non-infected distal sites. This result suggested to the authors that autophagy negatively regulates HR-mediated PCD (Liu et al., 2005). While the HR produces a very rapid form of plant cell death and utilises NO as a signalling molecule to induce reactive oxygen species, salicylic acid and Ca\(^{2+}\) influxes (Mur et al., 2006), the cell death associated with nutrient limitation is much slower and probably does not involve the same sequence of events.

High cytoplasmic Ca\(^{2+}\) levels have been associated with endoplasmic reticulum (ER) stress and subsequent autophagy in response to accumulation of unfolded proteins (Hoyer-Hansen and Jaattela, 2007). A similar event was theorised to occur in response to autophagy induced by sucrose starvation in BY-2 cells. Evidence supporting this was seen in aging sucrose starved cells, when Ca\(^{2+}\) baseline levels were higher than seen at earlier time points and higher than when cells were cultured in complete medium (as measured using the indo-1 fluorescence ratio) (figure 4.31 & 4.32). The failure of
Chapter 4: Carbon source availability and its effect on BY-2 cell growth...

Ionomycin to elicit Ca\(^{2+}\) uptake and the lack of a subsequent recovery phase suggests that sucrose starved cells do not have functional Ca\(^{2+}\) pumps or compromised cell membranes (Evans et al., 1994) by day 8 (figure 4.32). Chelation of intracellular Ca\(^{2+}\) by 1,2-bis(2-aminophenoxy)ethane-\(N,N,N',N'\)-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) has been shown to significantly block autophagy in murine cardiac myocytes. The authors of this study identified “calcium homeostasis as an essential component of the autophagic response to nutrient deprivation.” (Brady et al., 2007). While this statement was expressed based on evidence gathered from mammalian cell experiments, the conserved nature of autophagy suggests the likelihood of calcium fulfilling a similar role in plants that undergo sucrose starvation-induced autophagy.
5. The impact of nitrogen withdrawal on BY-2 cell growth and chromatin state

5.1 Introduction

The previous chapter considered the effect of sucrose deprivation on BY-2 cell growth, induction of autophagy and chromatin conformation changes as measured by propidium iodide (PI) fluorescence. In this chapter, parallel studies were conducted with respect to nitrate deprivation, as nitrogen starvation is known to induce autophagy in many cell types (Bassham et al., 2006; Patel et al., 2006; Klionsky et al., 2007).

Nitrites are essential for the production of amino acids and thus of protein synthesis in plants. Amino acid formation is achieved through the two step reduction of $\text{NO}_3^-$ to form $\text{NH}_4^+$, which is quickly incorporated into other organic compounds (due to its toxicity) such as $\alpha$-ketoglutarate. This is in turn converted to glutamate, which is used as the basis for synthesis of other amino acids (Dennis and Layzell, 1997).

Without the nitrate building blocks of amino acid synthesis in plants, cellular processes including cell division would come to a halt, save for the actions of the protein recycling process of autophagy. Nitrate deprivation has been successfully used as an initiator of autophagy in other model systems, providing vital insights into the role autophagy has in times of nutrient starvation (Bassham, 2007; Ma et al., 2007).

Autophagy defective *Saccharomyces cerevisiae* mutants were identified after their growth in nitrogen limited medium resulted in a hastened loss of viability when compared to the wild type. This led to the initial description of fifteen *APG* genes in yeast (Tsukada and Ohsumi, 1993). There is also evidence for selective autophagy in BY-2 cells grown under nitrate limited conditions. A cytochrome b5-red fluorescent protein (Cytb5-RFP) fusion was processed faster than other proteins and was co-localised with a tobacco Atg8 homolog-yellow fluorescent protein (NtAtg8-YFP) fusion in autophagosomes (Toyooka et al., 2006). This indicated specific targeting of the Cytb5 protein for degradation and amino acid recycling as a response to nitrate limitation.

The formation of starch granules has been observed in several plant cell culture systems exposed to varying experimental conditions (Chen et al., 1994; Smith et al., 1997; Miyazawa et al., 2002). These energy storage vessels develop from a specific type of leucoplast termed an amyloplast and usually form in storage tissues such as...
tubers, endosperm and cotyledons, as well as differentiated plant cells such as root caps. Their formation has been linked to sugar limitation and the subsequent autophagic condition in cultured rice cells (Chen et al., 1994). Preferential formation of these granules during nitrate starvation in BY-2 cells has not been previously described, although nitrate limitation has been shown to repress starch metabolism (Scheible et al., 1997) and the role of auxins in amyloplast formation has also been described (Miyazawa et al., 2002).

BY-2 cells are commonly propagated in a rich complete medium containing large amounts of nitrates (NH$_4$NO$_3$ and KNO$_3$). The molar substitution of the NO$_3^-$ anion present in complete medium, with the Cl$^-$ anion, along with the substitution of NH$_4^+$ with K$^+$ should allow for a suitable medium to study the implications of nitrogen starvation.

Aims of this chapter:

To measure changes in chromatin conformation, as determined by PI staining of the model plant cell line (BY-2) following withdrawal of nitrates; and to compare these changes with other physiological variations occurring after nitrate starvation, observing changes in cell growth and medium composition, with an attempt to describe any possible autophagic condition.

5.2 Results

5.2.1 DNA peak position changes during growth of BY-2 cells in nitrate starved media

When BY-2 cells were cultured under nitrate deficient conditions, there appeared to be a delay in the time when PI fluorescence intensity maxima were reached and these maxima were lower than that seen in the complete medium grown cells (data were included from a parallel experiment presented in chapter 3 (figure 3.6) for comparative purposes). Cells grown in KCl substituted medium reached a mean PI fluorescence intensity maximum of 278 channels by day 5 and cells grown in medium lacking nitrates reached a mean PI fluorescence intensity maximum of 305 channels by day 6 (figure 5.1).
Figure 5.1. Mean G₀/G₁ DNA peaks of BY-2 nuclei stained with PI, measured over time for three medium treatments. BY-2 cells were grown as 2 mL cultures in 12 well plates and harvested every day for production of protoplasts. Nuclei were isolated from the protoplasts during incubation with PI (18 μM) and Triton X-100 (0.1%) for 5 min, before flow cytometric analysis. Approximately 1 x 10⁵ protoplasts/mL were present in each sample. When compared to the complete medium control, fluorescence intensity also increased, then decreased over the course of culturing in media lacking nitrates, but the intensity maximum was lower and delayed by one day in KCl substituted medium and two days in medium lacking nitrates. Each data point represents the average of six replicate sample measurements. Error bars are included (± 1 standard error), but are not discernable at this scale.

When the changes in PI fluorescence were compared with cell counts (measured concurrently and presented from figure 5.6 for comparison) in the BY-2 cells grown in medium lacking nitrates, there appeared to be a close relationship, with the peak in cell population coinciding with the peak in PI fluorescence intensity (day 6) (figure 5.2).
Figure 5.2. BY-2 G₀/G₁ DNA peaks measured using PI staining and the corresponding cell proliferation curve over ten days, when grown in medium lacking nitrates. BY-2 cells grown in 12 well plates were converted to protoplasts, which were then incubated for 5 min with PI (18 µM) and Triton X-100 (0.1%), before analysis using flow cytometry. Each sample comprised of approximately 1 x 10⁵ protoplasts/mL and 50,000 events were captured during the flow cytometry run. Cell counts were performed using a haemocytometer. Maximum PI fluorescence and maximum cell concentration were both reported on day 6. Each data point represents the average of six replicate sample measurements. PI staining data replicated from figure 5.1 for comparative purposes. The included error bars are representative of ± 1 standard error.

G₀/G₁ DNA peak fluorescence shifts observed in BY-2 cells grown in medium where the nitrates were substituted with KCl, reached a maximum of 278 channels by day 5, which was a day earlier than when the cell population reached its maximum level of 1.1 x 10⁶ cells/mL. Regardless of this, the shifts in PI fluorescence intensity still closely modelled the proliferation profile (data gathered concurrently and presented from figure 5.6 for comparison) during these growth conditions (figure 5.3).
Figure 5.3. BY-2 G₀/G₁ DNA peaks measured using PI staining and the corresponding cell proliferation curve over ten days, when grown in KCl substituted medium. BY-2 cells grown in 12 well plates were converted to protoplasts, which were then incubated for 5 min with PI (18 µM) and Triton X-100 (0.1%), before analysis using flow cytometry. Each sample comprised of approximately 1 x 10⁵ protoplasts/mL and 50,000 events were captured during the flow cytometry run. Cell counts were performed using a haemocytometer. Maximum staining of the nuclei occurred on day 5, but the highest cell concentration was not attained until day 6. Each data point represents the average of six replicate sample measurements. PI staining data replicated from figure 5.1 for comparative purposes. Included error bars represent ± 1 standard error.

5.2.2 DNA peak position during growth of BY-2 cells in different media using Hoechst 33342 fluorescence

Duplicate samples to those presented in section 5.2.1 were stained with Hoechst 33342 (H342) and analysed using flow cytometry. There was little variation between media treatment groups and also over the course of culturing of the BY-2 cells in 12 well plates. Results presented in chapter 3 (figure 3.7) were reproduced here for comparative purposes, as these data sets were collected in parallel experiments (figure 5.4).
Figure 5.4. G₀/G₁ DNA peaks of BY-2 nuclei stained with H342, measured over time for three medium treatments. Growth of cells occurred in 12 well plates (2 mL culture volume) and protoplasts (approximately 1 x 10⁵ protoplasts/mL) were freshly made before incubation with H342 (16 μM) and Triton X-100 (0.1%) for 5 min. The released nuclei were then analysed using flow cytometry, with 50,000 events recorded in each sample. DNA peak position and therefore H342 fluorescence did not increase, then decrease over the course of culturing, instead remaining constant throughout. The mean of six replicate sample measurements was obtained (from each media treatment group, on each day) for display in this graph. Error bars (± 1 standard error) are included, but not discernable at this scale.

5.2.3 The effect of 2,4-dichlorophenoxyacetic acid supplementation on DNA peak position

Because 2,4-dichlorophenoxyacetic acid (2,4-D) was quickly depleted from the surrounding medium during culture growth (see section 5.13), daily supplementation was proposed to test the impact of this synthetic auxin on chromatin alterations (PI staining). Both of the nitrate deprived cultures displayed PI fluorescence intensity maxima at day six, one day later than with growth in complete medium when supplemented with 2,4-D. The experiment performed in section 4.2.3 was done in parallel and therefore the complete medium data set was included here for comparison with nitrate deprived culture results (figure 5.5).
Figure 5.5. Changes in the mean $G_0/G_1$ peak position of BY-2 nuclei grown in various media supplemented with a 2,4-D. Cells were grown in 12 well plates (2 mL of culture in each well) and supplemented with 700 nM of 2,4-D each day. After daily harvesting, cells were converted to protoplasts (sample concentration of approximately $1 \times 10^5$ protoplasts/mL) and incubated for 5 min with PI ($18 \mu$M) and Triton X-100 (0.1%). The released nuclei were then analysed using flow cytometry, where 50,000 events were recorded. A one day delay in the PI fluorescence intensity maxima was measured in the cultures lacking nitrates, when compared to the complete medium. Over ten days, daily replicate samples were measured in each of the medium treatments and the average PI peak position plotted in this graph. Error bars of $\pm 1$ standard error are included.

5.2.4 Growth curves of BY-2 cells grown in 12 well plates

Nitrate withdrawal caused a reduction in cell proliferation, with an initial doubling time of 16 h as compared to 12 h in complete medium (data included from chapter 3 for comparison). This decreased cell division time was not to the same degree as seen with sucrose withdrawal, with initial doubling of that population taking approximately 89 h (see section 4.2.4). Culture decline started a day later than was observed in complete medium grown BY-2 cells, suggesting that nitrate withdrawal facilitated slower dividing, longer living cultures (figure 5.6).
Chapter 5: The impact of nitrogen withdrawal on BY-2 cell growth...

Figure 5.6. Cell counts taken from BY-2 cells grown in various medium preparations and plotted over the course of ten days. The cells were grown as 2 mL cultures in 12 well plates and counted with the aid of a haemocytometer. Proliferation of the cells in media lacking nitrates was similar, with slower division times and lower maximum concentrations attained than the complete medium control. Each data point represents the mean of six counts. Error bars included represent ± 1 standard error.

Optical density measurements were recorded after cell counts were taken and although the two data sets were similar, optical density estimated higher cell mass at later stages of growth in the media tested. Cultures supplemented with complete medium recorded higher densities when compared to nitrate starved cultures (figure 5.7).
Figure 5.7. Optical density (620 nm) measurements were taken from BY-2 cells grown in different media, over a ten day growth period. The cells were grown as 2 mL cultures in 12 well plates and optical density was measured at 620 nm in a 96 well plate. Very similar density measurements were recorded for both of the nitrate starved cultures and their density curves were approximately 0.1 units less than the complete medium control on day 7, when the maximum density for all three cultures was obtained. Six replicate samples were averaged for each time point and the included error bars designate ± 1 standard error.

5.2.5 Cell viability assessment during growth of BY-2 cells in nitrate deprived cultures

BY-2 cells grown in media lacking nitrates were co-stained with fluorescein diacetate (FDA) and PI to assess the cell viability profile during growth in 12 well plates.

5.2.5.1 BY-2 cells grown in medium lacking nitrates

BY-2 cells grown in medium lacking nitrates exhibited a similar viability profile as was observed under complete medium conditions (figure 4.11), although culture viability decreased quickly beyond day 6 (37% decrease in viable cells from day 6 to day 7) and was extended slightly (FDA positive cells detected on day 13) (figure 5.8).
Figure 5.8. Proportion of live versus dead BY-2 cells as measured by co-staining with FDA and PI. Cells were grown as 2 mL cultures in 12 well plates, with samples taken daily, co-stained with FDA (2.4 μM) and PI (3.0 μM) for 10 min, before counts were taken under a haemocytometer grid. The highest proportion of viable cells was seen on day 3, before decreasing viability of this batch culture was observed. The largest attrition of viable cells occurred between day 6 and day 7, and from day 14, no viable cells were detected. Cells were sampled over the course of twenty days when grown in medium lacking nitrates, with the mean of five replicate counts presented for each time point. Error bars correspond to ± 1 standard error.

5.2.5.2 BY-2 cells grown in KCl substituted medium

When grown in KCl substituted medium, the BY-2 cell viability decreased from day 3 and culture viability was extended by one day when compared to the complete medium control (figure 4.11), as FDA positive cells were detected beyond day 12 (figure 5.9).
Figure 5.9. Proportion of live versus dead BY-2 cells as measured by co-staining with FDA and PI. Cells were grown as 2 mL cultures in 12 well plates, with samples taken daily, co-stained with FDA (2.4 μM) and PI (3.0 μM) for 10 min, before counts were taken under a haemocytometer grid. Viability was at its maximum on day 3, with the FDA positive (PI negative) cells decreasing in number after this. Most viable cells were lost between day 6 and say 7, with no viable cells observed from day 14. Cells were observed over the course of twenty days when grown in KCl substituted medium. Each sampling point is the mean of five replicate counts and the error bars represent ± 1 standard error.

5.2.6 The pH during growth of BY-2 cells in media lacking nitrates

Culture pH during growth without nitrates (medium lacking nitrates and KCl substituted medium) was measured as a possible contributor to observed fluctuations in PI staining. The pH of these two medium formulations was found to remain quite stable throughout growth, at a value between 5.4 and 5.6 (figure 5.10). This stability can be compared to the other medium types (complete medium included below for comparison), where the pH changed considerably during the course of growth (section 4.2.6). Note that all media were pH adjusted to 5.8 before autoclaving, after which the pH dropped by varying amounts in all of the medium types.
Chapter 5: The impact of nitrogen withdrawal on BY-2 cell growth...

Figure 5.10. The pH levels measured in various BY-2 cultures over the course of ten days. Cultures were grown in 12 well plates (2 mL culture volume), with their pH tested on a daily basis. All media were adjusted to pH 5.8 before autoclaving, with a reduction in 0.4 pH units in the media lacking nitrates post autoclaving. Throughout culturing, the pH of the nitrate lacking cultures ranged between 5.4 and 5.6, which at most times was more alkaline than the culture grown in complete medium. Samples were replicated six times and each data point represents the average of these six replicates. Included error bars represent ± 1 standard error.

5.2.7 Sucrose concentration in BY-2 12 well cultures grown in nitrate starved media

Nitrate starved cultures did not consume sucrose at such a high rate, when compared to cultures grown in complete medium (data replicated for comparison from a parallel experiment presented in figure 4.16). Levels of approximately 30 mM were still detectable at day 10 in both nitrate deprived treatments (figure 5.11), suggesting that sucrose was not a limiting medium component and the slower cell division times seen in section 5.2.4 were due to nitrate limitation. The persistence of sucrose may also explain the extended vitality seen in cultures exposed to nitrate withdrawal (figure 5.8 & 5.9).
Figure 5.11. Sucrose content in different BY-2 media over the course of ten days growth. Cells were grown as 2 mL cultures in 12 well plates. Samples were centrifuged and the supernatant treated with invertase, to convert sucrose to glucose. A PGO enzyme preparation (Sigma-Aldrich, Saint Louis, MO, USA) was used to quantify glucose as per the manufacturer’s instructions. By day 10, there was approximately 30 mM of sucrose remaining in the cultures lacking nitrates and sucrose depletion was slower when compared to a complete medium grown culture. Averages were taken from duplicate sample measurements for display in this graph and error bars signify ± 1 standard error.

5.2.8 Protein content in the different BY-2 media from 12 well BY-2 cultures

The nitrate starved cells showed similar trends in total protein content to cells grown in complete medium (data reproduced from figure 4.17 to allow comparison) when assayed using the BCA protein quantification method. A trend of decreasing protein content during culturing was observed until day 8, after which higher levels of protein were detected in the media of nitrate starved cells (figure 5.12). As with the parallel experiment performed in section 4.2.8, the nitrate deprived cell-free medium samples underwent the same dialysis treatment to ensure compatible results with sucrose containing samples. The pre- and post-dialysis results were similar for the nitrate deprived culture samples, confirming low protein loss to the dialysate (data not shown).
Figure 5.12. Total protein content in BY-2 media after dialysis, over the course of ten days. Cultures were grown in 12 well plates, with a volume of 2 mL per well. Supernatant samples were dialysed over 15 h using 6-8 kDa cut-off dialysis tubing, and the resulting dialysed liquid was tested for total protein content using a BCA protein assay (Pierce, Rockford, IL, USA) as per the manufacturer’s instructions. Similar levels of protein were observed in the cultures lacking nitrates and in the complete medium control. Initially, the trend displayed a decrease in protein present in the dialysed supernatant, but in the last 2-3 days, protein content increased, perhaps due to increased cell death and subsequent cell lysis. Each data point represents the average of duplicate samples and error bars of ± 1 standard error are reported.

5.2.9 Depletion of 2,4-D in different BY-2 media from 12 well BY-2 cultures

Deficiency of 2,4-D was thought to play a role in decreased cell proliferation and perhaps account for the altered PI staining profiles, therefore its concentration was monitored during BY-2 cell culturing. Initially added to achieve a final concentration of 900 nM in all the medium formulations, 2,4-D levels dropped dramatically within the first 24 h of growth in the KCl substituted medium (from 693 nM at day 0, to 177 nM by day 1) and beyond day 5 levels were undetectable (except for an elevated reading on day 8, which was perhaps contamination or some other measurement anomaly). This result was similar to the trend of 2,4-D depletion measured in complete medium grown cultures (data set replicated from figure 4.18 for comparison) (figure 5.13).
Figure 5.13. Depletion of 2,4-D levels in nitrate deprived medium (KCl substituted) and nitrate containing medium (complete medium) over the course of a ten day BY-2 batch culture. Samples from shaker cultures (100 mL culture volume) were centrifuged and passed through a 0.2 µm filter. The 2,4-D content was then quantified using HPLC. The utilisation of 2,4-D in the culture lacking nitrates followed a very similar pattern of depletion as seen in the complete medium control. An elevated level of 2,4-D was detected in the KCl substituted culture sample taken on day 8, but it may be attributed to a sampling error or carryover from a previous HPLC sample. Each data point represents a single sampling.

5.2.10 BY-2 cell morphology during nitrate deprivation

Withdrawal of nitrates from the medium caused major changes in BY-2 cell morphology. Widespread formation of transparent granular ‘vesicles’ after 4 days of culturing was the most obvious development. These vesicles were often situated around the periphery of the nucleus, and grew larger, reaching a maximum size between days 6 and 8. Some nitrate starved BY-2 cells also became elongated and swollen, forming giant cells up to four times their normal length. By day 12, few cells were viable, as the majority had shrunk, causing detachment of the cellular membrane from the cell wall (figure 5.14).
Figure 5.14. Morphology of BY-2 cells grown in medium lacking nitrates (KCl substituted) using phase contrast microscopy, over a time course of twelve days. Cells at day 0 (a) had distinct transvacuolar strands and appeared approximately rectangular when viewed from above. After day 4 (b), the cells started to develop vesicles surrounding the nucleus and lost the majority of their transvacuolar strands. The vesicles became larger and cells also became elongated, as seen at day 8 (c). By day 12 (d), many cells had become non-viable. Scale = 20 μm.

5.2.11 Starch granule development in nitrate deprived media

The vesicles formed during nitrogen deprivation were tested for the presence of starch by addition of an iodine solution. BY-2 cells grown without nitrates and in KCl substituted medium were positive for the presence of starch, whereas cells grown in all the other medium formulations tested negative for starch production. Upon microscopic observation, the distinctive black staining was found to be concentrated in the perinuclear vesicles (figure 5.15).
Figure 5.15. BY-2 cells stained with an iodine solution (0.017% iodine and 0.167% potassium iodide final concentration) after 4 days of culturing were positive for the presence of starch under nitrogen deprived (KCl substituted) conditions (a) & (b). There was no starch production noted in complete medium grown cells (a). Scale = 10 μm.

5.2.12 Transmission electron microscopy of starch granules in nitrate starved BY-2 cells

Further analysis utilising transmission electron microscopy (TEM) revealed an absence of starch granules in BY-2 cells grown in medium deprived of sucrose (data not shown) and in complete medium (figure 5.16).
TEM images of BY-2 cells grown in complete medium. Cells were fixed in glutaraldehyde (2.5%), stained with osmium tetroxide (1%), dehydrated in an increasing ethanol series and encased in epoxy resin. Ultra thin sections were applied to copper grids, then stained with uranyl acetate (5%), rinsed and further stained with Reynolds lead citrate solution (appendix 3.1). After a final rinse, the grids were viewed using a TEM and typical images of the cell cross sections recorded. No starch granules were identified in any of the complete medium grown sample preparations. Scale = 2 µm.

TEM gave insights into the nature of the starch granules that formed in nitrate starved BY-2 cells. The granules excluded both the uranyl acetate and lead citrate stains, but included a densely stained central strip. This stained region was perhaps an artefact of the dehydration and sectioning process that the cells underwent, and likely constitutes a fissure in the starch granule. Two or more granules were often found to be encapsulated within an organelle with a double membrane (figure 5.17).
Figure 5.17. Transmission electron microscopy images of starch granules in nitrogen starved (KCl substituted) BY-2 cells. Cells were fixed in glutaraldehyde (2.5%), stained with osmium tetroxide (1%), dehydrated in an increasing ethanol series and encased in epoxy resin. Ultra thin sections were applied to copper grids, then stained with uranyl acetate (5%), rinsed and further stained with Reynolds lead citrate solution (appendix 3.1). After a final rinse, the grids were viewed using a TEM and typical images of the cell cross sections recorded. The starch granules were visible surrounding the nuclear membrane (perinuclear) (a) and they were encapsulated in a membrane, often encompassing two or more granules (b) & (c). The casing appeared to be comprised of a double membrane structure (c) & (d). Scale = 0.5 µm

5.2.13 Formation of spherical bodies in response to protease inhibition

Nitrogen deprivation, combined with protease inhibition also resulted in the formation of spherical bodies after the first 24 h of exposure (figure 5.18), in a similar manner to that seen in complete medium grown BY-2 cells (see section 4.2.11).
Figure 5.18. BY-2 cells grown in nitrate deprived medium (KCl substituted) and treated with a cocktail of protease inhibitors over the course of fifteen days. The cells were grown as 2 mL cultures in 12 well plates. Following subculturing, the cells were treated with protease inhibitor tablets (Roche Diagnostics GmbH, Nonnenwald, Penzberg, Germany) at an equivalent dosage of 14 µM papain (the main protease inhibitor in the tablets). Samples were then taken daily and viewed under a microscope. The spherical bodies were present one day after treatment with protease inhibitors (a), persisting through day 4 (b) and day 8 (c). By day 15 (d), the majority of cells had become non-viable. Scale = 20 µm.

5.2.14 Staining of nitrate deprived BY-2 cells with monodansylcadaverine

To test for the appearance of acidic vesicles in nitrate deprived BY-2 cells, protoplasts were stained with monodansylcadaverine (MDC). In cells grown in KCl substituted medium, MDC positive vesicles were seen 3 days after transferral to new medium (subculturing), reaching a maximum density on day 4 (figure 5.19). Very few MDC positive vesicles were observed in BY-2 cells grown in complete medium at day 4, but such vesicles reached a similar maximum density after 8 days (data not shown).
Figure 5.19. MDC stained protoplasts obtained from BY-2 cells starved of nitrogen (KCl substituted). Cells were grown in 100 mL shaker cultures, converted into protoplasts at different sampling points, incubated for 10 min with MDC (40 µM), washed and viewed under a fluorescent microscope. This image above was taken four days after subculturing. Acidic vesicles are visible as green fluorescent spots scattered throughout the protoplast. Scale = 20 µm.

5.2.15 BY-2 cell area measured in two dimensions

Observations of BY-2 cell enlargement when grown in nitrate starved media (see section 5.2.10) were quantified with measurements of cell width and length. This allowed the estimation of the average space (in two dimensions) that each cell occupies, and from this trends in volume could be inferred. From day 5, cells grown in nitrate deprived media increased in size and by day 8 reached an average area of approximately 1750 µm$^2$, which was a 250 µm$^2$ larger than cells of the same age grown in complete medium (data included from parallel experiment presented in section 4.2.13 for comparative purposes). After day 8, the average cell area decreased in both nitrate deprived treatments, with a final increase in KCl substituted grown cells on day 10 (figure 5.20).
Figure 5.20. Cell areas based on two dimensional (length x width) measurements in BY-2 cultures grown in different media. Cultures grown in 12 well plates (2 mL per well) were sampled to view under a microscope and representative images taken to allow dimensional measurements. In the nitrate starved cells, the general trend was an increase of cell area up until day 8, at which time they were on average approximately 250 µm² larger than cells grown in complete medium. Each time point represents the average measurement of 12 cells chosen at random from a slide preparation. Error bars are representative of ± 1 standard error.

5.2.16 Total protein content within BY-2 cells during growth in nitrate deprived media

The total protein content of BY-2 cells (expressed as protein dry weight per litre of culture) was analysed over the course of 10 days, with an overall trend of increasing, then decreasing protein levels in cells grown in all the medium types tested. Cells grown in complete medium (data included from a parallel experiment presented in section 4.2.14) displayed significantly higher levels of protein when compared to nitrate starved cells (figure 5.21).
Figure 5.21. Total protein content per BY-2 cell grown in different media over the course of ten days. During culturing in 12 well plates, the samples taken were centrifuged, the supernatant discarded and the cell mass dried. The cell pellet was then frozen in liquid nitrogen, ground to a powder and resuspended in a protein extraction buffer. After vortex mixing and sonication, the cell homogenate was centrifuged. The resulting supernatant was used for protein determination using a BCA protein assay kit (Pierce, Rockford, IL, USA) as per the manufacturer’s instructions. Similar patterns of increasing, then decreasing cellular protein content (maximum reached on day 4) were seen in both nitrate starved cultures. Protein content at day 4 in the complete medium control was over twice that seen in the nitrate starved cultures. Protein content was measured from cell dry mass (excluding medium) and expressed as grams of protein per litre of culture. Error bars are included and represent ± 1 standard error from duplicated samples.

When protein levels were normalized to a per cell value (cell count data not shown), all of the cultures had approximately 690 pg/cell at day 0, with a decline to 340 pg/cell in the complete medium and approximately 150 pg/cell for the other nitrate deprived media by day 10 (figure 5.22).
Figure 5.22. Per cell protein content of BY-2 cells grown in various media over a ten day period. Previous total cell protein content measurements (see figure 5.21) were divided by cell counts taken earlier from the same samples (data not shown) to obtain a measurement of protein per cell. In the nitrate deprived cultures, the general trend was a depletion of cellular protein content, which was also observed to a lesser degree in the complete medium control. By day 10, the protein content per cell was over four times less than measured on day 0 in the nitrate deprived cultures.

5.2.17 Nitric oxide levels in nitrate deprived BY-2 cells

As referred to in chapter 4, nitric oxide (NO) plays an important role as a signalling molecule in plants. Its involvement in nitrate starvation and subsequent autophagy was assessed by using 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) fluorescence to track cellular NO levels over the course of culture growth. Histogram plot overlays of individual samples (figure 5.23) were not very useful when trying to ascertain trends in NO levels, but by plotting the mean x-axis channel numbers, changes in cellular NO became apparent (figure 5.24).
Figure 5.23. Flow cytometry histogram overlays representing BY-2 protoplasts stained with DAF-FM. Protoplasts were obtained from cells grown in 12 well plates (2 mL cultures) every two days, stained with DAF-FM (5 µM) and either left untreated (a) & (c) or pre-treated with 1 mM SNAP 24 h before sampling (b) & (d). While there were shifts in the DAF-FM histograms over time, they were difficult to interpret, so in another experiment the mean channel number of each histogram was recorded at different points during culture growth (see figure 5.24).

When stained with DAF-FM, BY-2 protoplasts grown in nitrate deficient media displayed similar fluorescence intensity profiles to that seen in cells grown in complete medium (data included from figure 4.28 for comparison). After a decrease in NO content during the first 2 days, there seemed to be an increase in NO content that
Chapter 5: The impact of nitrogen withdrawal on BY-2 cell growth...

reached a peak at day 4. A drop in NO content at day 5 was then followed by a second increase, reaching a second peak at day 9 (figure 5.24). This final increase was not seen in any of the cells grown in sucrose deprived media (see section 4.2.15).

Figure 5.24. NO levels as inferred by DAF-FM fluorescence in BY-2 cells grown over the course of ten days in various media. Cultures grown in 12 well plates (2 mL volume) were treated with DMSO (10 µL) 24 h before sampling. On the day of sampling, the cells were converted to protoplasts (approximately 1 x 10⁵ protoplasts/mL), stained with DAF-FM (5 µM), washed and analysed using flow cytometry. The pattern of changing DAF-FM fluorescence was very similar in all three culture treatments presented. Decreasing DAF-FM fluorescence during the initial phase of culturing was followed by increasing fluorescence from day 5. Overall, the nitrate deprived cultures displayed less DAF-FM fluorescence and therefore potentially lower NO content than the complete medium control. Data points represent the average of two replicate samples, where the DAF-FM fluorescence peak mean channel value was recorded. Error bars indicate ± 1 standard error from duplicated samples.

Duplicate fractions when treated with SNAP (24 h before DAF-FM staining) showed an initial decrease in fluorescence intensity over the first 2 days, which stabilised over the following 8 days. There seemed to be extra NO present from day 7 in the nitrate starved cells (figure 5.25), corresponding to the increases seen without SNAP treatment (figure 5.24). DAF-FM fluorescence intensities found in the nitrate deprived cells were lower, but changed over time in a similar manner to those measured in complete medium grown cells (data reproduced for comparison from the parallel experiment...
Figure 5.25. DAF-FM fluorescence inferring NO content in BY-2 cells. Cultures grown in 12 well plates (2 mL volume) were treated with SNAP (25 nM) 24 h before sampling. On the day of sampling, the cells were converted to protoplasts (approximately 1 x 10^5 protoplasts/mL), stained with DAF-FM (5 μM), washed and analysed using flow cytometry. After an initial large drop in DAF-FM fluorescence, the trend was of increasing fluorescence from day 5 to day 8 in the nitrate starved cultures. Patterns of changing DAF-FM fluorescence were similar in all media presented above, but levels were generally lower in the nitrate starved cultures when compared to those seen in complete medium control. Each data point for the various medium treatments was the mean of duplicate samples measuring the DAF-FM peak mean channel number. The included error bars represent ± 1 standard error from duplicated samples.

5.2.18 Intracellular calcium levels in BY-2 cells grown in KCl substituted medium

As proposed in chapter 4, intracellular Ca^{2+} concentrations may play a role in the response to stress induced by nutrient limitation and subsequent autophagy in BY-2 cells. To determine whether intracellular Ca^{2+} concentrations were affected by nitrogen deprivation, BY-2 cells were grown in KCl substituted medium, converted into protoplasts, stained with indo-1 AM and analysed using flow cytometry. Cells grown for 4 days had a mean intracellular [Ca^{2+}] of 551 nM as their baseline measurement. After stimulation with ionomycin, the final intracellular [Ca^{2+}] attained had a mean of 989 nM, with no recovery period noted. This indicated the presence of high intracellular Ca^{2+} levels (when compared to freshly subcultured cells analysed in section presented in figure 4.29).
Chapter 5: The impact of nitrogen withdrawal on BY-2 cell growth...

4.2.16.1) and no reuptake during the time frame of this analysis. The same culture tested at day 8 showed a higher baseline mean intracellular \([\text{Ca}^{2+}]\) of 925 nM. After ionomycin addition, a short recovery phase of 56 s was seen, before stabilising at a final mean intracellular \([\text{Ca}^{2+}]\) of 2044 nM (figure 5.25). The indication of high baseline \(\text{Ca}^{2+}\) levels and poor recovery from ionomycin addition in nitrate deprived (KCl substituted medium) cells, is evident when this result is compared to freshly subcultured BY-2 cells (see figure 4.30).

![Graph](a)

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Figure 5.26. Response of the indo-1 fluorophore to the addition of ionomycin, in nitrate limited (KCl substituted) BY-2 protoplasts at day 4 (a) and day 8 (b). Protoplasts isolated from 12 well plate cultures were incubated with indo-1 AM (1 µM) for 30 min, before washing and analysing using flow cytometry. From day 4 to day 8, the baseline [Ca^{2+}] increased and the ability of the protoplasts to recover their initial [Ca^{2+}] was very limited compared to that seen in freshly subcultured protoplasts (see section 4.2.16), although day 8 protoplasts did display a short recovery period. The ratio displayed on the y-axis is calculated from indo-1 fluorescence emissions at 400 nm and 475 nm. The ratio trace is separated into four sections; initial baseline, ionomycin uptake, recovery period and final baseline. The arrow represents the time point at which ionomycin (2 µM) was added and intracellular calcium concentration is measured in nM.

5.3 Discussion

The primary objective of this chapter was to assess the influence of nitrate withdrawal on chromatin conformation in BY-2 cells and to look for associated morphological and physiological changes. A secondary intention was to form a more complete picture of the inner workings of a model plant system during starvation and the subsequent autophagic processes. Nitrate starvation did not have a major effect on PI staining, except for a delay in the timing of the staining intensity maxima reached when compared to cells cultured in complete medium. Removal of nitrates shifted the maximum intensity reached by two days and substitution with KCl caused a one day
shift (figure 5.1). There was also a one day delay in the maximum PI peak intensity when both nitrate starved cultures were supplemented with 2,4-D (figure 5.5). As with all media treatments, H342 fluorescence was stable during culturing of the nitrate deprived cells (figure 5.4). These results do not support any strong causative link between nitrate withdrawal and the changes in PI stain fluorescence.

The role hormones play in growth and development of BY-2 cells has been well researched. BY-2 cells are autotrophic when it comes to cytokinin production and have therefore been valuable in the study of cytokinin kinetics during the plant cell cycle (Redig et al., 1996; Dobrev et al., 2002). Contrasting the ability to produce cytokinins, BY-2 cells are auxin auxotrophs, producing very low levels of indole-3-acetic acid (IAA) (Redig et al., 1996) and require the addition of 2,4-D, a synthetic auxin analogue to maintain suitable culture growth (Nagata et al., 1992). Cultures supplied with low auxin levels exhibit cell elongation, whereas high levels promote cell division (Hasezawa and Syono, 1983). Levels of 2,4-D in all of the BY-2 growth media tested, were very quickly depleted (figure 5.13) and therefore 2,4-D supplementation throughout culturing was investigated in relation to PI staining. While PI fluorescence intensity maxima were lower and delayed by one day in the complete medium and KCl substituted medium, there was no significant change in the medium lacking nitrates when 2,4-D supplemented culture results (figure 5.5) were compared to non-supplemented results (figure 5.1). With this in mind, it is unlikely that 2,4-D depletion plays a causative role in the fluctuating PI fluorescence during BY-2 culturing.

Withdrawal of nitrates from the medium had a negative effect on culture proliferation, with the initial doubling time extended to 16 h and the maximum cellular concentration reaching $1.1 \times 10^6 \text{ cells/mL}$ (figure 5.6). It was expected that nitrate withdrawal would have a more profound effect on culture proliferation and this result was surprising given that the two medium types were devoid of nitrates and therefore lacking amino acids for protein synthesis, required for cellular function and cell division. Perhaps sufficient nitrates were able to be scavenged from the medium, originating from dead and ruptured cells. Another theory is that the actions of autophagy allowed close to normal culture growth through efficient intracellular protein recycling.

A slight extension of vitality was also observed in nitrogen starved cells, with detection of live cells up until day 14 (figure 5.8 & 5.9). The rapid utilisation of sucrose in BY-2 cells grown in complete medium contrasted with a gradual uptake of sucrose in nitrate limited cultures. Whereas cells grown in complete medium exhausted their
Chapter 5: The impact of nitrogen withdrawal on BY-2 cell growth...

sucrose supply within 6 days, nitrate limited cultures retained approximately 30 mM of non-metabolised sucrose in the medium after 10 days of culturing (figure 5.11). A slowed rate of cell division and the extension of culture vitality (as assessed by FDA/PI co-staining) in the nitrate deprived cells would explain the lowered uptake and utilisation of sucrose.

When the media compositions were investigated further, it was clear that the exclusion of nitrates from the solution resulted in higher initial pH values, which remained stable during growth and reached a pH comparable to the culture grown in complete medium by day 5 (figure 5.10). Perhaps the cultures grown in complete medium manipulated the pH level to better suit growth conditions (i.e. pH rose after 2 days of growth) or alternatively, rapid growth caused the initial increase in pH by consumption of acidic species. A decreasing trend in protein content resident in the nitrate deprived media was observed during culture growth, with a spike seen at day 10 (figure 5.12), probably due to increased cell death and subsequent lysis releasing intracellular proteins into the surrounding medium.

In cells starved of nitrogen, there was a 13% increase in average cell footprint by day 8 (when compared to cells grown in complete medium) (figure 5.20), along with the appearance of starch granules (figure 5.15) and the loss of transvacuolar strands (figure 5.14). Loss of these strands appeared to result in the separate vacuoles merging into a singular entity. In a plant cell the vacuole normally occupies between 30% and 90% of cells volume and carries out several functions. Beyond providing volume and maintaining turgor pressure in the cell, the vacuoles main role is thought to be as a storage compartment, where hydrolytic enzymes carry out an analogous function to the lytic compartment in mammalian cells, the lysosome (Ryan and Walker-Simmons, 1983; Marty, 1999). Loss of the transvacuolar strands and consequent expansion of the vacuole seen during culture aging/starvation in BY-2 cells may be explained by increased sequestration of cytoplasmic substance due to increased autophagic activity.

Studies investigating the effects of sucrose starvation in BY-2 cells have focused on the targeting of autophagosomes to the vacuole for degradation. Since the vacuole of a mature plant cell can occupy up to 90% of the cell volume, viewing the formation and degradation of autophagosomes can be difficult. The use of cysteine protease inhibitors to block degradation of proteins within autophagosomes (therefore keeping them resident within the cytosol) has shed light on the chain of events that occurs during the autophagic process in plant cells (Moriyasu and Ohsumi, 1996). Two distinct organelle types were observed when comparing nitrate starved BY-2 cells with those starved of...
sucrose or grown in complete medium. During nitrate deprivation, starch granules formed which were approximately 5-7 µm in diameter, as confirmed by iodine staining (figure 5.15) and TEM (figure 5.17). When exposed to a cocktail of protease inhibitors, smaller vesicles (approximately 1-2 µm in diameter) formed in cells regardless of growth medium and these vesicles did not contain starch (iodine stain negative). When treated with the cocktail of protease inhibitors, the nitrate starved cells did not develop starch granules (figure 5.18). It is therefore proposed that these smaller organelles are the same structures identified by others (Moriyasu and Ohsumi, 1996; Toyooka et al., 2006) as autophagosomes (or the functional plant equivalent). One interesting observation discovered through TEM imagery was a double membrane surrounding the starch granules in nitrate deprived cells (figure 5.17). As autophagosomes are known to encapsulate material tagged for recycling in a double membrane (Juhasz and Neufeld, 2006), it was initially thought that these starch granules may represent autophagosomes. Later it was realised that they were in fact amyloplasts, a type of plastid that contains starch grains surrounded by a double membrane (Wise and Hoober, 2007).

MDC staining highlighted the appearance of acidic vesicles in both nitrate starved (figure 5.19) and sucrose starved BY-2 cells (figure 4.23). The emergence of MDC positive vesicles in cells grown in complete medium was delayed, reaching maximum levels by 8 days of culturing (data not shown). Autophagosomes have been successfully and specifically identified in Arabidopsis protoplasts by staining with MDC, and it was noted that as starvation progressed, the size of autophagosomes increased, possibly linked to the increased expression of the AtATG8e protein (Contento et al., 2005). MDC tends to stain mature autophagosomes (i.e. post acidification of the lumen) in mammalian cells, which further complicates the use of this stain for autophagosome identification purposes (Bampton et al., 2005). In BY-2 cells, MDC uptake was blocked by the cell wall and only reached the cytoplasm after enzymatically stripping away the cell wall, thus creating protoplasts. This caused problems when examining less robust older cells (>6 days old), with enzymatic treatment often leading to ruptured cell membranes before the cell wall removal was complete. The conversion of BY-2 cells to protoplasts also caused a redistribution of the cellular contents, with previously perinuclear organelles spread throughout the protoplast. This was perhaps due to damage to transvacuolar strands and their anchor points with the cell membrane, and may explain the scattered distribution of MDC positive staining vesicles (figure 5.19).

While following the total protein content in BY-2 cells grown in the different medium types, it became clear that nitrate starvation had the greatest effect on cellular
protein levels. All cultures showed a general trend of decreasing protein content per cell over the course of culturing, and without an available nitrogen source, the BY-2 cells grown in nitrate deprived media were left with approximately 150 pg of total protein content per cell by day 10 (figure 5.22). This result is suggestive of protein degradation and recycling as a result of autophagy (Klionsky and Emr, 2000).

The speculated role of NO in signalling during programmed cell death (PCD) as a result of autophagy (or otherwise) was investigated in nitrate starved BY-2 cells. An increase in NO production was seen in nitrate deprived cultures from day five and followed a similar trend to that seen in cells cultivated in complete medium (figure 5.24). This increase in intracellular NO concentration may be consistent with increasing cell stress and autophagy due to nitrate deprivation. While none of the current literature investigates the question of whether NO has a signalling role during autophagy triggered by nitrogen limitation, one study does show induction of autophagy in cardiomyocytes exposed to NO (Yuan et al., 2009). There is also evidence in mammalian cells to the contrary, that NO is not involved in the induction of autophagy, with the autophagy related genes beclin-1, Atg51 and Atg121 not up-regulated during NO stimulation in mouse cardiomyocytes (Rabkin, 2007).

Like sucrose starved BY-2 cells (figure 4.32), intracellular \( \text{Ca}^{2+} \) baseline concentrations were also higher in nitrate deprived cells during the later stages of growth (day 8), when compared to cells grown in complete medium (figure 4.31). Unlike sucrose starved cells, the response of 8 day old nitrate starved cells to ionomycin challenge (figure 5.26) was more akin to that seen in cells grown in complete medium (figure 4.31), with a discernible uptake phase and evidence of recovery, presumably through the efforts of active \( \text{Ca}^{2+} \) pumps (Evans et al., 1994). It seems that nitrate starvation has less of an impact on a cells ability to cope with \( \text{Ca}^{2+} \) influx than sucrose starvation. While there is currently no evidence in the literature to support the involvement of \( \text{Ca}^{2+} \) signalling in plants undergoing nitrate withdrawal-induced autophagy, the elevated intracellular \( \text{Ca}^{2+} \) concentrations seen in nitrate starved BY-2 cells does suggest a role for calcium during nitrate starvation. Whether this is a result of a general stress response (Mahalingam and Fedoroff, 2003) or a more specific signalling mechanism involved in autophagy remains to be seen.
6. Chromatin alterations during the growth cycle of BY-2 cell cultures

6.1 Introduction

The results of the previous three chapters indicate that the chromatin of BY-2 cells stains differently with PI at different stages during culturing. In complete medium, the transition to higher staining intensity occurred within four days of subculturing in fresh medium and the corresponding reverse transition occurs following depletion of sucrose as the carbon source (chapter 3). Growth under reduced nitrogen supply led to similar results (chapter 5) and growth in a medium containing an inferior carbon source led to the same chronological changes despite a much lower growth rate (chapter 4). In this chapter, potential changes in chromatin are considered in greater detail.

Chromatin present in eukaryotes can be categorized into two cytologically distinctive configurations; densely packed heterochromatin or less condensed euchromatin. Heterochromatin is further characterised as either facultative, where a transition to a more open arrangement can occur, and constitutive, which remains mostly static in its densely packed state. A less condensed state (as seen in euchromatin and facultative heterochromatin) is often associated with higher levels of gene expression and therefore DNA transcription (Dillon, 2004). The higher AT base pair content in heterochromatin is also a defining feature, which is revealed when stained with either DAPI or Hoechst dyes (Zimmer and Wahnert, 1986).

Perceived alterations in chromatin state and/or composition during cell cycling and in several forms of programmed cell death (PCD) have been previously described (O'Brien et al., 1998b). It is well known that chromatin becomes condensed in cells undergoing apoptosis (Oberhammer et al., 1993; Bursch, 2004), but the phenomenon of chromatin condensation has not been ascribed to cells involved in autophagic cell death (ACD). Previous studies into the existence of apoptosis in tobacco cells provided evidence of a relaxed chromatin state in cells undergoing active growth and a condensed state preceding the onset of PCD (O'Brien et al., 1998a; O'Brien et al., 1998b). Whether the chromatin condensation observed in BY-2 cells is associated with, or a result of ACD warrants further investigation.

DNA methylation is known to result in gene silencing. The process that leads to conversion of a silenced gene whose DNA is methylated and often in the form of
facultative heterochromatin, to an actively transcribed form is not fully understood. Demethylation and therefore potential gene activation have been described in *Arabidopsis* by the actions of DNA glycosylases DEMETER (DME) and REPRESSOR OF SILENCING1 (ROS1) (figure 6.1). Genomic imprinting in endosperm requires the actions of DME, while ROS1 is thought to be involved in trimming DNA methylation patterns in transposons and genic regions of vegetative tissues (Morales-Ruiz *et al.*, 2006).

![DNA Glycosylase](image)

Figure 6.1. During DNA demethylation in plants, DNA glycosylates (DME and ROS1) cut the N-glycosidic linkage between the DNA backbone and 5-methylcytosine. Adapted from Ikeda and Kinoshita (2009).

The exact process involved in plant DNA demethylation is not known, but a proposed model involves the recruitment of an unknown DNA demethylation complex by siRNA, that results in chromatin remodelling, 5-methylcytosine excision (by the AP lyase activity of DME or ROS1) and base excision repair. Chromatin remodelling is believed to involve histone modification proteins to convert the chromatin to an active state (figure 6.2) (Ikeda and Kinoshita, 2009).
Figure 6.2. Proposed model for DNA demethylation, involving target recognition, chromatin remodelling and the base excision repair system in plants. Adapted from Ikeda and Kinoshita (2009).
Chapter 6: Chromatin alterations during the growth cycle of BY-2 cell cultures

Markers that may be useful in understanding chromatin structure alterations (beyond DNA specific dyes) include indicators of histone modification, the status of DNA methylation (hyper- or hypomethylation) and nucleosome acetylation levels. Histones can be successfully labelled and tracked using antibodies tagged to reporter molecules (Swindle and Engler, 1998) and several molecular methods have been employed in the determination of DNA methylation status (Mills and Ramsahoye, 2002). In this chapter, selected methods for examining chromatin structure will be applied in the investigation of BY-2 cell chromatin remodelling.

Aims of this chapter:
To investigate changes in chromatin structure and composition within a plant model system (BY-2 cells), by measuring DNA methylcytosine content and histone analysis, with the intention of developing a model to explain these changes.

6.2 Results

6.2.1 Methylation-sensitive restriction fingerprinting of BY-2 genomic DNA

To investigate the effect of cell proliferation on DNA methylation, BY-2 cells were analysed either directly after harvest (day 0) or after growth in complete medium for 4 days. The methylation-sensitive restriction fingerprinting (MSRF) profile revealed alterations in the methylation status of the cultured BY-2 cells. Two DNA regions that displayed positive PCR amplification at day 0, failed to be amplified in DNA from 4 day old cultures, suggesting a disruption of binding sites for primer sets 1 and 2 by cutting from the methylation-sensitive BstU I endonuclease. Since BstU I cleavage is blocked by CG methylation at the 5’-CG►CG-3’ recognition site, it is reasonable to conclude that the previously methylated site has undergone demethylation (figure 6.3).
Figure 6.3. MSRF profile of BY-2 genomic DNA. Odd numbered lanes are digested with both BstU I and Mse I restriction enzymes, while even numbered lanes are digested with Mse I only. Lanes 1, 2, 7 & 8 are amplified with primer set 1, lanes 3, 4, 9 & 10 are amplified with primer set 2 and lanes 5, 6, 11 & 12 are amplified with primer set 3. The first 6 lanes contain DNA from cells at day 0 and the next 6 lanes contain DNA from day 4 (grown in complete medium). Arrows indicate missing bands in the MSRF profile at day 4, suggesting demethylation of genomic DNA. A PCR positive control (N. tabacum actin gene Tob103 fragment), a PCR negative control (positive control lacking genomic DNA) and a 1 kb DNA ladder (Invitrogen Corporation, Carlsbad, CA, USA) are also included.

6.2.2 Total 5-methylcytosine content of BY-2 cells during growth and starvation

The previous section suggested that some changes in cytosine methylation occurred during culturing in complete medium. To investigate the effect of cell proliferation and subsequent nutrient depletion on total cytosine methylation, BY-2 cells were analysed directly after subculturing into 100 mL shaker flask cultures (day 0) and after growth in
Chapter 6: Chromatin alterations during the growth cycle of BY-2 cell cultures

complete medium for 2, 4, 6 and 8 days. As measured using HPLC, the percentage of cytosine bases that were methylated in BY-2 genomic DNA changed during the course of growth and starvation. Cytosine methylation dropped from a mean of 31.5% immediately after subculturing, to a mean of 27.0% by day 4 of growth in complete medium. A paired $t$-test comparing these means gave a two tail p-value of 0.0007, confirming the difference as being significant. By day 6, the mean percentage of methylated cytosine bases in the BY-2 genome increased to 32.5% and remained at this approximate level when measured on day 8. A paired $t$-test comparing the means at day 4 and day 6 gave a two tail p-value of 0.0110, also confirming this difference as significant (figure 6.4).

Figure 6.4. Total 5-methylcytosine content of BY-2 genomic DNA. Raw genomic DNA (5 µg in 50 µL of distilled water) was treated with ammonium acetate (0.01 M) and nuclease P1 (1 unit), before being incubated for 2 h at 45°C. Ammonium bicarbonate (0.1 M) was then added, along with phosphodiesterase I (0.00002 units) before incubation for 90 min at 37°C. After addition of alkaline phosphatase (0.0004 units), the samples were further incubated at 37°C for 2 h. Finally, 40 µL of distilled water was added to each sample, before HPLC analysis using an ammonium acetate and acetonitrile gradient method. Methylated cytosine content decreased and then increased during culturing of BY-2 cells, with a minimum content of 27% measured on day 4. The cells were grown in complete medium for 8 days, with duplicate samples taken every 2 days. DNA was extracted from each sample, processed and the 5-methylcytosine content quantified using HPLC. Each data point represents an average of sample duplicates. Error bars represent ±1 standard error.
6.2.3 Anti-histone H3 mono-methyl K4 and anti-histone H1 antibody analysis

The results of the previous two sections showed that changes in DNA methylation appeared to be taking place, raising the question of whether this reflected concurrent changes in chromatin conformation. Such changes could be associated with histone DNA interactions and further experiments were carried out to determine whether specific histones were involved. As antibodies directed against plant histone H3 and plant histone H1 were not available, human directed antibodies were tested for their cross reactivity in BY-2 nuclei. Using fluorescent FITC labelled anti-IgG secondary antibodies directed against the anti-histone primary antibodies, localisation in the BY-2 nucleus was confirmed for both the anti-histone H3 mono-methyl K4 (H3K4me) and anti-histone H1 antibodies (figure 6.5). When the primary anti-histone antibodies were omitted from nuclei suspensions, no FITC fluorescence was seen after addition of the appropriate FITC labelled secondary antibodies and subsequent washing (data not shown).

![Figure 6.5](image)

Figure 6.5. FITC labelled anti-IgG secondary antibodies (bound to primary anti-histone antibodies) exhibiting fluorescence within BY-2 nuclei. BY-2 cells were harvested from 12 well plate cultures, converted to protoplasts, incubated with Triton X-100 (0.1%) and labelled with either anti-histone H1 coupled to anti-mouse IgG:FITC (a) or rabbit anti-histone H3K4me coupled to goat anti-rabbit IgG:FITC (b). The anti-histone antibodies were developed to target human histones, but appeared to be cross-reactive with BY-2 histones.

Once the histone labelling technique was observed by fluorescence microscopy in BY-2 nuclei, it was then applied using flow cytometry for quantitative analysis. BY-2 nuclei were analysed directly after subculturing (day 0) and on day 2, day 4 and day 8 in complete medium, sorbitol substituted medium, KCl substituted medium and medium substituted with both sorbitol and KCl. H1:FITC fluorescence was observed to reach a
minimum level 4 days post subculturing within nuclei from BY-2 cells grown in complete medium. By day 8, the FITC signal intensity had increased again to levels slightly less than seen at day 0. Similar trends were observed in sorbitol and KCl substituted treatments, with a FITC signal decrease associated with times of cell proliferation (day 2 and day 4). Anti-histone H1:FITC fluorescence did not alter as much over time in nuclei from cells grown in medium substituted with both sorbitol and KCl (figure 6.6).
Figure 6.6. Flow cytometry histogram overlays depicting shifts in anti-histone H1:FITC fluorescence intensity over the course of eight days. Cells were harvested from 12 well plate cultures, converted to protoplasts (approximately $1 \times 10^5$ protoplasts/mL) and incubated with Triton X-100 (0.1%). Anti-histone H1 antibodies (diluted 1:1000) were added and incubated at RT for 20 min, followed by anti-mouse IgG (Fc):FITC antibodies (diluted 1:1000) which were incubated a further 20 min at RT. After washing, nuclei grown in complete medium (a), sorbitol substituted medium (b), KCl substituted medium (c) and sorbitol and KCl substituted medium (d) were analysed using flow cytometry (50,000 total events recorded). Shifts in fluorescence over time were observed in the all of the treatments, but the changes measured in sorbitol and KCl substituted medium treated cells were less marked. Typically, more fluorescence was observed at day 0 and day 8, than at day 2 and day 4 when the cells were in their proliferative phase.
Chapter 6: Chromatin alterations during the growth cycle of BY-2 cell cultures

This experiment was then repeated, with a larger number of samples taken (duplicate samples taken each day) so as to gain a more detailed record of the anti-histone H1 labelling profile during BY-2 growth in the various media. When plotted over the course of 10 days, the trend in FITC signal loss, then subsequent recovery became more apparent (figure 6.7).

![BY-2 anti-histone H1 (FITC)](image)

Figure 6.7. Anti-histone H1:FITC fluorescence (FITC peak x-mean), measured over time for BY-2 nuclei that were grown in differing media. Cells were harvested from 12 well plate cultures, converted to protoplasts (approximately 1 x 10^5 protoplasts/mL) and incubated with Triton X-100 (0.1%). Anti-histone H1 antibodies (diluted 1:1000) were added and incubated at RT for 20 min, followed by anti-mouse IgG (Fc):FITC antibodies (diluted 1:1000) which were incubated a further 20 min at RT. The resulting labelled nuclei were washed and analysed using flow cytometry, capturing 50,000 total events. Minimal fluorescence was observed on either day 2 (sorbitol substituted and KCl substituted treatments) or day 4 (complete medium treatment). Cells starved of both nitrates and sucrose (KCl and sorbitol substituted medium) produced anti-histone H1:FITC fluorescent nuclei that did not fluctuate as much over time. Each point represents the mean value of duplicate samples. Included error bars represent ± 1 standard error.

Concurrent experiments were also carried out using antibodies directed against histone H3K4me. However, in these experiments H3K4me:FITC fluorescence did not change appreciably with culture time and did not follow the pattern observed in anti-histone H1:FITC labelled nuclei. This observation did not seem to be influenced by the growth medium, as all the types tested showed similar H3K4me:FITC fluorescence peak positions (figure 6.8).
Figure 6.8. Flow cytometry histogram overlays depicting shifts in anti-histone H3K4me:FITC fluorescence intensity over the course of eight days. Cells were harvested from 12 well plate cultures, converted to protoplasts (approximately $1 \times 10^5$ protoplasts/mL) and incubated with Triton X-100 (0.1%). Anti-histone H3K4me antibodies (diluted 1:1000) were added and incubated at RT for 20 min, followed by anti-rabbit IgG (H&L):FITC antibodies (diluted 1:1000) which were incubated a further 20 min at RT. After washing, nuclei grown in complete medium (a), sorbitol substituted medium (b), KCl substituted medium (c) and sorbitol and KCl substituted medium (d) were analysed using flow cytometry (50,000 total events recorded). Very little change was noted over the course of culturing in regards to the mean FITC signal in all of the medium treatments. Note that these histograms represent corresponding H3K4me:FITC labelled replicates of those presented in figure 6.6.
Chapter 6: Chromatin alterations during the growth cycle of BY-2 cell cultures

A repeat of the initial anti-histone H3K4me:FITC labelling experiment was also undertaken, with replicated, daily sampling to gain more resolution and potentially track small scale patterns of labelling over time. While there was variation in H3K4me:FITC labelling from day to day, changes in FITC fluorescence intensity were less than 100 channels (figure 6.9) and did not trend with respect to culture proliferation (data not shown).

![BY-2 anti-histone H3 (FITC)](image)

Figure 6.9. Anti-histone H3K4me:FITC fluorescence (FITC peak x-mean), measured over time for BY-2 nuclei that were grown in differing media. Cells were harvested from 12 well plate cultures, converted to protoplasts (approximately 1 x 10^5 protoplasts/mL) and incubated with Triton X-100 (0.1%). Anti-histone H3K4me antibodies (diluted 1:1000) were added and incubated at RT for 20 min, followed by anti-rabbit IgG (H&L):FITC antibodies (diluted 1:1000) which were incubated a further 20 min at RT. The resulting labelled nuclei were washed and analysed using flow cytometry, capturing 50,000 total events. The fluorescence signal reported from all of the samples taken ranged from channel 115 and channel 202, and there did not seem to be any obvious trend over time in any of the treatments. Each point represents the mean value of duplicate samples and this experiment was performed concurrently with that presented in figure 6.7. The error bars represent ± 1 standard error.

Replicate samples (of those presented in figures 6.7 and 6.9) were also stained with PI to allow direct comparison of either histone H1 or H3K4me labelling and PI staining. There was a strong negative relationship between PI staining and H1 labelling, with a coefficient of determination (R^2) of 0.75 and a corresponding p-value (two-tail) of 0.0005 (indicating a statistically significant difference). When PI staining was correlated with H3K4me labelling, the relationship was weakly positive, with a R^2 value
Chapter 6: Chromatin alterations during the growth cycle of BY-2 cell cultures

of 0.33 and a corresponding p-value (two-tail) of 0.0650 (indicating that the difference was not statistically significant) (figure 6.10).

![Graph](a)

**BY-2 anti-histone H1 (FITC) vs. PI staining**

![Graph](b)

**BY-2 anti-histone H3 (FITC) vs. PI staining**

Figure 6.10. Relationships between anti-histone H1:FITC or anti-histone H3K4me:FITC labelling and PI staining. There was a strong negative correlation between histone H1 labelling and PI staining ($R^2 = 0.75$, p-value = 0.0005) (a), whereas the relationship between histone H3K4me labelling and PI staining was weak ($R^2 = 0.33$, p-value = 0.0650) (b).
6.3 Discussion

The results in this chapter show that time-related changes in PI staining observed during BY-2 cell culturing, appear to correlate chronologically with both changes in DNA methylation and changes in the labelling profile of antibodies directed against histone H1. A decrease in total 5-methylcytosine content of DNA (figure 6.4) was accompanied by changes in methylation at specific sites, as detected by alterations in DNA fragmentation patterns brought about through the actions of methylation-sensitive restriction endonucleases (figure 6.3). A decrease in labelling of histone H1 during the cultures proliferative phase (figure 6.7), was strongly correlated with changes in PI staining of chromatin (figure 6.10). Histone H1 is a member of a family of linker histones that are involved in both nucleosome structure and in higher order chromatin structure. This structure has been described as a chain of nucleosomes organised as a solenoid in a 30 nm fibre, the condensation state of which is related to the presence of histone H1 (Thoma et al., 1979; Happel and Doenecke, 2009). Although it cannot be excluded that the decrease in histone H1 labelling is due to a change by protein modification or substitution with another member of the linker histone family, further work is warranted to determine whether the observed changes reflect actual reduction of histone H1 content, and whether similar changes occur in other plant species. Lack of suitable antibodies for modified plant histones has precluded studies here on histone modification, but this is an obvious area for further investigation.

Slight differences in the timing and extent of PI staining changes were seen when cells were cultured in modified growth media. Starving the cells of either a nitrogen or carbon source caused up to a two day delay in the PI peak maxima seen when compared to cells grown in complete medium (figure 4.2 & 5.1). This delay in chromatin ‘relaxation’ normally observed during the log phase of BY-2 culture proliferation (in complete medium), may be explained by nutrient limitation causing reduced expression of genes required in cell growth and division. Increased gene transcription is often associated with an increase of DNA accessibility and associated chromatin changes (Gorisch et al., 2005).

In plants, active demethylation occurs widely and is carried out as part of a DNA repair process utilising 5-methylcytosine glycosylases such as DME and DEMETER-LIKE proteins (Gehring et al., 2009b; Gehring et al., 2009a; Hsieh et al., 2009). These enzymes are not found in mammalian cells and in fact, plant chromatin has a very different pattern of methylation from mammalian chromatin (Zhang et al., 2010). An attractive hypothesis to explain why BY-2 chromatin may change its conformation...
during culture proliferation would be that histone H1 content is reduced during active growth (until day 4) and then regained as the growth rate diminishes (from day 5) (figure 6.7). This hypothesis may also explain the 4.5% decrease in DNA methylation seen between subculturing and day 4 of growth (figure 6.4). A more open or ‘relaxed’ chromatin conformation could result due to the loss of histone H1 (Tamakaka and Thomas, 1990) and as a consequence may allow improved access for demethylating enzymes (Gehring et al., 2006). Whether the condensed chromatin state is associated with over expression of histone H1 or ‘normal’ levels of H1 is not discernable from the results presented here, but there is evidence for involvement of H1 expression in regulation of genes involved in the control of specific developmental pathways in tobacco (Prymakowska-Bosak et al., 1996). A natural progression from these results would be to perform experiments utilising western blotting to allow quantification of H1 protein levels. Further to this, an investigation involving northern blotting or quantitative real time PCR (qPCR) would allow assessment of the H1 mRNA levels during BY-2 cell culturing. An initial chromatin ‘relaxation’ and later condensation, could explain the altered accessibility of PI to intercalate into the DNA and thus the difference in staining properties (figure 6.11).
Figure 6.11. Two models explaining the changes in PI staining observed during the culturing of BY-2 cells. Both models involve progressive dissociation of histone H1 during active cell growth, then re-association of H1 due to lower levels of proliferation in aging cultures. In the condensed chromatin state, model one has a single H1 linker protein per nucleosome, while model two has the nucleosome with excess H1 in the condensed chromatin state.
7. Overview and concluding discussion

7.1 Overview

It is postulated that the differential propidium iodide (PI) staining observed in BY-2 cells during the course of culturing is due to chromatin remodelling. A proposed model for the mechanism involved, centres around histone H1 dissociation and re-association with the nucleosome. The H1 protein may have progressively dissociated from the nucleosome following subculturing of the cells, with minimum levels of H1 association achieved four days later. If H1 dissociation is occurring, it appears to correspond to the active phase of culture proliferation and may result in the decondensation of heterochromatin i.e. a more open or ‘relaxed’ chromatin state. A less dense chromatin structure may allow the PI molecule better access to DNA binding sites and therefore account for the changing PI fluorescence. Concurrently, DNA demethylation was observed, perhaps because of better access to DNA for the actions of DNA glycosylases and the requirement of demethylation to activate genes required during cell proliferation.

Once the BY-2 cells came under metabolic stress, due to the depletion of nutrient resources, proliferation slowed and the H1 protein may have then been re-synthesised and re-associated with the nucleosomes, as labelled H1 directed antibodies reported higher fluorescence levels after slowing of culture proliferation. Also at this point, cytosine re-methylation was observed, perhaps heralding the return to a condensed chromatin state, as fewer loci would need to be accessed due to the drop in culture proliferation (figure 7.1). Under conditions of carbon and nitrogen starvation, the transition to the proposed ‘relaxed’ chromatin state was delayed and this was perhaps related to slower cell division and the extension of culture vitality.
Chapter 7: Overview and concluding discussion

Figure 7.1. Proposed model explaining differential PI staining during BY-2 cell culturing. In the condensed chromatin state, histone H1 is bound to the nucleosome, DNA is methylated, fewer genes are expressed and PI fluorescence intensities are low. During active growth, a ‘relaxed’ chromatin state is adopted due to dissociation of histone H1, allowing DNA demethylation, greater gene expression and an increase in PI fluorescence intensity. Nutrient limitation halts proliferation, the chromatin returns to its previous condensed state and autophagy commences.

7.2 Nuclear staining changes during BY-2 cell growth

The phenomenon of DNA content measurements appearing to change during the course of BY-2 cell culturing was shown to be predictable and consistent (chapter 3). Flow cytometry of the PI stained nuclei revealed a pattern of increasing fluorescence intensity during the initial proliferative phase of growth, reaching a peak at approximately four days post subculturing. Once this fluorescence intensity peak was attained, the PI stained nuclei became less fluorescent with age, reaching a stable intensity during the stationary and death phases of the culture (from approximately day eight post subculturing) (figure 3.6).

When replicate samples were stained with Hoechst 33342 (H342), no such fluorescence intensity fluctuations were observed over time (figure 3.7). The major point of difference between PI and H342 is their action of DNA binding, with PI intercalating between base pairs and H342 binding within the DNA helix minor groove (non-intercalating) (Shapiro, 2003). With this knowledge it was reasonable to assume
that DNA content is not changing, but the ability of PI to access DNA binding sites does change, and that these changes may result from chromatin modifications.

Further evidence of chromatin remodelling, beyond the flow cytometric studies, is the observation of nuclear swelling upon addition of PI. The pattern of swelling had the same chronological order as the DNA peak shifts seen with flow cytometry i.e. compact nuclei at early and late phases of culture and swollen nuclei during the initial proliferative phase. No changes in nuclei size were seen when BY-2 nuclei were stained with H342 (figure 3.8).

Although chromatin alterations were suspected to be causing these discrepancies in DNA content measurement, other explanations are possible. While few studies have reported similar observations, one such report mentions the potential for compounds (as yet unidentified) that interfere with PI staining in sunflower (*Helianthus annuus*) (Price *et al.*, 2000) and may explain how PI staining can fluctuate. To further investigate this feature of BY-2 nuclei staining, the conditions during culture growth were checked for correlating factors. With the monitoring of nutrients over the course of culturing, it was noted that sucrose was rapidly utilised by the cells, leading to the theory that the chromatin remodelling may be correlated with carbon starvation and subsequent autophagy. A series of experiments was devised to pursue this theory and characterise the autophagic condition in BY-2 cells.

### 7.3 Response to carbon deprivation in BY-2 cells

When deprived of sucrose, BY-2 cells displayed distinctive morphological changes, including a reduction in cell size, marginalisation of the nucleus, development of spherical bodies and the disappearance of transvacuolar strands. In the late stages of culturing, the cell membrane shrunk away from the cell wall or in some instances the nucleus disappeared and with loss of transvacuolar strands gave the appearance of total cell vacuolisation i.e. the cell appeared empty (figure 4.20 & 4.22). Some of these morphological features have been described previously in studies that tracked autophagy in BY-2 cells (Moriyasu and Ohsumi, 1996; Inoue and Moriyasu, 2006b).

BY-2 cultures grew very poorly when sucrose was omitted or substituted with sorbitol and displayed a similar pattern of PI staining to cultures provided with sucrose from the onset of subculturing. One subtle difference in PI staining of cells grown in sorbitol substituted medium (when compared to those grown in complete medium) was a delay in the maximum fluorescence intensity reached (figure 4.2). As with growth in complete medium, H342 staining did not change significantly during the course of
culturing BY-2 cells under sucrose starved conditions (figure 4.6). These results suggested that chromatin remodelling was also occurring in cultures starved of sucrose.

Medium composition was monitored and along with sucrose (in complete medium) (figure 4.16), the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) was depleted in a very rapid fashion, with levels falling sharply over the first day following subculturating (figure 4.18). Supplementation of 2,4-D throughout BY-2 cell culturing had the effect of delaying the PI fluorescence intensity maxima by one or two days (when compared to cells grown in complete medium without 2,4-D supplementation) in all media types tested (figure 4.7). The direct effect that auxins may have on chromatin structure is not known, but as the BY-2 cell line is essentially an auxin auxotroph the addition of 2,4-D (or another suitable substitute) is required for sustained mitotic activity (Nagata et al., 2004).

When viability studies were conducted it became clear that although the BY-2 cell cultures deprived of sucrose did not divide rapidly, their viability was extended by three days (figure 4.12 & 4.13) over cultures grown in complete medium (figure 4.11). There are many precedents for a similar observation in organisms placed on highly reduced caloric intakes. Extension of life due to low calorie diets has been observed in mammals (Masoro, 2009), Drosophila (Mair et al., 2003), Caenorhabditis elegans (Hansen et al., 2008) and yeast (Dilova et al., 2007). A similar outcome has been proposed in plant senescence, where signalling based on reduced energy availability causes inhibition of TOR (target of rapamycin), which is known to result in increased life span in other organisms (Wingler et al., 2009).

Monodansylecadaverine (MDC) staining was used to determine the presence of acidic vesicles in sucrose starved BY-2 protoplasts. MDC has been used as an indicator of autophagosomes in mammalian (Bampton et al., 2005) and plant cells (Contento et al., 2005) and therefore often utilised as a marker of autophagy. In sorbitol substituted medium, BY-2 cells developed MDC positive vesicles from three days post subculturating (figure 4.23), whereas BY-2 cells grown in complete medium showed a later appearance of such vesicles at day four. This result indicates an early onset of autophagy in sucrose starved BY-2 cells, but also demonstrates the autophagic condition is occurring when BY-2 cells are grown in complete medium.

The signalling molecule nitric oxide (NO) has been implemented in cell defence through the hypersensitive response (HR) (Mur et al., 2006; Mur et al., 2008), programmed cell death (PCD) (DeMichele et al., 2009) and gene regulation in plants (Grun et al., 2006). The NO sensitive dye DAF-FM was used to follow levels of NO in
BY-2 cells during the course of culturing in sucrose deficient media. Patterns of intracellular NO did not convincingly correlate with the PI staining changes observed in previous experiments, with DAF-FM signal intensities remaining stable for the first six days of growth in the various media tested. Of note, was a significant increase in DAF-FM signal during the late phase of culturing (from day six) in complete medium grown cells (figure 4.28). An explanation for this sudden increase in intracellular NO, might involve its use as a signalling molecule for the upregulation of genes involved in a stress response (Grun et al., 2006), which in this case correlates with an exhaustion of sucrose supply by day six (figure 4.16).

Another indicator of autophagy and ACD is the release of Ca$^{2+}$ from the endoplasmic reticulum (ER) into the cytosol during the stress response. The mechanism involved has not been fully elucidated in mammalian cells, but it seems Ca$^{2+}$ activates the CaMKK-β protein, which in turn, eliminates the autophagy repressing ability of AMPK and mTOR (Hoyer-Hansen and Jaattela, 2007). Investigations of this nature in BY-2 cells were undertaken in the hope that the autophagic condition could be further understood during carbon starvation. The calcium sensitive dye indo-1 AM was utilised in experiments that measured baseline Ca$^{2+}$ levels and then challenged BY-2 protoplasts with addition the calcium ionophore ionomycin. Their ability to recover after the challenge was also observed. Older cultures had elevated indo-1 AM baseline ratios, indicating higher intracellular Ca$^{2+}$ levels and had a decreased ability to recover from ionomycin challenge. Sucrose starved BY-2 protoplasts had very high baseline ratios when compared to protoplasts grown in complete medium of the same age (figure 4.31 & 4.32). These results indicate an increased propensity of calcium flux during the late stages of BY-2 cell culturing and during sucrose starvation.

### 7.4 The effect of nitrogen withdrawal in BY-2 cells

A similar hypothesis, that changes in PI staining are related to carbon starvation and subsequent autophagy was applied to a nitrogen starvation model in a set of parallel experiments. Very little research has been conducted in BY-2 cells concerning the withdrawal of nitrates from the growth medium. Morphologically, the nitrate starved cells showed distinct characteristics, including elongation and swelling, the loss of transvacuolar strands and the formation of starch granules (figure 5.14). At first appearance the starch granules were thought to be autophagosomes, and indeed when viewed using transmission electron microscopy (TEM) they are encapsulated within a double membrane vesicle (figure 5.17). A double membrane is regarded as one of the
defining features of autophagosomes, but these granules were large, iodine positive (figure 5.15) and similar in appearance to previous reports of starch granules contained within amyloplasts (Chen et al., 1994). Similar morphologies have been described in auxin deprived BY-2 cells (Sakai et al., 1996), but in this study, nitrate withdrawal seemed to have a stronger influence on starch granule formation, as 2,4-D was depleted quickly by BY-2 cells in all medium formulations (figure 4.18 & 5.13). Starch granule formation during nitrate withdrawal may be triggered by survival mechanisms that specify the caching of available sugars for later use. It is likely that starch accumulation is in response to intracellular ammonium or amino acid concentrations (Wang et al., 2004).

Proliferation of BY-2 cells cultured in the absence of nitrates proved to be better than seen in sucrose deprived media, with cultures attaining approximately 75% of the peak cell concentration that complete medium cultures attain (figure 5.6). This was surprising considering the requirement of nitrogen in amino acid synthesis, but perhaps intracellular nitrogen recycling (autophagy?) or environmental nitrogen scavenging (from the breakdown of cellular debris?) allowed near normal cell growth and division.

As with the sucrose starved BY-2 nuclei (figure 4.2), PI staining of the nitrate starved nuclei displayed a similar pattern to that seen in complete medium, with the exception of a delayed peak in PI fluorescence intensity (day six in medium minus nitrates and day five in KCl substituted medium) (figure 5.1). H342 staining of these same nuclei samples did not show any major fluctuations in fluorescence intensity during culturing in nitrate deprived medium (figure 5.4). It was apparent that PI staining fluctuations (and therefore possible chromatin remodelling) were independent of nitrate availability and occurred in all of the medium formulations tested. The delays in PI staining maxima during nitrate withdrawal could simply be the result of a delayed and extended cell proliferative period.

When nitrate derived media were supplemented with 2,4-D throughout culturing, no major deviations in the PI fluorescence patterns were observed, except for a delay in the maximum intensity reached by nuclei grown in KCl substituted medium (day six rather than day five) (figure 5.5). This result compared favourably with the same experiment on BY-2 cells grown in sucrose deficient media (figure 4.7). This delayed response is somewhat surprising, as the actions of 2,4-D promote cell growth and division (Nagata et al., 2004), and if these PI staining increases were related to chromatin modifications, an earlier peak in maximum PI fluorescence intensity might be expected.

Like carbon withdrawal, omission of nitrates from the BY-2 media extended the
viability of the culture beyond that seen when cells were grown in complete medium. A small percentage of FDA positive cells were seen at day thirteen in nitrate deprived cultures, whereas day twelve was the last time point that viable cells were observed in complete medium (figure 5.8 & 5.9). Nitrate limitation has not been reported to cause extension of life in plant cell cultures, but slower growth and lower cell density may have a rationing effect on available resources and therefore prolong the cultures viability.

Acidic vesicles positive for MDC staining were observed in nitrate limited cultures from three days post subculturing and reached a maximal density at day four (figure 5.19). The staining of starch granules was ruled out due to the smaller size and different distribution of the MDC stained vesicles. Previous reports propose a perinuclear pattern of autophagosome distribution in sucrose starved BY-2 cells (Moriyasu and Ohsumi, 1996), but the MDC positive vesicles seemed to be evenly spread throughout the BY-2 protoplast. Whether this was an artefact from the procedure of protoplast production or their actual allocation throughout the cell is unknown and could not be tested, as MDC did not migrate through the cell wall. These vesicles suggest an early onset of autophagy and represent the first evidence of autophagosomes in nitrate deprived BY-2 cells.

NO levels (inferred from DAF-FM fluorescence) in the nitrate deprived BY-2 cultures showed similar trends to those seen in complete medium cultured cells. An increase of intracellular NO was observed from day six, with higher levels seen throughout the stationary phase of the nitrate deprived cultures (figure 5.24). In plants NO is synthesised from arginine (or nitrite) by the family of enzymes called the nitric oxide synthases (NOSs) and there are four such enzymes in plants; iNOS (chloroplast), AtNOS1 (mitochondria), pNOS (peroxisome) and aNOS (leaf apoplast) (Shapiro, 2005). Evidence of NO having a role in regulating mitochondrial respiration was provided by exposure of plant mitochondria to NO and the subsequent inhibition of enzymes that catalyse the electron transport chain (Caro and Puntarulo, 1999). This may explain the increase of intracellular NO in the stationary phase of nitrate limited cultures, as growth slows and mitochondrial respiration is repressed by AtNOS1. Other studies have shown an opposite effect of NO, with stimulation of respiration by upregulating the alternative oxidase gene (Huang et al., 2002), possibly explaining a small increase of NO production mid-growth phase (day 4) in the nitrate limited cultures examined here (figure 5.24).

Intracellular calcium levels were monitored using indo-1 AM during the course of
growth, and as with the sucrose starved cultures, the baseline ratios of nitrate starved BY-2 cells were elevated in older samples. This suggests higher levels of intracellular Ca\textsuperscript{2+} in the older nitrate starved cultures, possibly as a result of an increased stress response and autophagic condition. Interestingly, after challenging with ionomycin, the sample taken at day four did not recover, whereas the day eight sample showed evidence of Ca\textsuperscript{2+} reuptake, as reflected in the drop in the fluorescence intensity ratio (400 nm:475 nm) post ionomycin addition (figure 5.26). This ability to recover did not match the results seen in sucrose starvation experiments (figure 4.32) and may be a sign of the comparatively better growth characteristics noted in nitrate starved cultures.

7.5 Chromatin remodelling

Methylation-sensitive restriction fingerprinting (MSRF) was employed as a technique to investigate the cytosine methylation status of BY-2 cells undergoing nutrient depletion. The results demonstrated that at least two CG base pair sites had undergone demethylation (figure 6.3). In plants, demethylation is associated with chromatin relaxation and subsequent gene activation (Finnegan et al., 1998; Vanyushin, 2006), which may draw a parallel with the increased cell division and differential PI staining by the fourth day of culturing BY-2 cells in complete medium. This outcome was explored further by directly measuring the extent of total methylation in the BY-2 genome using HPLC. With a 4.5% drop in methylated cytosine observed by day four, before a 5.5% increase at day six (figure 6.4), the previous MSRF result was supported and this provided more evidence toward the theory of differential PI staining being a symptom of chromatin remodelling.

With demethylation linked to active gene expression, it was theorized that histone modifications were also occurring during times of maximum PI fluorescence. Of specific interest was the histone H1, due to its function in nucleosome stabilization. By binding to DNA entering and exiting the nucleosome coil, H1 is thought to influence the folding state of chromatin. Because histones are highly conserved between kingdoms (Isenberg, 1979), labelled antibodies directed against human histones H1 and H3 mono-methyl K4 (H3K4me) were thought to be cross reactive with the respective BY-2 histones (figure 6.5). Nuclei labelled with anti-histone H3K4me did display changes during the course of culturing, but these changes did not seem to follow any particular trend (figure 6.9); whereas probing nuclei with the anti-histone H1 antibody revealed a pattern of change over time. In all of the media formulations tested, there appeared to be a trend of decreasing histone H1 availability after subculturing, reaching a minimum
level by day 3 or 4 (figure 6.7). The chronological pattern in this result correlated well with the timing of PI staining changes, suggesting a link between loss of H1 protein and increased PI fluorescence in the BY-2 nuclei.

Alterations in chromatin structure that are thought to occur during BY-2 cell culturing could involve at least two distinct events; demethylation and dissociation of histone H1. This likely causes chromatin relaxation and results in the phenomenon of higher intensity PI fluorescence during the exponential phase of BY-2 culture growth due to improved dye accessibility. The dynamic nature of chromatin organisation is a topic of increasing interest in plants, due to its role in gene expression and response to states of stress (Jarillo et al., 2009). While the link between chromatin remodelling and nutrient limitation was not fully demonstrated in this study, much was learned about BY-2 cells and their use as a model system for autophagy and autophagic cell death (ACD).

7.6 Problems inherent to this study and possible solutions

Do protoplasts offer a realistic model system to study plant cell physiology? The production of protoplasts was necessary for the study of BY-2 cells using the method of flow cytometry and provided a useful technique to effectively introduce intracellular fluorescent dyes that would be otherwise excluded by the cell wall. Physiologically, a protoplast cannot be regarded as a plant cell lacking a cell wall. The process of protoplast production involves enzymatic degradation of the cell wall and this renders the emerging protoplast sensitive to changes in the osmotic environment, which is of significance because they require an isotonic environment to avoid protoplast rupture or shrinkage. Exposure of the cell membrane to an altered environment is likely to influence the metabolism and structure of the resulting protoplast. Without the cell wall, ultrastructural changes are evident and there may be alterations to the cell membrane permeability, affecting the solute balance within the protoplast. Under the right conditions, BY-2 protoplasts can revert back to their previous state, by reforming a cell wall (Nagata et al., 2004), thereby highlighting the transient state that a protoplast represents. Given the artificial nature of protoplasts, experimental data gained from their study must be applied with caution to the assumed situation in an intact cell. With this caveat, protoplasts are still a widely utilised and valuable tool for the study of plant physiology.

While utilising flow cytometry to investigate dyes directed to the cytoplasm necessitated the production of protoplasts, experiments investigating nuclear
fluorescence may have been achieved by the production of nuclei obtained through the use of chopping techniques e.g. as used to release *P. sativum* nuclei from shoot apices (section 2.2.2). There are alternative techniques to measure physiological parameters in plants, such as intracellular calcium concentrations using vibrating electrodes (Antoine *et al.*, 2000) and chemiluminescent detection of gaseous nitric oxide (Mur *et al.*, 2011), but many still require the absence of a cell wall.

Beyond the issue of whether BY-2 protoplasts offer an applicable plant cell model is their production from aging cultures. In a batch culture, BY-2 cells harvested before day 7 were transformed into protoplasts after approximately 4 h of enzymatic treatment, with a high rate of transformation (typically 99%) and they possessed a robust cell membrane (few protoplasts ruptured after 24 h of incubation in the osmoticum). From day 7, the cells required shorter enzymatic treatment times, had lower rates of successful transformation (decreasing to approximately 70% by day 10) and the resulting protoplasts were more inclined to rupture after extended incubation periods. It is therefore possible that results gained from aging cultures could be confounded by selective protoplastability of the BY-2 cells.

In order to investigate the potential for changes to histone interaction with BY-2 DNA during nutrient depletion, fluorescently labelled antibodies were utilised. This allowed the monitoring of anti-histone binding not only through fluorescence microscopy, but also quantification using flow cytometry. It was unfortunate that antibodies specifically directed against plant histones were not available, but being a highly conserved family of proteins, anti-human histone H1 and anti-human histone H3K4me were chosen as reporter probes. The manufacturers of these antibodies did state a predicted cross-reactivity to plant histones and preliminary experiments displayed what appeared to be binding of these antibodies within the BY-2 nucleus, so further experimentation was performed. In hindsight, the specificity of these antibodies to BY-2 histones should have been further confirmed with a technique such as western blotting. Direct comparison of BY-2 whole cell and nuclear extracts, with known human H1 and H3 proteins would allow confirmation that BY-2 H1 and H3K4me proteins were being specifically labelled and assess any cross-reactivity of the applied antibodies to other BY-2 proteins. Western blotting would have also provided information on the relative amounts of H1 and H3 proteins present, and employment of this method could have provided supporting data to that obtained from flow cytometric analysis of H1 and H3K4me antibody binding over the course of BY-2 cell culturing. Further to this, northern blotting or quantitative real time PCR (qPCR) may have been
employed in a complimentary investigation, analysing whether H1 and H3 mRNA levels followed a similar temporal pattern to the flow cytometric study.

While no information was conveyed as to the specificity of H1 directed antibodies with respect to H1 modification, the anti-histone H3K4me antibodies were noted to bind specifically to H3 that has a mono-methylated lysine residue at position 4 (K4). This prerequisite to binding had the potential to confound results, given that methylation of the K4 residue of H3 has been associated with actively transcribed euchromatin (Probst et al., 2003; Tariq et al., 2003), although during the course of culturing BY-2 cells, there did not appear to be any significant changes in binding of the H3K4me directed antibodies as measured with flow cytometry (figure 6.9).

The use of PI to measure nuclear DNA (nDNA) content can give erroneous results, since the DNA intercalating dye can also bind to RNA. This problem can be rectified by addition of ribonuclease (RNase) to specifically target and enzymatically degrade any RNA present (Dolezel et al., 2007a). But RNase also degrades the ribonucleoproteins that contribute to the structure of chromatin (Nickerson et al., 1989). While RNase A was added prior to nuclei extraction from BY-2 protoplasts in an experiment investigating PI loading concentrations (section 3.2.1), it was not added in subsequent nuclei preparations. This was because it was found that PI fluorescence intensity within the nuclei was not influenced by inclusion or omission of RNase (data not shown). It is likely that the RNase A did not come in contact with the chromatin due to its encapsulation within the nuclear membrane, protecting the associated ribonucleoproteins from enzymatic degradation.

Another form of nucleic acid that has the potential to confound nDNA measurements of BY-2 cells is plastid DNA (ptDNA). Other studies have observed changes in ptDNA synthesis during the culturing of BY-2 cells (Yasuda et al., 1988; Nagata et al., 2004), and to exclude any potential contribution of ptDNA to nDNA measurements, a qPCR experiment could have been undertaken, since qPCR can provide copy number information of a targeted DNA sequence. Changes in copy number of ptDNA specific sequences could have been compared to nDNA specific sequences and the temporal pattern of these changes could then be examined. While it was deemed unlikely that ptDNA was contributing to changes in nDNA measurements (the smaller ptDNA fragments would have been gated out during flow cytometry runs), using a qPCR approach would have provided further information as to whether this was indeed true.
7.7 Implications and future directions

Does autophagy and subsequent ACD cause chromatin remodelling in BY-2 cells? This was the primary question that was proposed at the inception of this study, with many more questions arising during its course. Some of those questions have been answered by the results presented here, but of those that remain, more research would be necessary to delve into the processes involved. With finite time and resources devoted to this particular project, and the writing of this thesis defining the scope of research, where to from here?

The distinction between cell death arising from apoptosis (type I cell death) and autophagy (type II cell death) is not clear cut. There are several examples where both processes come into play during the course of a cell’s demise, with autophagy preceding apoptotic cell death or vice versa (Velentzas et al., 2007; Thorburn, 2008). It has become increasingly apparent that there is a certain amount of “cross talk” between organelles traditionally associated with executing cell death in each system, mitochondria in apoptosis and lysosomes/vacuoles in autophagic cell death (Kundu and Thompson, 2005). Evidence suggesting multiple links between death pathways is proving to be a common occurrence (Thorburn, 2008) and further blurs the definition of type I and type II cell death.

When comparing autophagy and apoptosis, it is widely understood that autophagy (along with ACD) is evolutionarily more ancient and this is reflected in the widespread and conserved nature of autophagy in eukaryotes. From single celled yeasts, to multicellular plants and animals, autophagy is conserved due to its role in cell survival during times of nutrient depletion. Certain forms of PCD observed in plant cells are likely to be a type of ACD (VanDoorn and Woltering, 2005), since this is an evolutionarily conserved and ancient exit strategy (Bassham, 2007). Plants do not utilise caspases when initiating PCD (Bonneau et al., 2007) (although caspase-like activity has been identified (Woltering et al., 2002)), and with caspase mobilisation central to most forms of apoptosis in mammalian cells (Lockshin and Zakeri, 2004a), it is unlikely that this process is occurring in plant PCD. Also, with the absence of macrophage removal of non-viable cells in plants, ACD is currently perceived as a prominent type of cell death in plant cells (Reape et al., 2008). There are studies that dispute the role autophagy plays in PCD, either advocating a role of cell death repression (Liu et al., 2005), or suggesting alternative death mechanisms (VanDoorn et al., 2011). A consensus on the mechanisms leading to PCD in plants will be reached, but further research is needed.
Defining nuclear events during the process of apoptosis in mammalian cells include; histone release from chromatin, internucleosomal DNA cleavage and chromatin condensation (Lockshin and Zakeri, 2004b). Full understanding of the molecular events that lead to these changes has yet to be attained, but it is possible that genome-wide alterations of DNA methylation and histone binding patterns are at least partly responsible for these changes. The fate of DNA structure during autophagy and ACD is largely unknown, but may involve alterations similar to those seen in apoptosis.

The models proposed to explain chromatin alterations in BY-2 cells raise further questions about the molecules involved and the exact mechanistic execution. Of the two models mentioned in chapter 6, there is evidence for model two, with over expression of histone H1 causing abnormal heterochromatinized nuclei in tobacco plants (Prymakowska-Bosak et al., 1996). Another study in a mammalian system (rat sarcoma XC10 cells) looked at the substitution of histone H1 with H5 (a replacement variant of H1) and concluded that this substitution resulted in greater density and stability of chromatin. Expression of the transfected histone H5 gene also resulted in the arrest of cell proliferation and changes in gene expression (Sun et al., 1990). Would the PI staining profile change in this transfected cell line? Such an experiment performed in BY-2 cells would provide further insight into the role histone H1 plays in gene expression and cell proliferation.

While the correlation between chromatin remodelling and autophagy was not definitively proven, the methods used and the results obtained in this study provide support toward the use of BY-2 cell cultures as a viable tool for study of autophagy and PCD in plants. The findings presented here also provide a useful marker for the study of overall chromatin function and form the basis for investigations in other plant systems, with potential application to other forms of metabolic or viral stress.
Appendices

Appendix 1: Plant liquid culture media

1.1 Murashige and Skoog (MS) micronutrient stock solution (200X)

\[
\begin{align*}
\text{H}_3\text{BO}_3 & : \quad 1.240 \text{ g} \\
\text{MnSO}_4.4\text{H}_2\text{O} & : \quad 4.460 \text{ g} \\
\text{ZnSO}_4.7\text{H}_2\text{O} & : \quad 1.720 \text{ g} \\
\text{KI} & : \quad 0.166 \text{ g} \\
\text{Na}_2\text{MoO}_4.2\text{H}_2\text{O} & : \quad 0.050 \text{ g} \\
\text{CuSO}_4.5\text{H}_2\text{O} & : \quad 0.005 \text{ g} \\
\text{CoCl}_2.6\text{H}_2\text{O} & : \quad 0.005 \text{ g} 
\end{align*}
\]

Add salts one at a time and fully dissolve before adding the next one, make up to 1 L with distilled water. (Murashige and Skoog, 1962).

1.2 Murashige and Skoog (MS) macronutrient and micronutrient stock solution (20X)

\[
\begin{align*}
\text{NH}_4\text{NO}_3 & : \quad 33.0 \text{ g} \\
\text{KNO}_3 & : \quad 38.0 \text{ g} \\
\text{CaCl}_2.2\text{H}_2\text{O} & : \quad 8.8 \text{ g} \\
\text{KH}_2\text{PO}_4 & : \quad 3.4 \text{ g} \\
\text{MgSO}_4.7\text{H}_2\text{O} & : \quad 7.4 \text{ g} \\
\text{MS micronutrient stock solution (200X)} & : \quad 100 \text{ mL}
\end{align*}
\]

Add salts one at a time and fully dissolve before adding the next one, make up to 1 L with distilled water and adjust to pH 4.0-4.2. (Murashige and Skoog, 1962).
1.3 Murashige and Skoog (MS) macronutrient (KCl substituted) and micronutrient stock solution (20X)

KCl ............................................................................................................................ 58.8 g

CaCl$_2$$\cdot$2H$_2$O ....................................................................................................... 8.8 g

KH$_2$PO$_4$ ................................................................................................................. 3.4 g

MgSO$_4$$\cdot$7H$_2$O ..................................................................................................... 7.4 g

MS micronutrient stock solution (200X) ................................................................. 100 mL

Add salts one at a time and fully dissolve before adding the next one, make up to 1 L with distilled water and adjust to pH 4.0-4.2. (Murashige and Skoog, 1962).

1.4 BY2 medium (complete)

MS macronutrient and micronutrient stock solution ................................................. 50 mL

EDTA ferric-sodium salt ........................................................................................... 0.04 g

KH$_2$PO$_4$ .................................................................................................................. 0.30 g

Thiamine ................................................................................................................... 1.00 mg

Myo-inositol ............................................................................................................. 0.10 g

2,4-D ......................................................................................................................... 200 µg

Sucrose .................................................................................................................... 30.00 g

Make up to 900 mL with distilled water, adjust the pH with KOH to 5.8 and bring volume up to 1 L and autoclave.

1.5 BY2 medium (sorbitol substituted)

MS macronutrient and micronutrient stock solution ................................................. 50 mL

EDTA ferric-sodium salt ........................................................................................... 0.04 g

KH$_2$PO$_4$ .................................................................................................................. 0.30 g

Thiamine ................................................................................................................... 1.00 mg

Myo-inositol ............................................................................................................. 0.10 g

2,4-D ......................................................................................................................... 200 µg

Sorbitol ..................................................................................................................... 16.76 g

Make up to 900 mL with distilled water, adjust the pH with KOH to 5.8, and bring volume up to 1 L and autoclave.
Appendices

1.6 BY2 medium (KCl substituted)
MS macronutrient (KCl substituted) and micronutrient stock solution ............ 50 mL
EDTA ferrie-sodium salt ................................................................. 0.04 g
KH$_2$PO$_4$ ................................................................................ 0.30 g
Myo-inositol ............................................................................. 0.10 g
2,4-D ....................................................................................... 200 µg
Sucrose ..................................................................................... 30.00 g
Make up to 900 mL with distilled water, adjust the pH with KOH to 5.8 and bring volume up to 1 L and autoclave.

1.7 BY2 medium (KCl and sorbitol substituted)
MS macronutrient (KCl substituted) and micronutrient stock solution ............ 50 mL
EDTA ferrie-sodium salt ................................................................. 0.04 g
KH$_2$PO$_4$ ................................................................................ 0.30 g
Myo-inositol ............................................................................. 0.10 g
2,4-D ....................................................................................... 200 µg
Sorbitol ..................................................................................... 16.76 g
Make up to 900 mL with distilled water, adjust the pH with KOH to 5.8, and bring volume up to 1 L and autoclave.

Appendix 2: Protoplast production solutions

2.1 BY2 protoplast test buffer
MS salts (Sigma, MO, USA) ................................................................. 4.30 g
KH$_2$PO$_4$ ................................................................................ 0.10 g
Mannitol ..................................................................................... 36.44 g
Dissolve in 400 mL of distilled water, adjust pH to 5.8 with KOH and bring solution volume up to 500 mL before filter sterilising.
2.2 BY2 protoplast enzyme solution

Cellulase R-10 ................................................................. 2.00 g
Pectolyase Y-23 ................................................................. 0.20 g

Dissolve each enzyme one at a time in 150 mL of BY2 protoplast test buffer by stirring gently. Adjust the pH to 5.8 before making up to 200 mL, filter sterilising and storing at -20°C as 10 mL aliquots.

Appendix 3: TEM staining solution

3.1 Reynolds lead citrate solution

\[
\text{Pb(NO}_3\text{)}_2 ................................................................. 1.33 \text{ g} \\
\text{Na}_3(\text{C}_6\text{H}_5\text{O}_7)\cdot2\text{H}_2\text{O} ................................................................. 1.76 \text{ g} \\
\]

Add to 30 mL of distilled water and after 30 min of stirring, add 8 mL of NaOH (1 M). Continue to stir for 10 min and once clear, add a further 15 mL of distilled water. Solution can be stored for up to 6 months at 4°C. (Reynolds, 1963).

Appendix 4: DNA staining solutions

4.1 Galbraith’s buffer

\[
\text{Magnesium chloride} ................................................................. 2.14 \text{ g} \\
\text{Sodium citrate} ................................................................. 4.41 \text{ g} \\
\text{4-morpholinepropane sulfonate (MOPS)} ................................................................. 2.09 \text{ g} \\
\text{Triton X-100} ................................................................. 500 \mu\text{L} \\
\]

Make up to 500 mL with distilled water, stir to dissolve components and adjust the pH to 7.0. Autoclave and store at 4°C. A DNA staining solution is made up fresh by addition of 4 \( \mu\text{L} \) of PI stock solution (1 mg/mL) to every mL of Galbraiths buffer required. Protect from light. (Galbraith et al., 1983).
4.2 PBS/Triton X-100 solution
PBS tablet solution (Sigma, MO, USA) ................................................................. 99.9 mL
Triton X-100 ........................................................................................................... 100 µL

Dissolve a PBS tablet in 200 mL of distilled water (as per manufacturer’s instructions) and add the triton X-100 to 99.9 mL of the resulting PBS solution. Stir well until the triton X-100 is dissolved, autoclave and store at 4°C. A DNA staining solution is made up fresh by addition of 4 µL of PI stock solution (1 mg/mL) to every mL of PBS/Triton X-100 solution required. Protect from light.

Appendix 5: DNA extraction and purification solutions

5.1 CTAB buffer
CTAB ................................................................................................................... 10 g
Tris-HCl (1 M) ..................................................................................................... 50 mL
EDTA (50 mM) .................................................................................................... 20 mL
NaCl (5 M) ......................................................................................................... 140 mL

Make up to 500 mL with distilled water and adjust pH to 8.0.

5.2 CTAB extraction buffer
CTAB buffer ........................................................................................................ 48.5 mL
2-mercaptoethanol .............................................................................................. 1.0 mL
Proteinase K (10 mg/mL) ................................................................................... 500 µL

5.3 TE buffer
Tris-HCl (1 M) ................................................................................................... 2.5 mL
EDTA (1 mM) .................................................................................................... 500 µL

Make up to 250 mL with distilled water, adjust pH to 8.0 with HCl and filter sterilize. Store at RT.

5.4 RNase solution
RNase A ............................................................................................................. 50 mg

Make up to 5 mL with distilled water, boil for 10 min, aliquot and store at -20°C.
References


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