THE CELLULAR AND PHYSIOLOGICAL BASIS FOR THE EFFECTS OF THE *Rht3* ALLELE ON SHOOT DEVELOPMENT IN NEAR-ISOGENIC LINES OF WHEAT (*Triticum aestivum*.L)

by

Claire L. Tonkinson

A thesis presented in fulfilment of
the requirements for the degree of
Doctor of Philosophy

University of Edinburgh 1992

An ordinary day

I took my mind a walk
or my mind took me a walk -
whichever was the truth of it.

....

And my mind observed to me,
Or I to it, how ordinary
Extraordinary things are or

How extraordinary ordinary
Things are, like the nature of the mind
And the process of observing.

Norman McCraig

DECLARATION

I hereby declare that this thesis was composed by myself and the work contained herein to be my own, except where indicated otherwise.

ACKNOWLEDGEMENTS

It is a pleasure to thank my supervisor, Dr. Robert Lyndon for his constant interest, advice and friendship during the whole period of my study at Edinburgh. I am also grateful to Dr. Stephen Fry for his helpful advice and discussion during my experimental work.

I was fortunate, during the period of my research, to work at Long Ashton Research Station, Bristol. I would especially like to thank my CASE award supervisor Dr John Lenton for his useful comment, both in the preparation of this thesis and particularly during my stay at Long Ashton which ensured that my visit was so successful. I would like to thank Ms Gillian Arnold for her statistical analysis of my leaf growth data, Steve Croker for his advice on HPLC, Nigel Appleford for locating an eye-piece graticule, Ken Williams for the colour photographs and Sean Richardson for his gibberellin analyses.

I would also like to thank all members of the Plants and Soils group at the Welsh Plant Breeding Station, Aberystwyth for my both enjoyable and useful visit to the laboratory. In particular, I would like to thank Dr. Chris Pollock for inviting me to use the extensiometer and for the helpful hints on its use, and Dr. Helen Ougham for 'putting up with me' during my visit to Aberystwyth.

My time within the Botany department at Edinburgh University has been very happy, for this I would like to thank all members, both past and present.

I am grateful to the Science and Engineering Research Council for financial assistance.

It is to Patrick, Flower and Ronny that I dedicate this thesis in grateful recognition of their love and encouragement, particularly during the preparation of this thesis.

GLOSSARY

ABA	abscisic acid
AIR	alcohol insoluble residue
ATP	adenosine triphosphate
AU	absorbance units
Bq	Bequerel(s)
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
ca	circa, approximately
CA	caffeic acid
Ca^{2+}	calcium ion
cf.	confer
cm	centimetre(s)
cpm	counts per minute
cv	cultivar
d	day(s)
°	degree centigrade
DFA	di-ferulic acid
DM	dry matter
DNA	deoxyribonucleic acid
E_{ex}	elastic extensibility
eqn	equation
et al.	*et alia*
FA	ferulic acid
g	gram(s)
GA	gibberellin
GA_3	gibberellic acid
h	hour(s)
H^+	hydrogen ion
ha	hectare(s)
HPLC	high pressure liquid chromatography
IAA	indolacetic acid
kBq	kilo Bequerel(s)
λ	wavelength (nm)
ln	natural logarithm
L1	first leaf (lamina + sheath)
L2	second leaf (lamina + sheath)
LER	leaf elongation rate
Lp	hydraulic conductivity
LVDT	linear variable displacement transducer
m	cell wall extensibility
mm	millimetre(s)
MeOH	methanol
MF	microfibril
min	minute(s)
MPa	mega pascal(s)
N	nitrogen

NaCl	sodium chloride
NaOH	sodium hydroxide
N°	number
n.s.	not significant
P	probability
%	per cent
Pac	2*S*,3*S* paclobutrazol (PP333)
PCA	para-coumaric acid
pers. comm.	personal communication
pH	negative log of H+ ion concentration
PHBA	para-hydroxybenzoic acid
P	turgor pressure
p	para-
π	osmotic pressure
P_{ex}	plastic extensibility
PGR	plant growth regulator
Rf	relative to solvent front
REGR	relative elemental growth rate
rht3	wild-type, normal, tall
Rht3	mutant, dwarf
SA	specific activity
s.e.	standard error
t	tonne(s)
TBq	Tera Bequerels
TLC	thin layer chromatography
t_r	retention time
WLF	wall loosening factor
XET	xyloglucan endotransglycosylase
Y	cell wall yield threshold
Ψ	water potential
*	significant ($P < 0.05$)
**	significant ($P < 0.01$)
***	significant ($P < 0.001$)

CONTENTS

CHAPTER 1 1
INTRODUCTION 1
DWARFING GENES IN WHEAT; THE PLEIOTROPIC NATURE OF THEIR EFFECTS 1
1.1 Historical origin of wheat semi-dwarfing and dwarfing alleles 1
1.2 Genetics of the *Rht* dwarfing alleles 2
1.2.1 Formation of an active product by the Rht alleles 3
1.3 Basis for the success of wheat semi-dwarfing alleles, on a world-wide scale 3
1.4 Pleiotropic effects of the *Rht* allele on wheat physiology 5
1.4.1 Reduced stem height 5
1.4.2 Effect on the development of the root system 7
1.4.3 Reduced coleoptile length 7
1.4.4 Reduced lamina and sheath length 7
1.4.5 Reduced aleurone response to application of gibberellin in the *Rht3* line 8
1.5 Insensitivity to gibberellin 8
1.5.1 Different classes of gibberellin sensitivity mutants 9
1.5.2 The gibberellin biosynthetic pathway 10
1.6 Insensitivity to growth retardants 11
1.6.1 Paclobutrazol and mode of action 11
1.7 *Rht* alleles and growth at low temperature 13
1.8 The unknown physiological and biochemical basis for the action of the *Rht* alleles 13
1.9 Summary of research objectives 14

CHAPTER 2 15
GENERAL MATERIALS AND METHODS 15
2.1 Plant material 15
2.2 Growth conditions 15
2.2.1 Sowing conditions 16
2.2.2 Treatment of seedlings with gibberellin and paclobutrazol 16
2.3 Sampling of L2 16
2.4 Estimation of growth parameters from freehand curves and regression analysis 17
2.5 Statistical analysis 17

CHAPTER 3 19
EFFECTS OF THE *Rht3* MUTANT ALLELE ON THE GROWTH AND DEVELOPMENT OF WHEAT 19
3.1 INTRODUCTION 19
3.1.1 Effects of the *Rht3* allele on the formation and growth of main stem internodes and leaves 19
3.1.1.1 Effect of the *Rht3* allele on extension of internodes within the ear 20
3.1.2 Effect of *Rht* alleles on apical development during the period when stem internodes are initiated 20

3.2 MATERIALS AND METHODS 22
3.2.1 Plant material and growth conditions 22
3.2.2 Apical dissection 22

3.2.3 Preparation of apical sections 22
3.2.3.1 Fixation 22
3.2.3.2 Sectioning 23
3.2.3.3 Staining and mounting 23
3.2.3.4 Measurement of parameters from the stained apical sections 23

3.3 RESULTS 26
3.3.1 The rate of primordium initiation 26
3.3.2 Early internode development 26
3.3.2.1 Length, width and sectional area of developing internodes 26
3.3.2.2 Cell number within internodes of the wild-type and mutant genotypes 29
3.3.2.3 Cellular dimensions within internodes of the wild-type and mutant genotypes 34
3.3.2.4 Summary of the genotypic effects on early stem internode development 34
3.3.3 Time course of internode elongation 36
3.3.4 Conclusions: lack of effect of the *Rht3* allele on apical development 38

3.4 DISCUSSION 39
3.4.1 Effect of the *Rht3* allele on shoot apical development and the initiation of stem internodes 39
3.4.2 When in development does the *Rht3* allele first initiate a measurable effect on stem internode extension ? 39
3.4.3 Choice of a model system for the effects of the *Rht3* allele 41

CHAPTER 4 42
GROWTH OF THE SECOND LEAF (L2): EFFECTS OF THE *Rht3* ALLELE AT THE CELLULAR LEVEL 42
4.1.1 Characteristics of growth within a graminaceous leaf 42
4.1.1.1 Analysis of the pattern of growth within the extension zone 43
4.1.1.2 Effects of the *Rht* alleles on growth of wheat leaves 44
4.1.2 The relationship between the *Rht3* allele and endogenous gibberellins 44
4.1.2.1 Growth at low temperature and gibberellin responsiveness 45
4.1.3 Research objectives from the L2 model system 46

4.2 MATERIALS AND METHODS 47
4.2.1 Growth conditions 47
4.2.2 Sampling for leaf length, dry weight and surface area analysis 47
4.2.2.1 Surface area and dry weight determination 47
4.2.2.2 Analysis of L2 extension, dry weight and surface-area data 47
4.2.3 Measurement of the length of the basal meristem in L2 47
4.2.4 Measurement of the length of the extension zone of the second leaf 48
4.2.4.1 Calculation of the relative elemental growth rate (REGR) 48
4.2.4.2 Calculation of the cell residence time within the L2 extension zone 49
4.2.5 Measurement of abaxial cell lengths and widths 50
4.2.5.1 Preparation of epidermis 50
4.2.5.2 Measurement of cell lengths and widths 50
4.2.5.3 Calculation of total cell number per stomatal epidermal cell file 52
4.2.6 Statistical tests of significance 52
4.2.7 Measurement of the endogenous levels of gibberellin 52

4.3 RESULTS 53
4.3.1 Characterisation of the L2 model system 53
4.3.1.1 Growth pattern of L2 53
4.3.1.1.1 Comparison of growth parameters of L2 in the *rht3* (wild-type) and the *Rht3* (mutant) lines 53
4.3.1.1.2 Length of L2 basal meristem 56
4.3.1.2 Cellular and temporal pattern of L2 growth 57
4.3.1.2.1 Estimation of the length of the L2 extension zone 57
4.3.1.2.2 Relative elemental growth rate through L2 extension zone 57
4.3.1.2.3 Epidermal cell elongation rates 60
4.3.1.2.4 Residence time of cells in the extension zone of the wild-type and mutant 62
4.3.1.3 Effect of the *Rht3* allele on epidermal cell dimensions and number 63
4.3.1.4 Conclusions; the influence of the *Rht3* allele on growth of L2 64

4.3.2 The effect of applied growth regulators on L2 morphology and growth characteristics 65
4.3.2.1 Effect of applied gibberellin and paclobutrazol on the cellular and temporal development of L2 68
4.3.2.1.1 How do gibberellin and paclobutrazol affect the length of the extension zone ? 68
4.3.2.1.2 Effect of gibberellin and paclobutrazol on cell length and residence time within L2 meristem and extension zone 72
4.3.2.2 Effect of paclobutrazol and gibberellin on cell dimensions and cell numbers 74
4.3.2.3 Effect of gibberellin and paclobutrazol on mass per unit length of L2 75
4.3.2.4 Conclusions 75

4.3.3 Effect of low temperature; modification of L2 growth 78
4.3.3.1 Genotypic comparison between the *rht3* (wild-type) and the *Rht3* (mutant) lines grown at 10 °C 78
4.3.3.1.1 Length of second leaf basal meristem 80
4.3.3.2 How does low temperature affect the cellular and temporal pattern of growth along L2 ? 81
4.3.3.2.1 Does growth at 10 °C affect the cell residence time within the extension zone ? 82
4.3.3.3 Effect of growth at low temperature on epidermal cell dimensions and number 84
4.3.3.4 Can the application of gibberellin reverse the effect of low temperature in the wild-type ? 85
4.3.3.5 Measurement of the concentration of endogenous GA 88
4.3.3.6 Conclusions 89

4.4 DISCUSSION 90
4.4.1 Possible basis for the effect of the *Rht3* allele on the growth of L2 90
4.4.2 Effects on cell numbers 90
4.4.2.1 Measurement of epidermal cell length as a means of calculating cell number 92
4.4.2.2 Effects of growth regulators, the *Rht3* allele and low temperature on final cell number 93
4.4.3 Control of the length of the extension zone 93
4.4.3.1 Measurement of the length of the extension zone 93
4.4.3.2 Influence of plant growth regulators on the length of the extension zone in the *rht3* wild-type line 95
4.4.3.3 Influence of low temperature on the growth of the L2 extension zone 95

4.4.4 Extent of meristem activity 96
4.4.5 Final conclusions 97

CHAPTER 5 99
THE BIOPHYSICAL CONTROL OF CELL EXTENSION 99

5.1 INTRODUCTION 99
5.1.1 Equations describing the process of cell extension 99
5.1.1.1 Mechanism of cell expansion 101
5.1.1.2 Measurement of the parameters described in the Lockhart equation 101
5.1.2 Effect of low temperature on cell extension 102
5.1.3 Effect of plant growth regulators on cell extension 103
5.1.4 The effect of the *Rht3* allele on cell extension 104

5.2 MATERIALS AND METHODS 104
5.2.1 Growth conditions 104
5.2.2 Measurement of the plastic extensibility (P_{ex}) of the cell wall 104
5.2.2.1 Sampling procedure for tissue in determination of wall extensibility 104
5.2.2.2 Edinburgh extensiometer 105
5.2.2.3 Conditioning of tissue segments prior to use in the extensiometer 105
5.2.2.4 Aberystwyth extensiometer 105
5.2.2.5 Correction factors for the plastic extensibility 106
5.2.3 Estimation of osmotic pressure 106
5.2.4 Estimation of the wall yield threshold 110

5.3 RESULTS 111
5.3.1 Measurement of the cell wall extensibility 111
5.3.1.1 Cell wall extensibility of L2 material grown at 20 °C 111
5.2.1.2 Cell wall extensibility of L2 material grown at 10 °C 112
5.3.2 Measurement of cell turgor pressure 113
5.3.2.1 Measurement of the osmotic pressure of expressed cell sap 113
5.3.2.2 Osmotic pressure of plants treated with gibberellin and paclobutrazol 115
5.3.3 Measurement of the cell wall yield threshold 115
5.3.4 Conclusions 118

5.4 DISCUSSION 119
5.4.1 Effect of the *Rht3* allele on cell wall extensibility 119
5.4.1.1 The effect of low temperature growth on P_{ex} in the mutant and the wild-type genotypes 119
5.4.1.2 Influence of the physical condition of tissue samples being stretched in the extensiometer 120
5.4.2 Difficulties in measurement of turgor pressure 121
5.4.2.1 The effect of the *Rht3* allele on the measured osmotic pressure of cell sap 121
5.4.3 Effect of the *Rht3* allele on the wall yield threshold 122
5.4.4 Effect of low temperature and plant growth regulators on the water relations within the expansion zone of L2 124
5.4.5 Conclusions 124

CHAPTER 6 **126**

POSSIBLE INVOLVEMENT OF PHENOLIC ACID CROSS LINKS IN MODIFYING CELL WALL EXTENSIBILITY IN THE *Rht3* MUTANT **126**

6.1 INTRODUCTION 126
6.1.1 Occurrence of phenolic acid cross-links within the primary cell wall 127
6.1.1.1 The formation of phenolic acid cross-links; the role of peroxidase 127
6.1.1.2 The negative correlation of plant height and peroxidase concentration 128
6.1.1.3 The role of phenolics and peroxidase in cell extension and the effects of the *Rht3* allele 128

6.2 MATERIALS AND METHODS 131
6.2.1 Plant material and growth conditions 131
6.2.2 Sampling of leaf tissue 131
6.2.3 Extraction and analysis of the phenolic acids bound to the cell wall 131
6.2.3.1 Thin layer chromatography (TLC) 131
6.2.3.2 High pressure liquid chromatography (HPLC) 132
6.2.3.3 Chemical synthesis of ferulic acid oxidation products 132
6.2.4 Labelling of cell wall phenolics with [^{14}C]cinnamic acid 132
6.2.4.1 Feeding of labelled [^{14}C]cinnamic acid to basal L2 segments 132
6.2.4.2 Autoradiography of [^{14}C] TLC plate and subsequent scintillation counting of the resolved phenolic compounds 132
6.2.5 Extraction and analysis of enzyme activity within the cell wall 133
6.2.5.1 Extraction of peroxidase activity 133
6.2.5.2 Peroxidase assay 133
6.2.5.3 Fumarase assay 133

6.3 RESULTS 135
6.3.1 Analysis of phenolic acids 135
6.3.1.1 Analysis of phenolic acids using TLC 135
6.3.1.2 Analysis of phenolic acids using HPLC 135
6.3.2 Incorporation of [^{14}C]cinnamic acid into cell wall phenolics of wild-type and mutant lines 140
6.3.3 Effects of gibberellin and paclobutrazol on bound phenolic acids of the cell wall 143
6.3.4 Estimation of apoplastic peroxidase activity in the wild-type and mutant lines 145
6.3.5 Conclusions: possible involvement of phenolic acid cross-links in rigidifying the cell wall of the *Rht3* mutant 146

6.4 DISCUSSION 147
6.4.1 The possible involvement of phenolic acid wall cross-links in rigidifying the cell wall of the mutant genotype 147
6.4.1.1 What quantitative difference in cross-linked phenolic acids would be sufficient to produce a significant effect on the extensibility of the cell wall ? 149
6.4.2 The nature of the relationship between final plant height and the level of peroxidase activity 149
6.4.2.1 Localisation of phenolic acids and peroxidase 150
6.4.2.2 The relationship between gibberellins, peroxidase and the rate of growth 150
6.4.3 Is there a causal relationship between phenolic acid cross-linking and the reduced wall extensibility present in the *Rht3* mutant ? 151

CHAPTER 7 153
DISCUSSION 153

7.1 Summary of the present work 153
7.2 The control of cell extension: Difficulties in the interpretation of P_{ex} 153
7.2.1 Is P_{ex} really an estimation of m, in the Lockhart equation ? 153
7.2.2 The heterogeneous nature of the leaf segment 154
7.2.3 Are tissue tensions important in the L2 system ? 155
7.2.4 Wall synthesis and wall loosening 157
7.2.5 Involvement of specific cross-links or enzyme activities in stiffening the cell wall of the *Rht3* mutant line 158
7.2.5.1 β-glucans 159
7.2.5.2 Extensins 160
7.2.5.3 Xyloglucans 160
7.2.5.4 Deposition of the secondary cell wall 161
7.3 Possible involvement of fructans biosynthesis in the control of cell extension by the *Rht3* allele 161
7.4 Could the *Rht3* allele induce a reorientation of growth? 162
7.5 The importance of GA in controlling cell extension 164
7.5.1 What processes of L2 growth may be blocked by the *Rht3* allele ? 165
7.5.2 How may GA affect cell extension in the *rht3* wild-type - what does the *Rht3* mutant line not do ? 166
7.5.3 Involvement of GA in the extension of stem internodes 167
7.5.4 How important is the GA concentration in the intact plant ? 167
7.5.5 Extension of internodes within the rachis of the ear; the effect of the *Rht3* allele 168
7.5.6 Possible evidence that the *Rht3* genotype is not totally insensitive to GA 170
7.6 Signal transduction: possible involvement of second messengers and the *Rht3* allele 170
7.7 Is the *Rht3* mutant more of a high temperature insensitive mutant than a GA-insensitive mutant ? 171
7.8 Is the control of growth regulated by the *Rht3* allele adequately defined by premature wall stiffening, or is there an additional controlling factor ? 174
7.9 Final conclusions: the mechanism of action of the *Rht3* allele ? 176

CHAPTER 8 178
REFERENCES 178

APPENDIX 208

ABSTRACT

The aim of this study was to identify the cellular and physiological mechanism(s) for the action of the *Rht3* (dwarfing) allele in near-isogenic lines of wheat (*Triticum aestivum* L. *cv* Maris Huntsman).

The *Rht3* allele did not affect the rate of initiation, or total number, of new leaf primordia and therefore stem internodes, formed by the shoot apex. The first observable effect of the *Rht3* allele was to reduce stem internode lengths, compared to those in the *rht3* wild-type, beginning *ca* 15 d after sowing.

The second leaf (L2) was chosen as the model system in which to investigate the cellular basis for the effects of the *Rht3* allele.

The *Rht3* allele did not reduce the maximum relative elemental growth rate (REGR) in L2 grown at 20 °C compared to the *rht3* wild-type or the distance from the leaf base at which this was achieved. However, distal to the position of maximum REGR within the L2 extension zone the *Rht3* allele significantly reduced both the REGR and the rate of cell extension compared to the *rht3* wild-type. At 20 °C, the *Rht3* allele reduced the length of the L2 extension zone from *ca* 20 mm to *ca* 13 mm. The *Rht3* allele reduced both the rate of cell extension and final epidermal cell length, but did not cause any significant reduction in total cell number. In the *rht3* wild-type the length of the extension zone could be modified by the application of growth regulators, GA_3, 2*S*,3*S* paclobutrazol and/or growth at 10 °C. However, the *Rht3* allele appeared to fix the upper limit of the extension zone between very narrow limits. The effect of the *Rht3* mutant allele could be mimicked in the *rht3* wild-type by the application of 2*S*,3*S* paclobutrazol and/or growth at 10 °C.

The *Rht3* allele prematurely restricted continued cell extension along the longitudinal axis of the leaf. At 20 °C the *Rht3* allele caused a significant reduction in the plastic extensibility of the cell walls, while having a lesser effect in reducing cell turgor pressure and no obvious effect on the wall yield threshold compared to the *rht3* wild-type. When L2 was grown at 10 °C, there was no significant difference in the plastic extensibility of the cell walls or cell turgor pressure between the two genotypes.

The possible involvement of phenolic acid cross-links in the reduced wall extensibility of the *Rht3* mutant line has been investigated. There were no obvious qualitative or quantitative differences between the two lines in the phenolic acid residues covalently

bound to the cell wall or, in apoplastic peroxidase activity. However, the *Rht3* allele was associated with a faster rate of incorporation of ^{14}C label from cinnamic acid into cell wall bound phenolics acids. It is proposed that the formation of cell wall phenolic cross-links is not the principal factor responsible for the reduced wall extensibility associated with the *Rht3* allele.

The results are discussed with reference to gibberellin and temperature sensitivity. It is suggested that both the *Rht3* allele and endogenous gibberellin may influence the same processes through which cell extension is promoted. The possibility that the *Rht3* allele is essentially a high-temperature insensitive mutant is also discussed.

CHAPTER 1

INTRODUCTION

DWARFING GENES IN WHEAT; THE PLEIOTROPIC NATURE OF THEIR EFFECTS

The incorporation of semi-dwarfing alleles into modern wheat varieties has been a major contributory factor in the "green revolution" which has occurred over the last thirty years (Gale & Youssefian, 1985). The first semi-dwarf variety, Fundin, was only released in Britain in 1974. Eight years later wheat varieties carrying a Norin 10 semi-dwarfing allele (see sections 1.1 & 1.2.1) were estimated to account for three quarters of total British wheat crop production (Gale & Youssefian, 1985). Their effect on grain yield in Britain has been both dramatic and rapid. It is estimated that of the 167 % increase in British grain yield over the period 1947-1983, nearly half could be associated directly with the introduction of new varieties of wheat carrying the *Rht1* and *Rht2* alleles (see section 1.1) (Scarisbrick *et al.*, 1987). The remaining increase in yield is attributable to the introduction of more effective fertilisers, herbicides and fungicides that has occurred simultaneously with the exploitation of the new semi-dwarfing alleles.

1.1 Historical origin of wheat semi-dwarfing and dwarfing alleles

The genetic source of most modern short-strawed wheats can be traced back to two Japanese varieties, Daruma and Akakomugi, which were discovered by Western wheat breeders in the 1920's (Reitz & Salmon, 1968; Gale & Youssefian, 1985). The Daruma variety was crossed with Turkey Red in 1924 to produce the Norin 10 variety that has provided the semi-dwarfing alleles, *Rht1* and *Rht2*. These have formed the basis of modern wheat production on a global scale (Reitz & Salmon, 1968; Gale & Youssefian, 1985). The high yield potential of such short strawed varieties under irrigation, heavy fertilisation and resistance to lodging were first noted by S. C. Salmon. Grain was exported to North America where O. A. Vogel used it extensively in a crossing programme which began in 1949 (Khanna, 1991). The resultant varieties later formed the basis of the important Mexican wheat breeding programme from which lines were selected for insensitivity to day length and a responsiveness to fertilisers. These were subsequently exported to over 20 countries (Reitz & Salmon, 1968). The Akakomugi variety has provided the dwarfing alleles that have

formed the basis of the Italian wheat breeding programme (Gale & Youssefian, 1985).

The original source of the more potent Tom Thumb dwarfing allele, *Rht3*, present in both D6899 (Fick & Qualset, 1973), and Minster Dwarf (Gale & Marshall, 1973b) is less certain. The *Rht3* allele may have originated either from an old English hybrid Carter G (Dalrymple, 1978), or from wheat samples collected in Tibet in the 1930's (Gale & Marshall, 1973a & b; CIMMYT, 1967).

1.2 Genetics of the *Rht* dwarfing alleles

The present work is based upon the bread wheat *Triticum aestivum* L. which is hexaploid and has three diploid genomes; A, B and D. The formal genetics can be written as ($2x = 6n = 42$). Alleles on homeologous chromosomes segregate independently of one another. Therefore, the effects of polyploidy are not on gene segregation but on gene expression (Snape, 1987). The polyploid nature of modern wheats means that recessive or null mutations in one genome may be easily buffered by functional alleles within the remaining two genomes. Variation in genetic backgrounds only becomes detectable when the mutation is active, usually dominant, and occurs in unique untriplicated loci (Gale & Youssefian, 1985). To identify a source of genetic variation a common approach has been to increase the homozygosity of the background genotype by the production of near-isogenic lines. Such lines allow individual plants which differ in a single allele, but with otherwise equivalent genetic backgrounds, to be compared (Snape, 1987). Near-isogenic lines of wheat have been used in the present study. They should allow any differences between the mutant and wild-type lines to be ascribed directly to an effect induced by the mutant allele.

The symbol *Rht* is used to denote a reduced height dwarfing allele in wheat. However, unlike the normal convention for assigning gene symbols the upper case R does not necessarily imply dominance. Instead it indicates that one of the effects of the allele is to reduce stem height. To be classified as *Rht*, the allele must (1) occur at a single locus which can be demonstrated by segregation and (2) have a reducing effect on plant height (Gale & Youssefian, 1985).

Over ten different major alleles for dwarfism have been described in wheat. They are all regulated by single genes located on chromosome 4B or 4D (as reviewed by Stoddart, 1988). Not all of the mutations produce desirable effects; *Rht5* and *Rht7* have a reduced yield potential in comparison with the *rht* wild-type (Gale & Youssefian, 1985). The dominant *Rht10* (Ai-biàn) dwarfing gene, located on the short arm of chromosome 4D (Wang *et al.*, 1982; Izumi *et al.*, 1981) produces a more severe reduction in stem height than does the *Rht3* allele (Gale & Youssefian, 1985).

Of the two Norin 10 alleles *Rht1* is located on chromosome 4B (Gale & Marshall, 1976) and *Rht2* on chromosome 4D (Gale *et al.*, 1975). The *Rht1* and *Rht2* alleles are

members of a homeologous series located on the α arm of chromosome 4B and on the short arm of chromosome 4D (MacVittie *et al.*, 1978). The more potent Tom Thumb allele, *Rht3*, is also located on chromosome 4B (Morris *et al.*, 1972). Hence, *Rht1* and *Rht3* are alternative alleles at the same locus on chromosome 4B (Gale & Marshall, 1976). Similarly, *Rht2* and *Rht10* are alternative alleles on chromosome 4D (Gale & Youssefian, 1985). Unlike the *Rht1* and *Rht2* alleles which are incompletely recessive *Rht3* is partially dominant (Morris *et al.*, 1972).

The effects of the *Rht* alleles are additive: the combination of *Rht1* + *Rht2* produces a more severe reduction in final stem height than either allele individually (Allan, 1990). Similarly, the *Rht2* + *Rht3* gene combination results in an extreme dwarf phenotype (Plate 1.1).

1.2.1 Formation of an active product by the *Rht* alleles

Lines of wheat lacking entire chromosomes exhibit the same phenotype as the *rht* wild-type implying that the *Rht* alleles are 'active' and encode products which are responsible for the reduction in stem height (Gale & Marshall, 1975). It has been suggested that the product of the *Rht* allele, as yet unidentified, may be an inhibitor of gibberellin action (Gale & Youssefian, 1985). However, the level of the endogenous gibberellin antagonist, abscisic acid (ABA), is similar in the wild-type and *Rht3* mutant lines (Ho *et al.*, 1981; King *et al.*, 1983).

1.3 Basis for the success of wheat semi-dwarfing alleles on a world-wide scale

The widespread adoption of the *Rht* alleles has been based on two major factors. (1) The alleles reduce stem height and produce a stiffer-strawed plant which is less prone to lodging prior to harvest. Lodged crops represent a major economic loss as they are difficult or impossible to harvest. In addition, the increased humidity surrounding the ear near the ground makes the grain more susceptible to pre-harvest sprouting which can reduce grain viability by promoting early α-amylase activity (Flintham & Gale, 1982; Gale & Youssefian, 1985). (2) The alleles are associated with increased grain yields especially under conditions of high soil fertility (Rawson & Evans, 1971; Fick & Qualset, 1973; Jain *et al.*, 1974; Law *et al.*, 1979; Gale, 1979). The *Rht* alleles are thought to cause both direct and indirect effects on grain yield. The exact mechanisms through which this effect is achieved are incompletely understood although they are believed to affect all three components of yield: grain number per ear, grain size and tiller number (Gale & Youssefian, 1985).

A positive relationship has been demonstrated between decreasing plant height and decreased grain yield in wheat (Reddi *et al.*, 1969; Law *et al.*, 1979), barley (Riggs & Hayter, 1975) and oats (Sampson, 1971; Rosielle & Frey, 1975). The increase in yield associated with the *Rht* alleles is associated with increased tiller number and floret survival resulting from a redistribution of assimilate as a consequence of a reduction in plant height (Brooking

& Kirby, 1981; Borrell *et al.*, 1991; Youssefian *et al.*, 1992b).

Several studies have attributed the physiological basis of the increased grain yields to a more favourable partitioning of assimilate towards the ear from the stem (Thorne & Welbank, 1969; Fisher, 1973; Brooking & Kirby, 1981; Fischer & Stockman, 1986; Youssefian *et al.*, 1992b), particularly in the period of maximum stem extension, 17 days prior to anthesis (Borrell *et al.*, 1991). The *Rht1* allele increased grain number per ear (McClung, 1986; Borrell *et al.*, 1991), while the *Rht2* allele increased the number of ears per plant (Borrell *et al.*, 1991). An increase in the numbers of fertile tillers in *Rht* genotypes has also been associated with the increased grain yields (Jain *et al.*, 1974; Borrell *et al.*, 1991). The *Rht3* allele increased floret survival at anthesis resulting in a higher grain number following increased grain set in the distal florets of the spikelet (Flintham & Gale, 1983). However, other studies, particularly in warmer climates, have reported no yield advantage associated with semi-dwarf genotypes compared to wild-type lines (Joppa, 1973; Joppa & Walsh, 1974; Pepe & Heiner, 1975; Bush & Chamberlain, 1981), demonstrating that the increased yield was a function of specific environmental interactions as suggested by Adams (1967).

The *Rht* alleles have been associated with a reduction in the protein content of the grain (Flintham & Gale, 1983; Gale & Hanson, 1982; Gale & Youssefian, 1985; Pinthus & Gale, 1990). This has been demonstrated in studies in which the *Rht* alleles both increased (Gale, 1979; Brandle & Knott, 1986), and decreased (Zaccai *et al.*, 1987) grain yield. *Rht* alleles markedly reduced both the percentage of grain protein and grain size in a recent study based on a near-isogenic series of wheat carrying the *Rht1*, *Rht2* and *Rht3* alleles (Pinthus & Gale, 1990). In addition, the reduction in protein level was related to overall grain yield. Higher-yielding semi-dwarf lines were associated with a greater reduction in grain protein compared to lower-yielding dwarf lines.

In recent years the ability of the Norin 10 semi-dwarfing alleles to cause increased yield under differing environmental conditions has been examined in more detail (Gale & Youssefian, 1985). The semi-dwarfing alleles have been associated with an increased susceptibility to drought (Liang & Fischer, 1977) and to male sterility induced by high temperature during the critical, pre-anthesis, growth stages. Such factors have restricted their use in southern European countries (Worland, 1986). Indeed, to achieve the maximum yield potential associated with the *Rht1* and *Rht2* genotypes a full nutrient supply is required (Law, 1989). It has been argued that the greater response in absolute terms to added fertilizers and herbicides of the semi-dwarf genotypes demonstrates a reduction in stability in these varieties compared to the less responsive, taller, older varieties (Austin & Arnold, 1989).

Concern is increasing that the intensive breeding of semi-dwarf varieties may be restricting the genetic base of modern wheats between very narrow limits. The decrease in the genetic diversity compared to that present in older varieties may lead to unpredicted

susceptibility to changing climatic conditions or disease (Simmonds, 1979). Such a situation was exemplified by the susceptibility to southern leaf blight, which affected the North American maize crop during the 1970's (Simmonds, 1979; Rhoades, 1991).

1.4 Pleiotropic effects of the *Rht* allele on wheat physiology

1.4.1 Reduced stem height

The most obvious manifestation of the presence of the *Rht* allele is a reduction in final stem height (Thorne & Welbank, 1969; Evans *et al.*, 1973; Youssefian, 1986; Lenton *et al.*, 1987; Hoogendoorn *et al.*, 1990). Near-isogenic lines of a winter wheat *cv* Maris Huntsman carrying the *Rht1* and *Rht3* alleles were reduced in height by 18 % and 56 % respectively compared to the *rht* wild-type (Lenton *et al.*, 1987). The Norin 10 alleles, *Rht1* and *Rht2*, have similar effects on plant height although absolute height is also modified by genetic background (Gale & Youssefian, 1985; Youssefian *et al.*, 1992a). The *Rht3* allele produces too severe an effect on plant height to make the genotype commercially viable. Modern harvesting techniques require a crop to be at least 70 cm in height in order to achieve maximum yields without causing damage to machinery (Flintham & Gale, 1983). However, for the purposes of this research, the more potent Tom Thumb *Rht3* allele has been used on the pretext that it should amplify any pleiotropic effects induced by the mutant allele (Plate. 1.2).

The reduction in plant stature could be achieved by varying the number and/or the degree of extension of sub-apical internodes, which themselves would be related to fundamental effects on the processes of cell division and/or cell extension (Stoddart, 1987). Many studies have confirmed that, while the number of stem internodes may vary between individual cultivars, within a given cultivar the *Rht* alleles do not affect the total number of stem internodes (Thorne *et al.*, 1969; Youssefian, 1986; Lenton *et al.*, 1987; Webb, 1987; Youssefian *et al.*, 1992a). Whether the effect of the *Rht* alleles in reducing internode length depends on shorter or fewer cells is more controversial. A reduction in internode length was associated with reduced epidermal cell length while parenchymal cells were reduced in both size and number in varieties carrying Norin 10 alleles (Nilson *et al*, 1957). A more recent study using near-isogenic lines carrying the *Rht1*, *Rht2* and *Rht3* alleles showed that reduced peduncle length was associated mainly with a reduction in epidermal and mesophyll cell length but, in the *Rht3* line, also with a reduction in cell number (Hoogendoorn *et al.*, 1990).

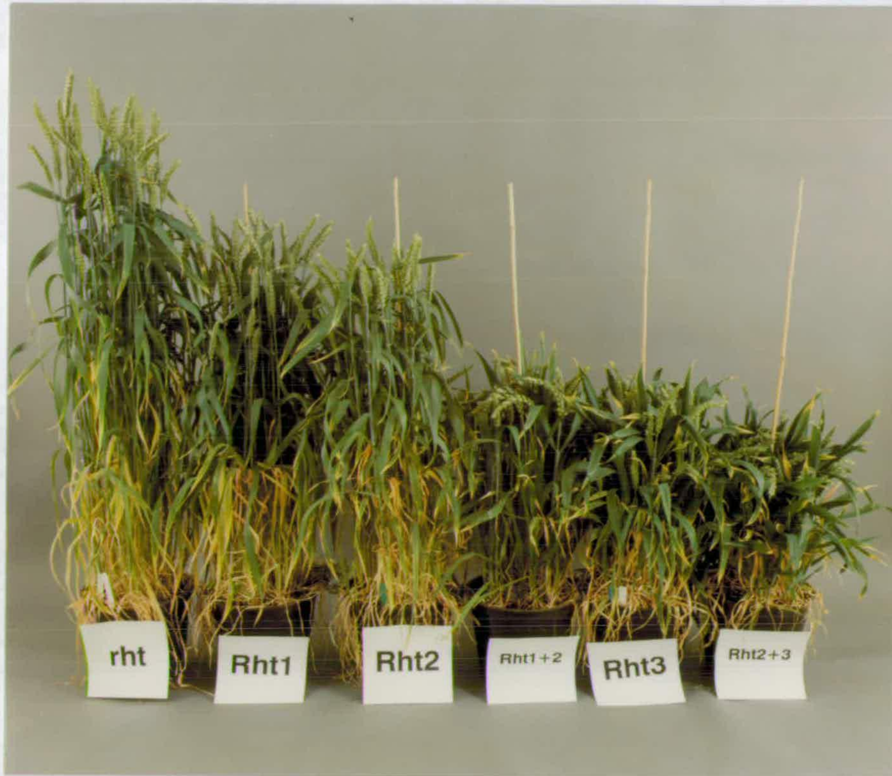


Plate 1.1 Near-isogenic series of wheat *cv* Maris Huntsman showing all possible combinations of the dwarfing alleles *Rht1*, *Rht2* and *Rht3*. (Plant material grown by Dr J. R. Lenton at Long Ashton Research Station, Bristol).

Plate 1.2 Near-isogenic lines of the *rht3* (wild-type) and *Rht3* (mutant) *cv* Maris Huntsman. (Plant material grown by Dr. J. R. Lenton at Long Ashton Research Station, Bristol).

1.4.2 Effect on the development of the root system

Clear evidence of an effect of the *Rht* alleles on root development is lacking. Gupta & Virami (1973) demonstrated a correlation between plant height, root weight and rooting depth with the *Rht1* and *Rht2* alleles. Other studies have shown no consistent effect of the *Rht* alleles in altering either the extent or the depth, of rooting compared to wild-type controls (Cholick *et al*, 1977; Bush & Evans, 1988). Lupton *et al.* (1974) suggested that although there was no difference in rooting depth between semi-dwarf and wild-type varieties, the semi-dwarf varieties have a more extensive root system.

Overall, it appears that the effect of the *Rht* alleles on roots may be regulated more by varietal and environmental differences than by the *Rht* alleles *per se*. However, if it is assumed that the *Rht* alleles have a similar effect on root:shoot ratios as they do in ear:shoot ratios then it may be expected that the *Rht* alleles would be associated with a more extensive root system than the *rht* wild-type genotypes.

1.4.3 Reduced coleoptile length

The *Rht* alleles usually cause the same proportional effect in reducing coleoptile length as they do on final stem height (Gale & Youssefian, 1985). The potency of the dwarfing allele has been positively correlated with the reduction in coleoptile length in lines carrying the *Rht3* (Flintham, 1981), *Rht1* and *Rht2* alleles (Allan *et al.*, 1962; Bains & Gill, 1973; Gale *et al.*, 1975; Allan, 1980). However, there are exceptions, as the *Rht4* dwarfing allele has no effect in reducing coleoptile length (Gale & Youssefian, 1985; Konzak, 1976).

1.4.4 Reduced lamina and sheath length

The *Rht1, 2* and *3* alleles do not reduce total main stem leaf number (Youssefian, 1986; Youssefian *et al.*, 1992a). However, the *Rht* alleles are associated with a significant reduction in final leaf length (King *et al.*, 1983; Youssefian, 1986; Keyes *et al.*, 1989) and sheath length (Keyes *et al.*, 1989; Appleford & Lenton, 1991). The reduction in leaf length has also been associated with an increase in leaf width (King *et al.*, 1983; Bush & Evans, 1988). Increasing *Rht* dosage resulted in a progressive reduction in the rate of first leaf extension and final epidermal cell length in near-isogenic lines carrying 0, 2 or 4 *Rht* gene doses (Keyes *et al.*, 1989). Although the maximum rate of extension of the first leaf was maintained for longer in *rht* wild-type lines, the total duration of extension was not affected by the *Rht* alleles. The effect of the *Rht* alleles on cell number was less clear. Cell numbers appeared to be regulated more by environmental conditions and the specific organ and surface in which it was measured than by the *Rht* alleles themselves. Extension of the laminae is discussed in more detail in Chapter 4, section 4.1.

1.4.5 Reduced aleurone response to the application of gibberellin in the *Rht3* line

The GA-induced synthesis and release of α-amylase by the aleurone layer in

germinating grains is much reduced in the *Rht3* mutant compared to the *rht* wild-type (Fick & Qualset, 1975; Gale & Marshall, 1975). However, the *Rht1* and *Rht2* genotypes do not show a graded response as they do for stem height; they are just as responsive as the *rht* wild-type to applied GA. Compared to the *rht3* wild-type the *Rht3* allele both increases the lag phase and reduces the rate of secretion of α-amylase (Fick & Qualset, 1975; Ho *et al.*, 1981). However, aleurone layers from *Rht3* lines become sensitized to GA_3 by prior treatment at low temperature (5 °C) (Singh & Paleg, 1984a, b & c), or by preincubation with indolacetic acid (IAA) (Singh & Paleg, 1986a). The production of GA_3-induced α-amylase by the aleurone cells was preceded by a change in the composition of the membrane phospholipids (Singh & Paleg, 1986b; Hetherington & Laidman, 1991). Treatment of *Rht3* de-embryonated half grains with IAA and low temperature restored the capacity of membranes to synthesize phospholipids (Singh & Paleg, 1986c). Paleg (1986) cited this as evidence that the GA receptor may be a membrane phospholipid. However, while more recent experiments have failed to repeat the induction of GA sensitivity in the *Rht3* line by either the incubation of half grains at 5 °C and/or prior treatment with IAA (R. Hetherington pers. comm.), treatment of *Rht3* with GA_3 inhibited the uptake of choline and its subsequent incorporation into phosphatidyl-choline which suggested that phosphatidyl-choline turnover may be an integral part of the GA_3 signal-transduction pathway (Hetherington & Laidman, 1991).

1.5 Insensitivity to gibberellin

It was initially assumed that insensitivity to applied GA and reduced stem height in wheat were controlled by different but linked alleles (reviewed by Gale & Youssefian, 1985). Hence, a second set of gene symbols *Gai1*, *Gai2* and *Gai3* (McIntosh, 1979) were assigned to lines which were both reduced in height and insensitive to gibberellin carrying the *Rht1*, *Rht2* and *Rht3* alleles respectively. However, subsequent research showed that a reduction in stem height and insensitivity to applied GA were pleiotropic effects of the same alleles (Gale & Marshall, 1975 & 1976; Gale & Gregory, 1977). Application of GA_3 has provided the basis for a screening programme in which lines homozygous for the *Rht* alleles of interest can be distinguished from lines which are heterozygous by their insensitivity to the applied GA_3 and lack of any commensurate growth response (Gale & Gregory, 1977).

Application of gibberellin (GA) to the *Rht1* and *Rht3* wheat genotypes does not initiate the characteristic acceleration in the rate of growth or the increase in stem and leaf lengths, that is observed in wild-type lines (Allan *et al.*, 1959; Gale *et al.*, 1975). Paradoxically, *Rht* genotypes contain higher levels of endogenous GA_1 in vegetative tissues than *rht* wild-type lines (Radley, 1970; Gale & Marshall, 1973; Romanova & Prilyuk, 1980; Stoddart, 1984; Lenton *et al.*, 1987; Appleford & Lenton, 1991). Moreover, in near-isogenic lines carrying the *Rht1* and *Rht3* alleles, the accumulation of GA_1 is inversely related to the

final sheath (Appleford & Lenton, 1991) and stem internode length (Lenton *et al.*, 1987). The accumulation of GA_1 was related to the potency of the dwarfing allele and to a reduction in the maximum rate of growth (Appleford & Lenton, 1991). The accumulation of GA_1 was initially interpreted as a block in the metabolism or utilization of the growth regulator initiated by the *Rht* alleles (Radley, 1970). Subsequent work has shown that there is little difference in the rate of metabolism of GA_1 between the *rht3* wild-type and *Rht3* mutant lines (Ho *et al.*, 1981; Stoddart, 1984). It has recently been suggested that the elevated levels of biologically active GA_1 in *Rht* lines may occur due to a block in the GA_1 utilization pathway (Scott, 1990).

1.5.1 Different classes of gibberellin sensitivity mutants

The *Rht* alleles of wheat represent one example from a whole spectrum of stature mutants of higher plants which differ in their sensitivity to GA. Such mutants can be divided into three main groups (Marx, 1983; MacMillan & Phinney, 1987; Hedden & Lenton, 1988):

1. Those with a dwarf phenotype in which the mutation has affected the biosynthetic pathway of endogenous gibberellins. They are sensitive to applied GA or GA precursors and thus may be restored to the stature of the wild-type (Table 1.1).

2. Mutants which are dwarf in stature, but do not respond to applied GA. The mutation has disabled the GA response pathway. The wild-type phenotype cannot be restored by the application of exogenous GA as it can in the group 1 mutants (Table 1.1).

3. Overgrowth, slender or giga mutants, which are insensitive to applied GA; the increase in growth appears to be independent of GA concentration. These are often recessive mutations in the signal transduction response pathway (Table 1.1).

Table 1.1 *Different groups of GA-insensitive mutants. Description of group numbers given in the text.*

Group N°	Species	mutant	Reference
1	pea	*Le, Na, Ls, Lh*	Potts *et al.*, 1982
			Potts & Reid, 1983
	maize	*dwarf-1, 2, 3, 5*	Phinney & Spray, 1982
	rice	*dx, dy*	Mukrami, 1972
	tomato	*ga-1, 2, 3*	Karssen *et al.*, 1985
	bean	*masterpiece*	Goto & Esashi, 1973
	Arabidopsis		Koorneef & Van der Veen, 1980
	lettuce	*dwf1, 2, 3*	Waycott & Taiz, 1991
2	wheat	*Rht1, 2, 3*	Radley, 1970
			Gale *et al.*, 1975
	maize	*dwarf-8*	Phinney, 1956
			Phinney & West, 1957
	Arabidopsis	*Gai*	Koorneef *et al.*, 1985
3	barley	*slender*	Foster, 1977
	tomato	*procera*	Jones, 1987
	pea	*la cry*	Potts *et al.*, 1985

For a more comprehensive review of the range of GA sensitivity stature mutants the reader is referred to review articles by Marx (1983), Stoddart (1987) and Graebe (1987).

1.5.2 The gibberellin biosynthetic pathway

Over 70 different GAs have been identified from plants and fungi (Hedden & Lenton, 1988). To account for such diversity it is thought that many GAs may be intermediates for a much smaller number of physiologically active forms, while others may be deactivation products from the active forms (Phinney, 1984; Potts & Reid, 1983). Studies of single gene recessive dwarf mutants in maize, rice and pea have established that, in these plants, GA_1 is the only active gibberellin which controls stem extension (Ingram *et al.*, 1984; Phinney & Spray, 1982; Phinney, 1984; Spray *et al.*, 1984; MacMillan, 1987; MacMillan & Phinney, 1987). In association with this, the 3β and 2β hydroxylase enzymes are considered to be the key enzyme activities in GA metabolism as they are directly responsible for the formation and deactivation of the physiologically active GA_1 (Fig. 1.1) (Hedden & Lenton, 1988).

More recently GA_3 has been identified in wheat (Appleford & Lenton, 1991), maize (Fujioka *et al.*, 1988b) and barley (Croker *et al.*, 1990) and shown to arise from GA_{20} via GA_5 (Fujioka *et al.*, 1990) (Fig. 1.1). Hence, it has recently been suggested that there may be two functionally active GAs in vegetative shoots of these species, provided that no interconversion between GA_1 and GA_3 can be shown to occur (Appleford & Lenton, 1991).

1.6 Insensitivity to growth retardants

GA biosynthesis inhibitors are useful tools to study the involvement of GAs in developmental processes. In wheat they can be used to produce phenocopies of the *Rht* alleles in responsive, wild-type lines in a controlled way (Hedden & Lenton, 1988). In near-isogenic lines of wheat the growth retardant chlormequat chloride (CCC) significantly reduced stem height in the *rht* wild-type with little effect in the *Rht1* semi-dwarf lines (Lovett & Kirby, 1971; Gale & Youssefian, 1984). Leaf growth in *Rht3* wheat seedlings was also relatively insensitive to both CCC and paclobutrazol compared to the effect in the responsive *rht* lines (Lenton *et al.*, 1987).

1.6.1 Paclobutrazol and mode of action

The broad-spectrum triazole growth retardant paclobutrazol (PP333) blocks the biosynthesis of endogenous GA by inhibiting three steps in the sequential oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Hedden & Graebe, 1985). The paclobutrazol molecule contains two asymmetric C atoms (Fig. 1.2) and so the compound can exist in 4 different configurations (Lenton, 1987), including 2 geometrical and 2 optical isomers (Izumi *et al.*, 1988). The commercially available product contains 98 % of the 2*RS*,3*RS* diastereoisomer (Hedden, 1988). However, the 2*S*,3*S* enantiomer shows the most potent growth retardant activity (Sugavanam, 1984) and is a potent inhibitor of *ent*-kaurene oxidase in pumpkin endosperm (Hedden & Graebe, 1985), while the 2*R*,3*R* enantiomer has quite different, fungicidal properties (Sugavanam, 1984).

In near-isogenic lines of wheat, application of 1 µM 2*S*,3*S* paclobutrazol to the *rht* wild-type reduced the endogenous GA_1 concentration 10-fold in basal sections of developing leaves and reduced final leaf length by 30 % (Lenton *et al.*, 1987). Conversely, application of the same paclobutrazol concentration to the *Rht3* mutant genotype reduced the endogenous GA_1 concentration in developing shoots and grains by 70 % and 86 % respectively, without affecting leaf growth. The result with the *Rht3* genotype suggests that the primary effect of paclobutrazol was to inhibit the production of endogenous GA and that the reduction in GA_1 concentration was not a consequence of reduced leaf growth caused by some other mechanism (Lenton *et al.*, 1987).

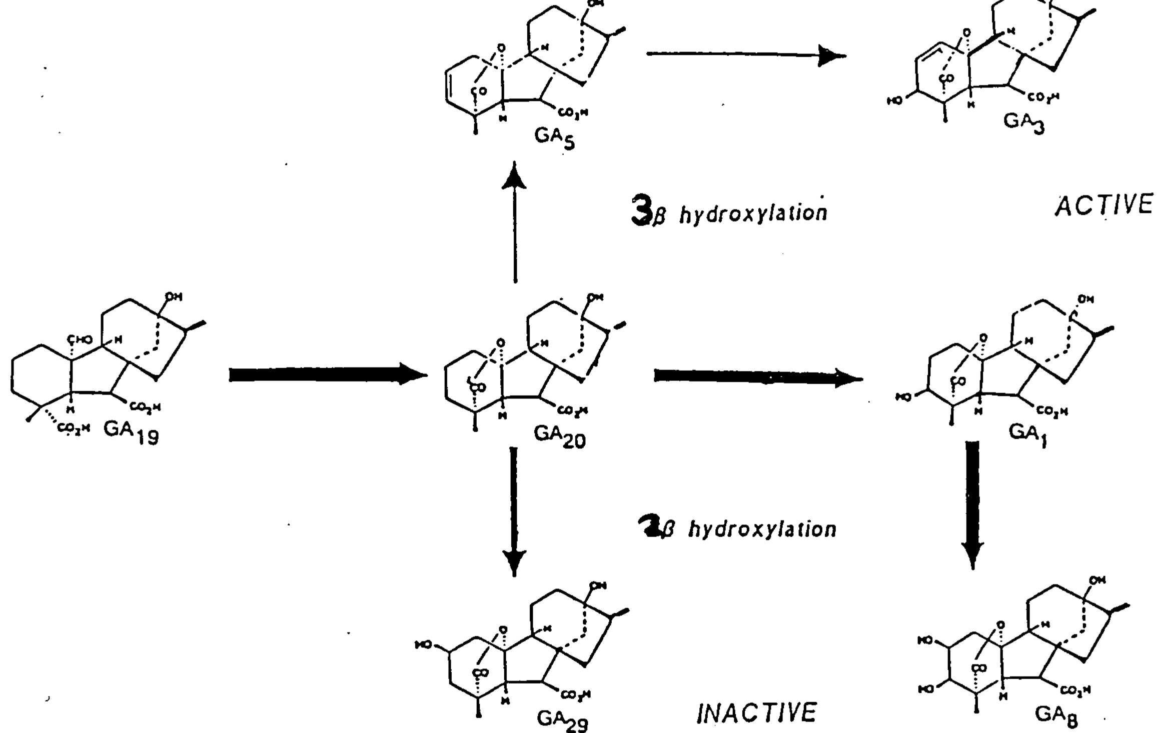


Fig. 1.1 Later stages in the 13-hydroxylation pathway from GA_{19} to the active GA_1 and GA_3. Thicker arrows indicate more major pathways.

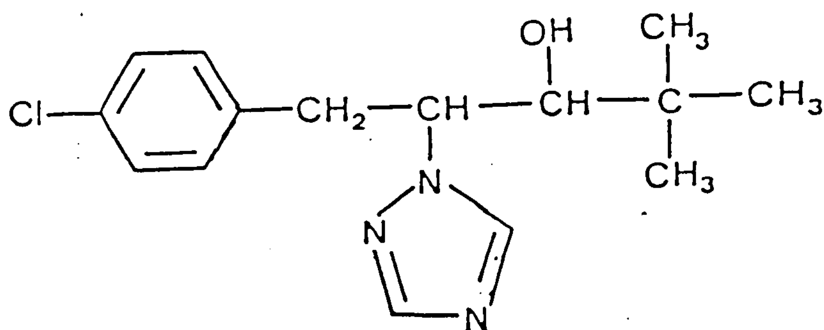


Fig. 1.2 Chemical structure of the GA-biosynthesis inhibitor, paclobutrazol.

1.7 *Rht* alleles and growth at low temperature

The responses of plants to changes in temperature are considered to be mediated via the interactions between the temperature (T) and enzyme-catalysed biosynthetic reactions (Berry & Bjorkman, 1980; Graham & Patterson, 1982; Pollock & Eagles, 1988). The rate of extension of wheat leaves was linearly related to 1/T in the range 5 ° to 26 °C (Kemp & Blacklow, 1980) whereas the duration of wheat leaf extension was inversely related to the growing temperature (Dale, 1982). The optimum growth temperature for most plants lies in the region 20 °C to 30 °C. Temperature affects leaf growth more by regulating the expansion of leaf surface area rather than by altering photosynthetic rate (Pollock *et al.*, 1983; Terry *et al.*, 1983; Pollock *et al.*, 1984). A reduction in the growth temperature can produce significant morphological and anatomical effects, often including the production of thicker, wider and shorter leaves (Terry *et al.*, 1983; Thomas, 1983b).

Studies of near-isogenic wheat lines carrying the *Rht1*, *Rht2*, and *Rht3* alleles have shown that temperature affects both the phenotypic expression of these alleles and the degree of responsiveness of the tissue to applied GA_3. The *rht* line was equally responsive to applied GA_3 at 11 ° and 25 °C and all *Rht* genotypes tested showed some responsiveness to applied GA_3 at the lower temperature (Pinthus *et al.*, 1990).

Such observations would be compatible with a suggested mechanism of GA action that involves thermal transitions within lipid membranes as occurs in liposomes (Wood & Paleg, 1972; 1974), in segments of *Avena* (Jusaitis *et al.*, 1982) and on synthetic phospholipids membranes (Pauls *et al.*, 1982; Singh & Paleg, 1984).

1.8 The unknown physiological and biochemical basis for the action of the *Rht* alleles

Despite the global adoption of semi-dwarfing alleles into commercial wheat cultivars the cellular and biochemical mechanisms underlying their pleiotropic effects are unknown.

The product(s) of the *Rht* alleles represent natural regulators of plant growth (Gale & Youssefian, 1985). If the mechanism(s) through which the *Rht* alleles operates were understood and the gene product identified, it might then be possible to transfer the alleles into other plant species in which they may also regulate growth in a predictable way and increase yields.

Clearly, there are good agronomic and economic reasons for investigating the mechanistic basis through which the *Rht* alleles operate.

1.9 Summary of research objectives

The objectives of the present work were to determine the cellular and physiological basis for the effects of the *Rht3* dwarfing allele in extension growth in wheat. To do this it is necessary:

1. To identify when in development the effect of the *Rht3* allele is first observed.
2. To determine the cellular basis for the action of the *Rht3* allele.
3. To determine how the biophysical properties of the cell walls in the leaf extension zone are affected by the *Rht3* allele.
4. To examine the possible biochemical basis for changes in cell wall extensibility.

The effects of the *Rht3* allele will also be compared with the effects of the growth retardant 2*S*,3*S* paclobutrazol, and the effect of low temperature. Both of these treatments produce phenocopies of the mutant in the *rht3* wild-type. A mechanistic basis for the effect of the *Rht3* allele may then be described. In addition, the properties of the *Rht3* mutation caused by treating the *rht3* (wild-type) with the GA-biosynthesis inhibitor 2*S*,3*S* paclobutrazol, and by a reduction in temperature will be examined. Such comparisons may provide clues to the mechanism of action of the *Rht3* gene product and also show how GAs are functioning in the *rht3* wild-type plants.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Plant material

Near-isogenic seed lines of the winter wheat *Triticum aestivum* L. *cv* Maris Huntsman were supplied by Dr J. R. Lenton of Long Ashton Research Station, Bristol. The lines were homozygous for the *rht3* wild-type and *Rht3* mutant alleles.

2.2 Growth conditions

Throughout the duration of the present research plants were grown under five different conditions, and in three different laboratories. All plants were grown at either 10 ° or 20 °C using a 16-h light/8-h dark cycle supplied by fluorescent tubes supplemented with tungsten bulbs. The different photon fluence rates of each growth condition have been summarised in Table 2.1. Growth conditions will be referred to in subsequent chapters according to their condition number in Table 2.1. Plants were watered and fed nutrient solution on alternate days, sufficient to maintain a moist soil or vermiculite surface.

Table 2.1 *Summary of growth conditions used in the present work, to be described in subsequent chapters in which they will be referred to according to the condition number.*

Condition number	Temperature (° C)	Photon fluence rate (μmol $m^{-2}s^{-1}$)	Watering solution
1	20	285	Hoagland[a]
2	20	265	LARS solution[b]
3	10	265	LARS solution
4	20	220	Hoagland
5	10	300	Hoagland

[a] = half strength nutrient solution was used as described Hoagland & Arnon (1938)
[b] = LARS, Long Ashton Research Station, nutrient solution was made up according to recipe shown below.

Table 2.2 Composition of Long Ashton nutrient solution

Chemical	Final concentration in solution (M)
KNO_3	0.23
$MgSO_4$	0.18
NaH_2PO_4	0.17
Fe EDTA	1.16×10^{-2}
$Ca(NO_3)_2$	0.33
Micronutrients	
$MnSO_4\ 4H_2O$	1.17×10^{-3}
$CaSO_4\ 5H_2O$	1.52×10^{-4}
$ZnSO_4\ 7H_2O$	1.14×10^{-4}
H_3BO_3	5.82×10^{-3}
$NaMoO_4\ 2H_2O$	5.87×10^{-5}
$NaCl_2$	1.17×10^{-3}

2.2.1 Sowing conditions

All grain was sown crease down in different size containers and medium depending upon its use.

1. Grain used for apical developmental analysis were sown 4 in each (80x80 x80 mm deep) polypropylene pot, in John Innes N° 1 potting compost.

2. Grain used for second leaf, growth analysis, dry weight and surface area determinations was sown 100 per polypropylene box (130x80 x80 mm deep), in John Innes N° 1 potting compost with a top layer of vermiculite.

3. Grain used for all other experiments based on the second leaf was sown 9 in each (50x50 x80 mm deep) polypropylene pot, in vermiculite alone.

2.2.2 Treatment of seedlings with gibberellin and paclobutrazol

The response of the second leaf to 5 or 10 µM GA_3, and 5 or 10 µM *2S,3S* paclobutrazol was tested by applying the compounds as a root drench, 100 ml of test solution to the (80x80 mm) trays and 50 ml to the smaller (50x50x80 mm) pots. An equivalent volume of water was applied to the control plants. Compounds were applied on d 2 and 5 at 20 °C and d 5 and 10 at 10 °C.

2.3 Sampling of L2

Each seedling was excised at the shoot/grain junction using a sharp single-edged razor blade. A fine needle was used to make an incision through the coleoptile and outer leaf sheath. The entire second leaf was then gently removed from the central hollow tube formed

by the encircling first leaf and coleoptile.

2.4 Estimation of growth parameters from freehand curves and regression analysis

Growth parameters were estimated from freehand curves fitted to extension data.

Estimation of the duration of the initial and extension phases of growth was made from the fitted curves between the time of sowing and 20 and 80 % final organ extension, as described in Fig 2.1 and legend.

Regression analysis was performed to estimate the maximum rate of organ extension by fitting regression lines to the period of organ growth between the points of 20 and 80 % final organ extension as shown in Fig. 2.1 and legend. Equations of regression lines fitted to extension data have been presented in the Appendix.

2.5 Statistical analysis

Students's t test analyses were performed on the data by the method of Clarke (1980). In all cases where where a t test was performed, unless otherwise indicated, comparisons were between the *rht3* wild-type and the *Rht3* mutant lines.

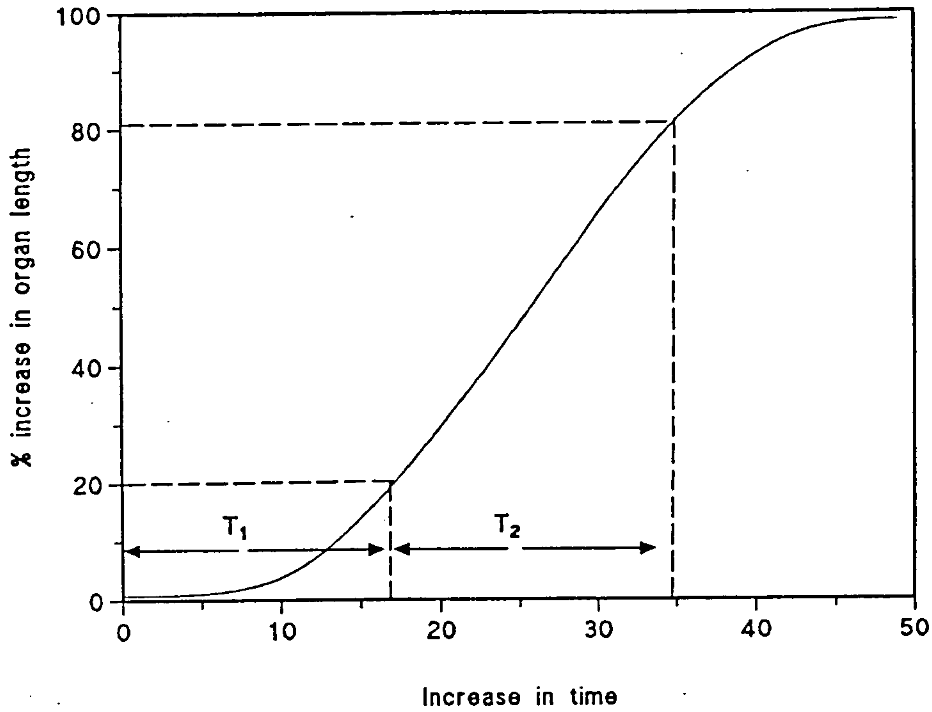


Fig. 2.1 Estimation of growth parameters from curves, of extension growth data, fitted by eye. Two horizontal lines were drawn from the y axis to intersect the curve at the points when the lamina had reached 20 and 80 % final extension respectively. Duration of the initial phase of growth (T_1) was the time taken from sowing, for the lamina to reach 20 % final length. Duration of the extension phase of growth was T_2, the time for the lamina to grow from 20 to 80 % final length. A linear regression line was fitted to data points lying between the two points corresponding to 20 and 80 % final extension, to estimate the maximum rate of lamina extension.

CHAPTER 3

EFFECTS OF THE *Rht3* MUTANT ALLELE ON THE GROWTH AND DEVELOPMENT OF WHEAT

3.1 INTRODUCTION

The most readily observable effect of the *Rht3* allele is a significant reduction in stem height (Gale & Youssefian, 1985; Youssefian, 1986; Lenton *et al.*, 1987; Webb, 1987; Hoogendoom *et al.*, 1990; Youssefian *et al.*, 1992b). However, both the timing and cellular basis of the reduction in internode length caused by the *Rht3* allele is unknown. A prerequisite to the understanding of the cellular and biochemical mechanism(s) underlying the effect(s) initiated by the *Rht3* allele was, therefore, a detailed analysis of growth in both the *rht3* (wild-type) and *Rht3* (mutant) lines. The study was begun from the onset of germination so that early stages of seedling growth, involving apical development in which stem internodes were initiated, could be compared between the two genotypes.

3.1.1 Effects of the *Rht3* allele on the formation and growth of main stem internodes and leaves

The *Rht3* allele reduces both the maximum rate of extension and the final length reached by individual stem internodes (Youssefian, 1986; Lenton *et al.*, 1987; Webb, 1987; Hoogendoom *et al.*, 1990; Youssefian *et al.*, 1992b). However, the allele does not affect the total number of main stem internodes formed. The later formed (i.e upper) stem internodes contribute proportionally more to final stem height in the *Rht3* mutant than in the *rht3* wild-type (Youssefian, 1986; Lenton *et al.*, 1987; Youssefian *et al.*, 1992b). Thus, although the absolute difference in stem internode length between the mutant and wild-type is greater in the upper internodes, the effect of the *Rht3* allele is relatively greater in the lower main stem internodes.

In the *Gramineae* stem internode extension begins around the time of floret or stamen initiation within the floral apex (Chinoy, 1949; Chinoy & Nanda, 1951; Tottman, 1977; Kirby & Appleyard, 1981). The period of rapid stem extension usually begins just before the terminal spikelet stage and continues until after anthesis (Youssefian, 1986; Youssefian *et al.*, 1992b). Significant extension of the stem (i.e internode extension > 1 cm) usually starts in the 5th and 6th internodes. The basal 3 or 4 internodes remain very short throughout the

whole growth of the plant (Langer, 1972; Kirby & Appleyard, 1981; Youssefian, 1986; Youssefian *et al.*, 1992b). Although the stem is pliable, as it extends it gains support from the surrounding leaf sheaths (Evans *et al.*, 1973; Niklas, 1990). A stem internode extends rapidly when the leaf and sheath which are inserted above it have finished extending (Evans *et al.*, 1973). The internodes extend in an ordered sequence. As one internode has reached approximately half its final length the one immediately above it begins to extend (Kirby & Appleyard, 1981). Each more distal internode becomes successively longer, with the peduncle usually showing the greatest final length (Kirby & Appleyard, 1981; Youssefian, 1986; Lenton *et al.*, 1987; Webb, 1987; Tottman, 1987; Youssefian *et al.*, 1992b). Between successive internodes the base of each leaf sheath on the extended stem grows to become swollen and thickened to form a hard joint, or node (Kirby & Appleyard, 1981). The nodes can perform an important role if the stem becomes lodged. When the stem is bent over, the nodes all grow on their lower side. The combined effect may bring the stem back up to the vertical position (Kirby & Appleyard, 1981).

The *Rht3* allele does not affect the total number of main stem leaves initiated at the apex, although it does reduce the maximum rate of elongation and final length of both the laminae and sheaths, compared to the *rht3* wild-type (Youssefian, 1986; Youssefian *et al.*, 1992b). The *Rht3* allele was expressed most actively in the lower leaves where the reduction in length associated with the mutant genotype was, as in the internodes, comparatively greater than in the upper, later formed leaves (King *et al*, 1983; Youssefian, 1986; Youssefian *et al.*, 1992). A study of isogenic lines of spring wheat carrying the *Rht2*, *Rht1+Rht2* and *Rht2+Rht3* alleles, showed that leaf lengths of the uppermost leaves of the mutant lines could be greater than those of the wild-type, especially when the plants were grown at cooler temperatures of 15 °C (Bush & Evans, 1988).

3.1.1.1 Effect of the *Rht3* allele on extension of internodes within the ear

Although the later-formed internodes of the stem and those of the rachis of the ear are extending at the same time there is no effect of the *Rht1* and *Rht3* alleles on either the final length, or rate of extension, of rachis internodes within the ear (Youssefian, 1986). Several suggestions have been made which may explain why the *Rht3* allele has such different effects on extension of ear and stem internodes; the lateral appendages of the rachis may physically restrict the extension of pith cells, tissues within the ear may be a rich source of auxin, which suppresses GA-promoted growth in *Avena* (Kaufman, 1967), or a source of abscisic acid, a natural antagonist of GA action (Lenton *et al.*, 1987). Alternatively, there could be different mechanisms which control stem and rachis internode extension (Kirby & Pharis, 1970).

3.1.2 Effect of *Rht* alleles on apical development during the period when stem internodes are initiated

There are different cell types present within developing stem internodes of the shoot apex. While all cell types may be necessary for an internode to extend, the effect of the *Rht3* allele may be to suppress the extension of particular cells, or, prevent certain cells ever being formed at all. Cell types which potentially may be affected in this way could be those which show increased extension growth in response to endogenous, or applied gibberellin in the *rht3* wild-type line. Thus, cells which in the wild-type extend in response to gibberellin may never be initiated or may be aberrant in some way in the mutant genotype.

Evidence from *Sambucus racemosa* L. suggested that stem internodes originated from a single cell layer within the apex (Zobel, 1985). Work on *Silene coeli-rosa* attempted to answer the question, why internodes were not formed in the flower. Within the flower of *Silene*, cell layers that normally give rise to internode cell files in the vegetative apex were not initiated in association with the petals or stamens. Hence, differences in final growth characteristics of the developing internodes could be detected at the cellular level within the apex, very early in development (Lyndon, 1987). In four different varieties of *Avena sativa* varying in several respects, including final height and diameter of the stem, characteristics typical of each variety were observed from the earliest stages of shoot apical development. Variations in width, and rate of elongation were observed in different tissues within developmental phytomers, just behind the apical meristem (Hamilton, 1948). Work on *Sambucus, Silene* and *Avena* suggests that an examination of the early pattern of differentiation in the shoot meristem could yield potential clues to the effects of the *Rht3* allele on later extension of stem internodes. Whether the *Rht3* allele may affect specific cells (or layers of cells), within the *rht3* wild-type apex, which subsequently divide and extend to form files within the mature stem internodes is not known. This possibility will be examined by measuring cell numbers and dimensions during internode initiation and early growth at the shoot apex in both the mutant and the wild-type lines.

While there are already comprehensive studies relating to the effects of the *Rht3* allele in both a spring and a winter wheat cultivars (Youssefian, 1986; Youssefian *et al.*, 1992a & b), the possible effects of the mutant alleles on very early developmental events, affecting the initiation of stem internodes, were not measured. In the work to be described early growth of the wheat plant will be measured in the wild-type and mutant lines in order to;

(a) investigate the effects of the *Rht3* allele on stem internode initiation and early development;

(b) determine when the effect(s) of the *Rht3* allele could first be observed.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions

All plant material was grown at 20 °C in a 16 h light/8 h dark photoperiod under the set of conditions N° 1 described in Table 2.1, Chapter 2.

3.2.2 Apical dissection

Plants were excised at the junction of the shoot and grain using a sharp razor blade. Excess soil was rinsed off the base of the plants in a stream of running tap water. All encircling laminae and sheaths were carefully stripped off the main stem to reveal the shoot apex.

Measurements of internode lengths were made using a Nachet dissecting microscope fitted with an eye-piece graticule. Progressively lower objective magnifications were required as the internodes matured and increased in length. Internode lengths were measured in the same order as they were formed by the shoot apex. Leaf primordia, positioned around the apical dome were carefully clipped off at their base using two sharp dissecting needles. The ridge or lesion which was left was used as a point of reference for the measurement of internode length. Internode lengths were measured from either this point or lesion, or the axillary bud associated with the excised primordium, whichever was easier, to the next successively higher leaf base.

The criterion used to decide whether a leaf primordium existed was based on measurement of the axillary distance (Hussey, 1971), described in Fig. 3.1 and legend. If an axillary distance could be measured then a leaf primordium was considered to be present.

3.2.3 Preparation of apical sections

3.2.3.1 Fixation

Three shoots of each genotype were collected every 2 or 3 d for a period of 45 d. Plants were excised with a razor blade at the junction of the shoot and grain. The roots and leaf material > 1 cm above the shoot base were excised. The remaining shoot stub was fixed immediately in 5 ml of 3:1 (v/v) ethanol:acetic acid. Samples were left in the fixative for a period of not less than 3 d, but often for 2 weeks until a sufficient number of samples had been collected to proceed to subsequent steps. The fixative was replaced with xylene through a graded series of 3:1, 2:1, 1:1, (v/v) ethanol:xylene, finishing with three changes of pure xylene. The final change of xylene was removed and wax pellets (Paraplast Plus, Monoject Scientific Inc.) were added to glass tubes containing the samples. The tubes were then placed in an oven maintained at 60 °C and the xylene was gradually replaced through a series of 3:1, 2:1, 1:1, (v/v) of xylene:wax changes to pure wax. The final change of pure

wax was replaced three times. Each fresh change was left to infiltrate overnight. Tissue samples were finally embedded in 4x2x1 cm depth, wax blocks and were stored at 4 °C prior to sectioning. Throughout the fixation procedure, the glass tubes containing the samples were shaken daily to aid the effective penetration of the fixative.

3.2.3.2 Sectioning

10 µm thick longitudinal sections were cut using a rotary Beck microtome. Approximately median, longitudinal and serial sections of the shoot apex were cut and flattened, by floating out in a bath of warm water maintained at 40 °C, and collected on glass microscope slides which had been pre-coated with a smear of egg albumin. The slides were dried overnight on a warm plate maintained at 40 °C.

3.2.3.3 Staining and mounting

The sections were stained using the periodic acid-Schiff's reaction (Pearse, 1968). Sections were dewaxed in xylene and hydrated through an ethanol series, (% ethanol: 100/90/70/50/30/10), to distilled water. The sections were rinsed in 2 changes of distilled water and were then hydrolysed in a solution of 0.5 % periodic acid (Sigma) for 10 min. The acid was completely removed by placing the sections in a stream of running tap water for 10 min. Sections were stained in Schiff's reagent for 12 min and then washed immediately in three changes of a solution made up from equal volumes of 0.1 M HCl and 1 % potassium metabisulphite (BDH), for 2 min. The sections were rinsed in a stream of running tap water for a further 10 min prior to being dehydrated through the same ethanol series as described above (in reverse) rinsed with xylene and finally mounted in Canada Balsalm under a glass coverslip.

3.2.3.4 Measurement of parameters from the stained apical sections

Slides were viewed on a Patholux microscope. Outlines of the sections were made on paper using an attached camera lucida drawing tube. Additional measurements of internode surface area were made by image analysis using a light microscope linked to an Apple computer and digitised drawing tablet. Image analysis was used to measure the section area of developing stem internodes using Vids II '2-dot' and 'general measurements' software packages. Stem internode section area was measured by tracing around the boundary of each individual internode (as described in Fig. 3.2) on a projected image of the apical section.

Measurements taken from the stained apical sections, using the camera lucida, were internode length and width, and cell dimensions within the central pith cells of the developing internode. For a description of how measurements were made refer to Fig 3.3 and legend.

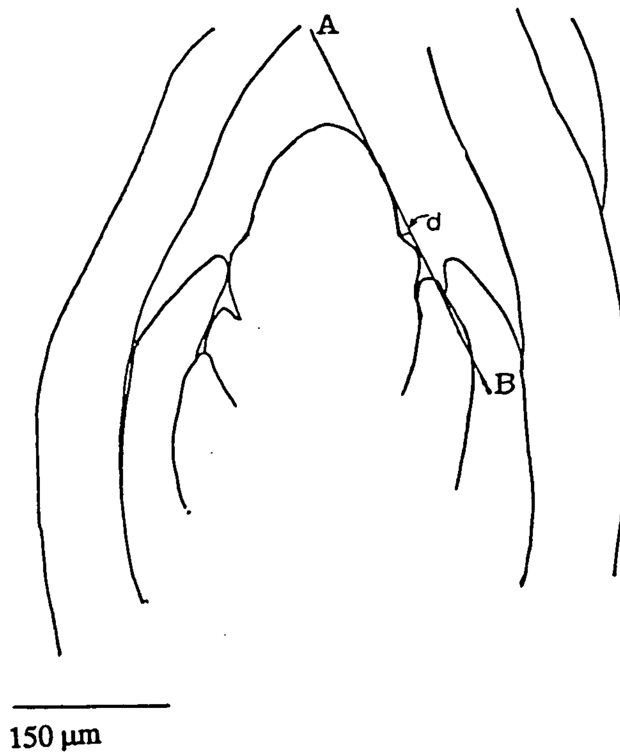


Fig. 3.1 Basis for recording the presence of a leaf primordium on a transverse section through the shoot apex by the method of Hussey (1971). A straight line AB, was drawn that touched the tip of the primordium and the apical dome. The axillary distance d = shortest distance between the leaf axil and the line AB. A leaf primordium was considered to exist if d > 0.

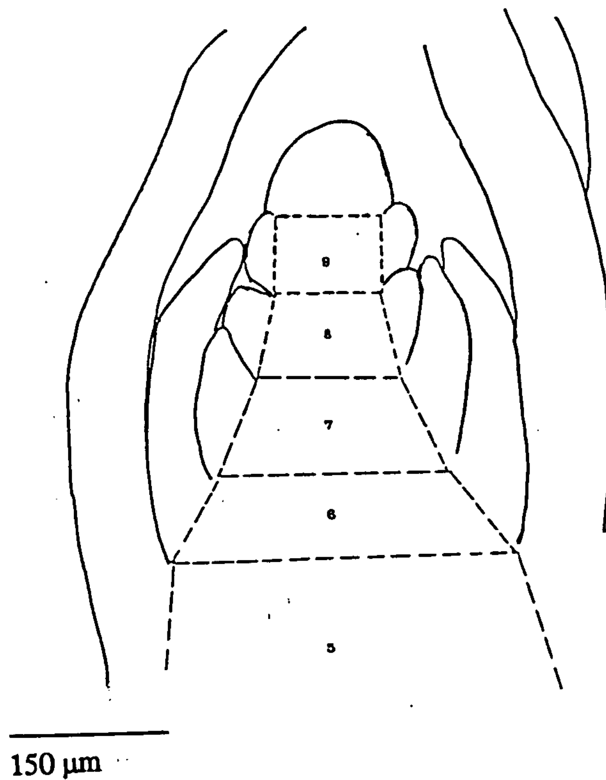


Fig. 3.2 Identification of internode cells, within a median, longitudinal, section through the shoot apex. Horizontal lines were drawn between the axils of the same leaf primordium on either side of the shoot apex. Each internode consisted of the region enclosed when a diagonal line was drawn on either side of the stem between two successive horizontal lines (or, that part of the phytomer which excluded the lamina).

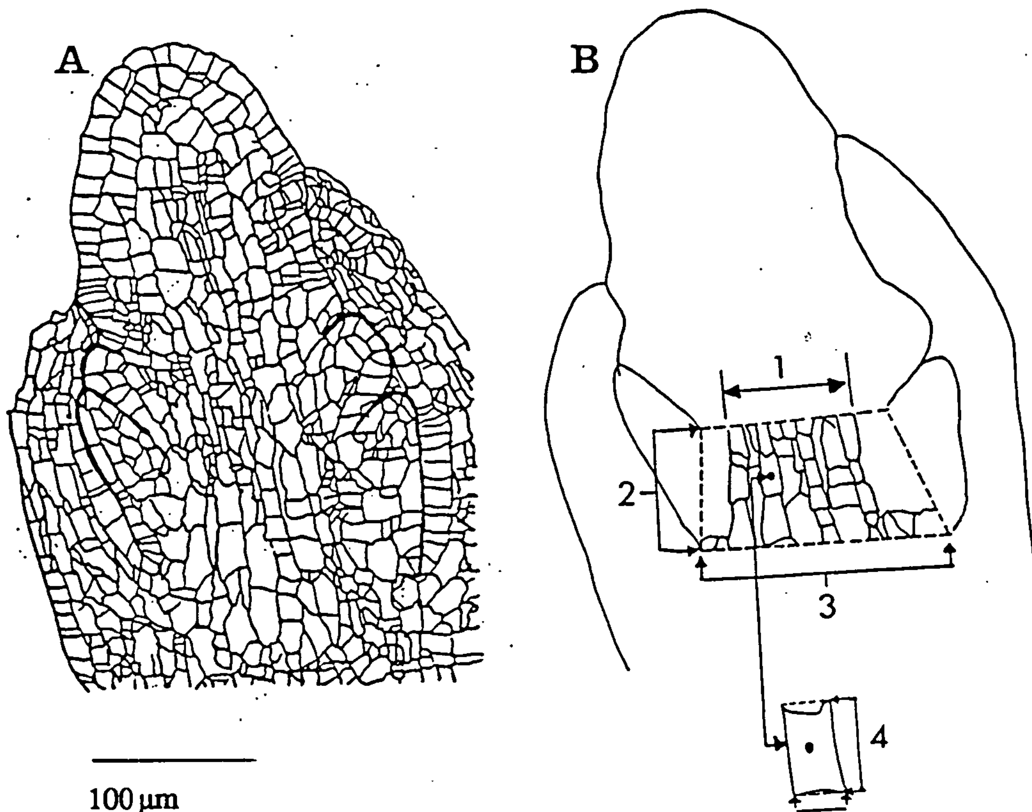


Fig. 3.3 Measurement of cell dimensions within individual internodes of the shoot apex. (A) Transverse section through a vegetative shoot apex showing cell outlines. (B) The outlines of internodes were drawn using a camera lucida drawing tube. Measurements were then made on the scaled drawing so produced.

In all measurements, only the large central pith cells, within distance (1) were included.

Internode length was measured as the distance between lines drawn connecting two successive leaf axils on either side of the stem , distance (2). Internode width, or stem width was measured as distance between the leaf axils at the base of the internode, distance (3). Refer to Fig. 3.2 for definition of individual internodes.

The number of cells across the internode base (or stem width), was counted along (1). The number of cells down the length of the internode was counted, as the number of cross walls passed down a line drawn between the upper and lower limit of the internode, distance (2).

Individual cell dimensions were measured by considering each cell as a regular shape, usually a square or a rectangle as shown (4). Cell length and width were measured as the length and width of the shape so drawn.

3.3 RESULTS

3.3.1 The rate of primordium initiation

Four leaf primordia were most commonly present within the grain embryo of both the *rht3* wild-type and *Rht3* mutant lines. In randomly selected samples, each of 9 grain, the mean number of leaf primordia present within the embyro was: 4.5 ±0.53 and 4.3 ±0.46 in the *rht3* wild-type and *Rht3* mutant lines respectively.

The *Rht3* allele did not affect the rate of initiation of new leaf primordia, and by implication, new stem internode initials, within the shoot apex. Both the wild-type and the mutant genotype initiated a new leaf primordium *ca* every 3 d at 20 °C in a 16 h photoperiod (Fig. 3.4). In both genotypes, the maximum complement of *ca* 12 leaf primordia was reached *ca* 30 d after sowing. Approximately 45 d after sowing, the formation of double ridges was observed on the shoot apices of both the *rht3* wild-type and *Rht3* mutant lines, indicating that the apex had switched from the initiation of leaf primordia to the initiation of floral, spikelet primordia.

Prior to the double ridge stage of apical development, the *Rht3* allele showed no obvious effect on the overall morphology of the shoot apex, which was very similar between the two genotypes (Plate 3.1). Had the microscope slides containing the stained apical sections not been labelled it would have been impossible to identify from which genotype they had originated.

3.3.2 Early internode development

Three different developmental stages were chosen to compare median sections through the apex of the *rht3* wild-type and *Rht3* mutant lines. The stages were those in which the apices had initiated 4, 6, or 9 leaf primordia, which corresponded to samples taken *ca* 1, 10 and 22 d respectively after sowing. In actual fact, the chronological and developmental scales were very similar between the two genotypes. (All stem tissue between the axils of 2 successive leaves has been referred to as an internode).

3.3.2.1 Length, width and sectional area of developing internodes

Although some differences were seen at the 6-leaved stages there was no consistent effect of the *Rht3* allele on either the length or width of stem internodes within shoot apices of the 3 developmental stages studied (Fig. 3.5). Curves shown in Fig. 3.5 were compared using analysis of variance to determine whether there were any significant effects of genotype on either internode number or internode dimensions. There was a strong effect of internode number (position within the developing stem) on both internode length and width ($P < 0.01$) (Statistical analysis presented in Table A.1 of Appendix).

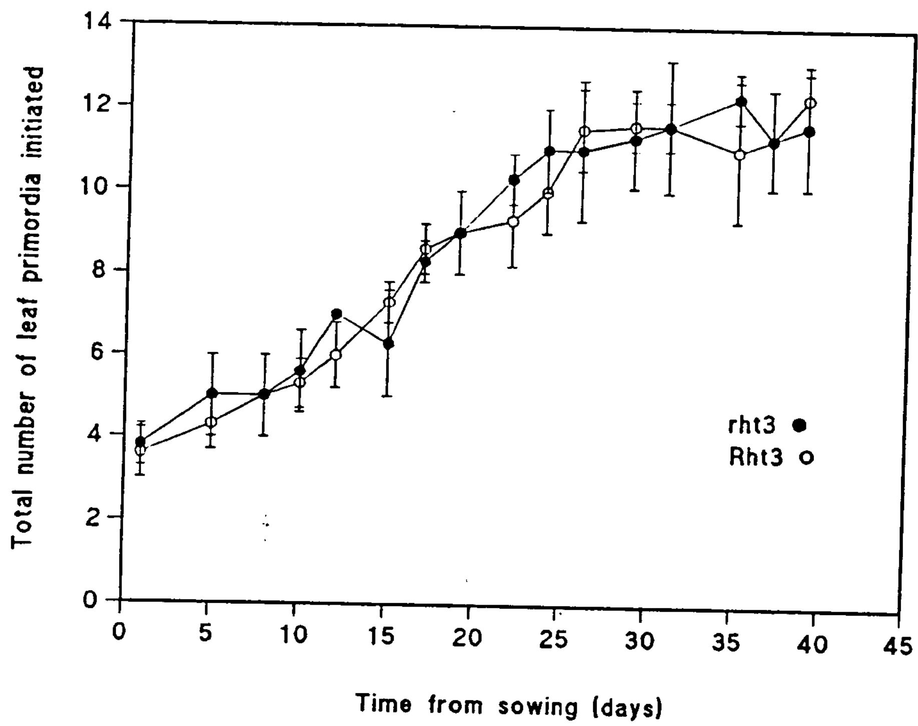
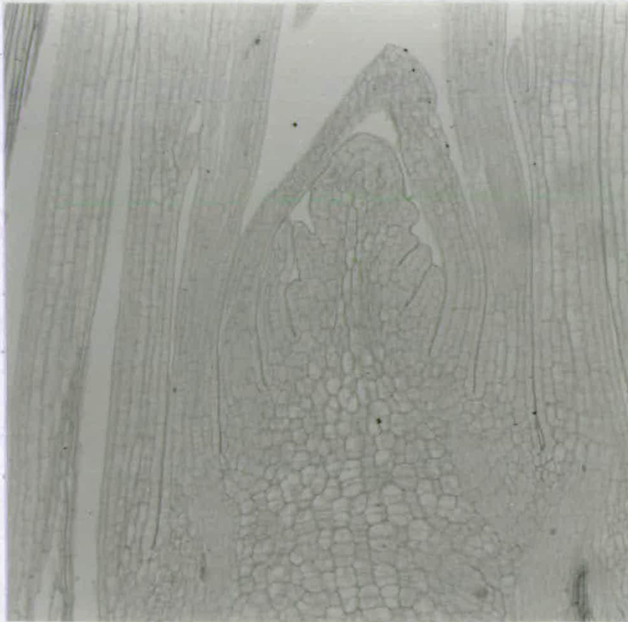


Fig. 3.4 Formation of new leaf primordia by the shoot apex of *rht3* (wild-type) and *Rht3* (mutant) lines (± s.e., n=3) of Maris Huntsman. Plants grown at 20 °C.

a

100 μm

b



100 μm

Plate 3.1 Morphology of the vegetative shoot apex in (a) *rht3* (wild-type) and (b) *Rht3* mutant lines of Maris Huntsman. Apices of both lines at 9/10 leaf primordia stage, sampled *ca* 26 days after sowing

This showed that internodes, in both genotypes, increased in length and width as they grew, over the 22 d period of the study. There was no significant genotypic effect, except in the 6-leaved shoot apices, where internode lengths were significantly ($P < 0.05$) longer in the *Rht3* mutant compared to the *rht3* wild-type line. This implies the possibility that during this early stage of internode development, growth in the *Rht3* mutant line may be faster than that in the *rht3* wild-type, but this effect did not continue.

The longitudinal sectional area of developing internodes was measured up to 25 d after sowing. In both genotypes, internode sectional area increased approximately exponentially as the internodes grew. Analysis of variance showed that there were no significant differences in overall internode size, or pattern of development between the wild-type and the mutant lines (Fig. 3.6) (Statistical analysis is presented in Table A.2 of Appendix). However, a difference in sectional area between the two genotypes in internodes 4, 5 and 6 began to be apparent *ca* 15 d after sowing (Fig. 3.6) which was confirmed in other later measurements *cf.* Fig. 3.9.

Thus, during the early growth and development of the first 9 stem internodes the products of the *Rht3* allele did not appear to have any obvious effects either on the length, width or sectional area of stem internodes compared to the *rht3* wild-type line except in internodes 4, 5 and 6 after *ca* d 15. However, it is important to realise that this study is based upon early stem internode development, (up to *ca* 26 d after sowing), prior to the major phase of stem elongation when the main genotypic effect of the *Rht3* allele becomes highly significant in reducing the length of distal internodes, as described by Youssefian (1986), Webb (1987) and Youssefian *et al.* (1992b).

3.3.2.2 Cell number within internodes of the wild-type and mutant genotypes

The effect of the *Rht3* allele on the length and width of developing internodes was investigated in terms of cell numbers within apices at the same developmental stages as described in section 3.3.2. In both lines, stem internode files appeared to originate from just 2 or 3 cell layers within the shoot apex (Fig. 3.7, 4a). The values shown in Fig. 3.7 were tested for any significant genotypic effect using analysis of variance. While there was a strong effect of internode number with cell number ($P < 0.01$) there was no obvious, consistent genotypic effect attributable to the presence of the *Rht3* allele in the mutant plants. Only in 6-leaved shoot apices was there any genotypic effect ($P < 0.05$) on cell number within longitudinal files of the internodes (Statistical analysis has been presented in Table A.3 of Appendix).

Therefore, no obvious genotypic effects of the *Rht3* allele were observed in the overall steady increase in cell number both across internode width and in longitudinal files as the internodes began to grow over the *ca* 22 d period of the study (Fig. 3.7).

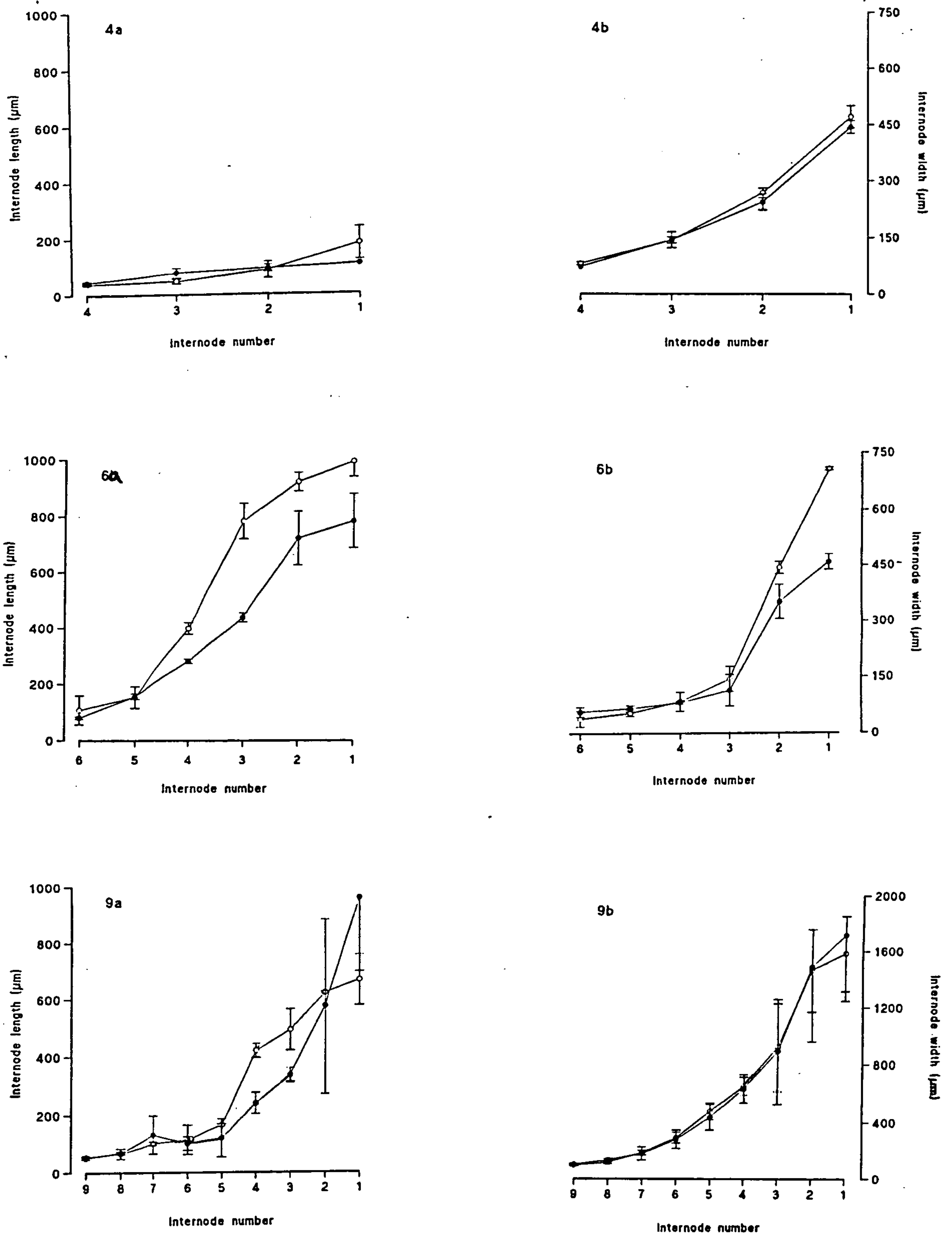
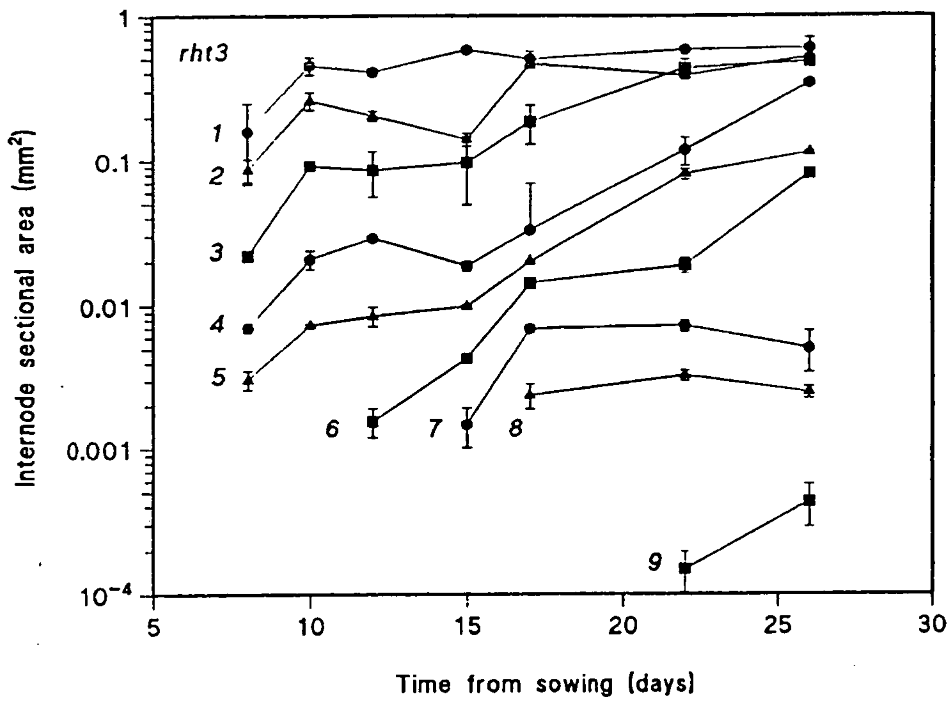


Fig. 3.5 Comparison of internode length (a) and width (b) (± s.e., n=3) in three developmental stages of the shoot apex of the *rht3* (wild-type) (closed circles ●) and *Rht3* (mutant) lines (open circles ○) of Maris Huntsman grown at 20 °C. The developmental stages used were 4-leaved, 6-leaved and 9-leaved shoot apices. Internodes numbered in order of initiation.



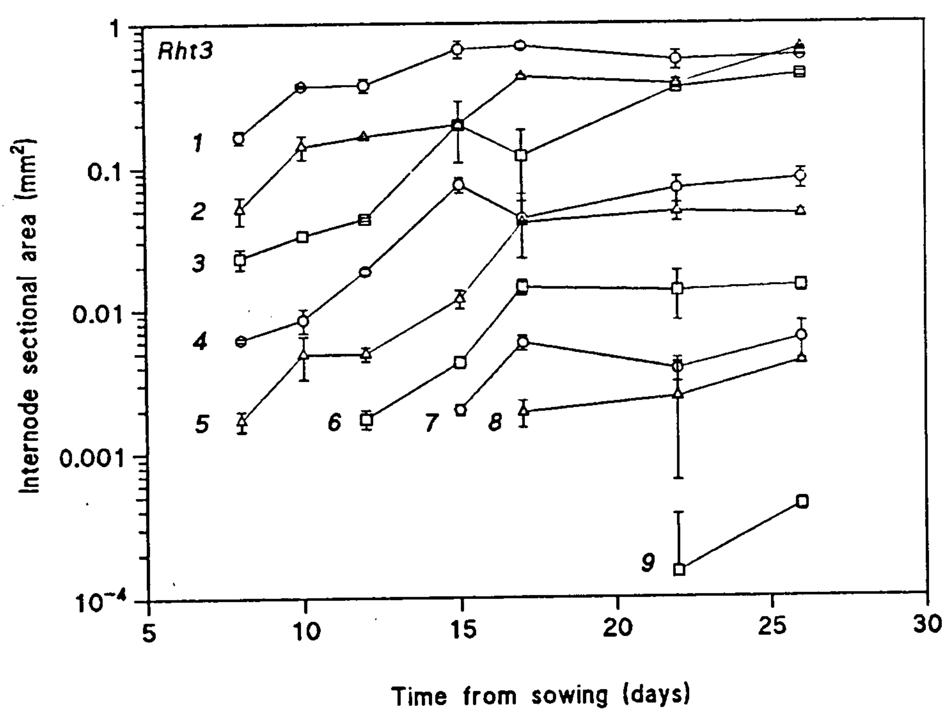


Fig. 3.6 Comparison of internode sectional area with time in the *rht3* (wild-type) and *Rht3* (mutant) lines (± s.e., n=3), of Maris Huntsman grown at 20 °C. Internodes numbered in order of initiation.

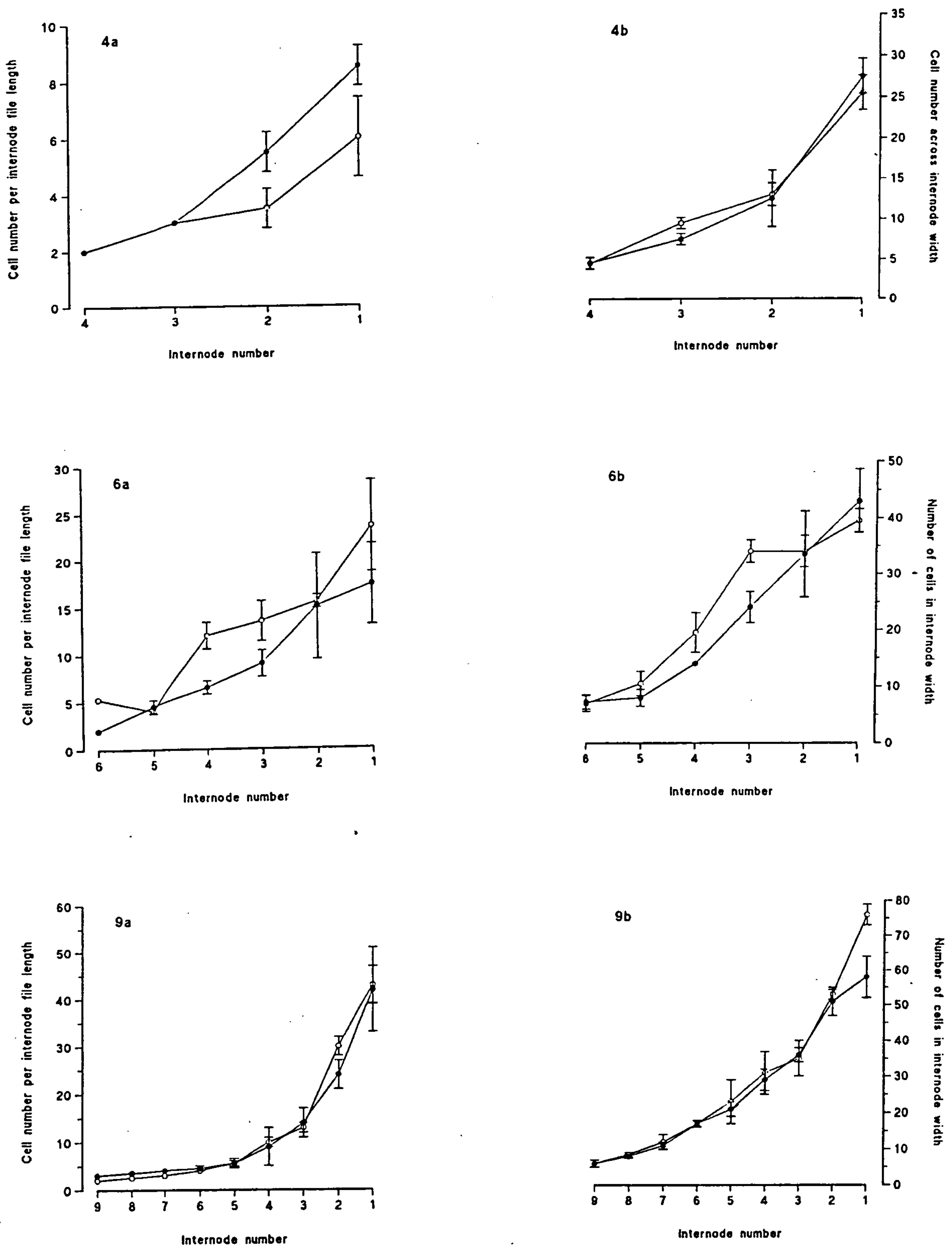


Fig. 3.7 Comparison of cell numbers (a) across the internode base and (b) in longitudinal files in shoot apices of the *rht3* (wild-type), (closed circles ●) and *Rht3* (mutant), (open circles O) lines (± s.e., n=3), of Maris Huntsman grown at 20 °C. Apices were compared at three different developmental stages when the apex had initiated 4, 6 or 9 leaves. Internodes numbered in order of initiation.

3.3.2.3 Cellular dimensions within internodes of the wild-type and mutant genotypes

In both genotypes, as the internodes developed, cell length remained essentially the same although cell width increased (Fig. 3.8), showing that, over the period in which developing internodes were studied, cell division kept pace with cell extension such that mean cell length remained unchanged. When the values shown in Fig. 3.8 were compared using analysis of variance there was a slight genotypic effect on cell length ($P < 0.05$) in 9-leaved shoot apices while there was a stronger genotypic effect on cell width ($P < 0.01$) in 6-leaved ($P < 0.05$) and 9-leaved ($P < 0.01$) apices. However, the results are difficult to interpret as different genotypes were having a different effect at different times. It was concluded therefore that there was no consistent effect of genotype on cell width. The statistical analysis, (presented in Table A.4 of Appendix), also indicated that there was less of an interaction between internode number and cell length, than was demonstrated in section 3.3.2.1, on internode dimensions, and in section 3.3.2.2, on cell number. Overall, this could suggest that cells within developing internodes up to *ca* d 22 are predominantly involved in cell division with cell extension and that the two processes are continuing at the same rate in both genotypes.

3.3.2.4 Summary of the genotypic effects on early stem internode development

The effects of genotype, internode number and their interaction in the first 4 internodes have been analysed using two-way analysis of variance and summarized in Table 3.1. The results show that while the particular stem internode has a significant effect on cell length and cell number per internode file, there was no significant effect of genotype on any of the measured parameters. In addition, there was only evidence ($P < 0.05$) of any interaction at all between genotype and internode number on cell width (Table 3.1).

Overall, it may be concluded that until d 26 of growth, within the first 3 stem internodes there is no consistent significant effect of the *Rht3* allele on internode length, width, cell numbers or cellular dimensions.

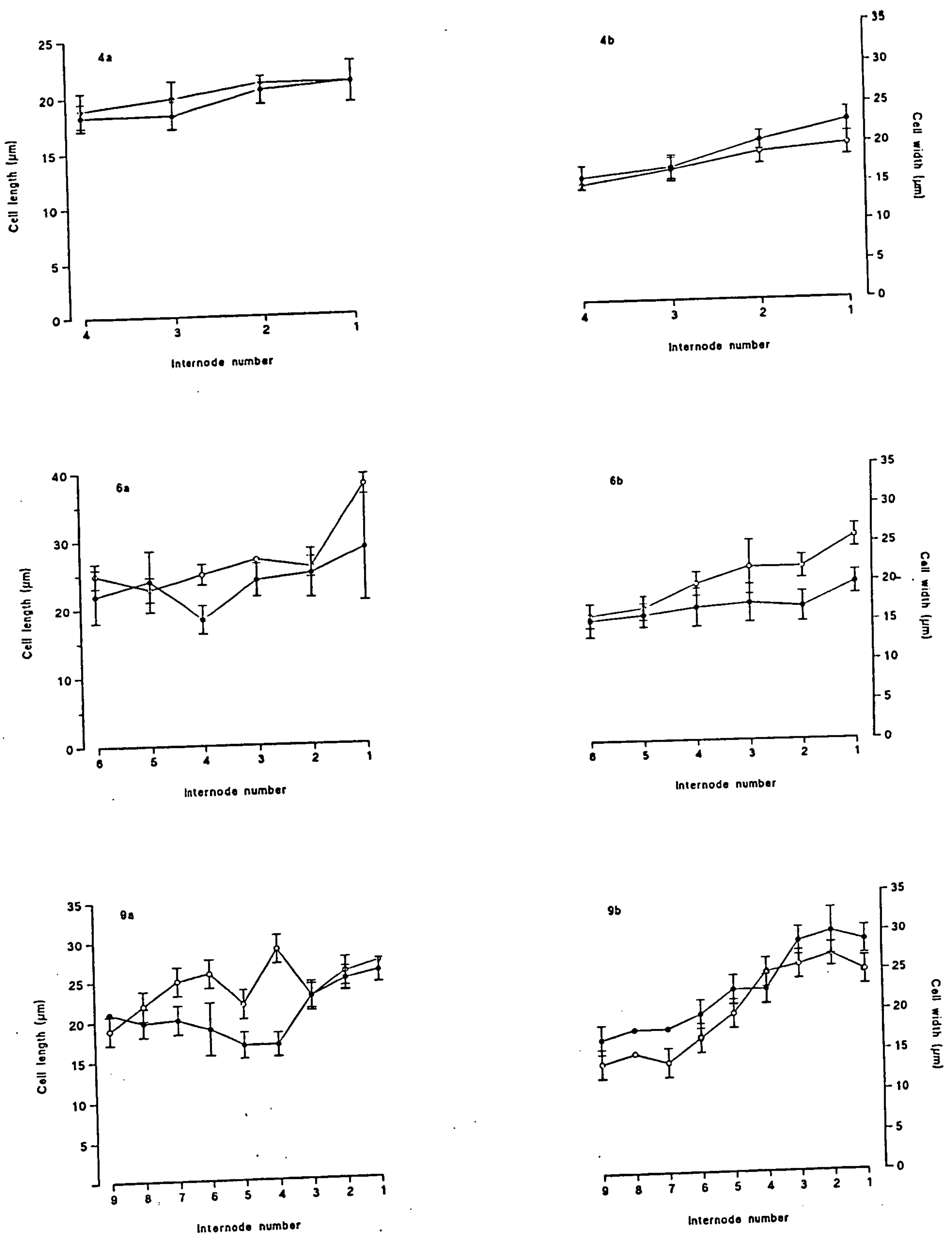


Fig. 3.8 Comparison of cell lengths (a) and widths (b), within individual internodes of shoot apices of the *rht3* (wild-type), (closed circles ●) and *Rht3* (mutant), (open circles ○) lines (± s.e., n=3), of Maris Huntsman grown at 20 °C. Apices were compared at three different developmental stages corresponding to the number of leaf primordia initiated by the shoot apex; 4, 6 or 9. Internodes numbered in order of initiation.

Table 3.1 *Summary of two factor analysis of variance within the first 4 developing stem internodes showing effect of genotype and internode number and their interaction on internode length and width, cell length and width and cell number. (DF = degrees of feedom, SS = sum of squares, MS = mean square, F = F value, sig = significance, n.s = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. Data of Figs 3.5, 3.7 & 3.8 respectively.*

Source	DF	SS	MS	F	Sig
Internode length					
Genotype	1	41168	41168	0.99	n.s.
Internode	2	1492562	746281	18.10	***
Interaction	2	56032	28016	0.68	n.s.
Error	18	740278	41227		
Total	23	2331841			
Internode width					
Genotype	1	4374	4374	0.04	n.s.
Internode	2	4382620	2191310	21.15	***
Interaction	2	15073	7536	0.07	n.s.
Error	18	1864518	103584		
Total	23	6266585			
Cell length					
Genotype	1	55.8	55.8	4.19	n.s.
Internode	2	180.2	90.1	6.77	*
Interaction	2	19.0	9.5	0.71	n.s.
Error	18	239.3	13.3		
Total	23	494.3			
Cell width					
Genotype	1	1.13	1.13	0.19	n.s.
Internode	2	307.24	153.62	26.81	***
Interaction	2	54.25	27.13	4.73	*
Error	18	103.18	5.73		
Total	23	465.80			
Cell numbers per internode file					
Genotype	1	15.40	15.40	0.18	n.s.
Internode	2	1435.30	717.60	8.39	**
Interaction	2	28.2	14.1	0.16	n.s.
Error	18	1539.7	85.5		
Total	23	3018.5			
Cell number across internode base					
Genotype	1	24	24	0.15	n.s.
Internode	2	4717	2359	14.84	***
Interaction	2	62	31	0.19	n.s.
Error	18	2865	159		
Total	23	7668			

3.3.3 Time course of internode elongation

The length of stem internodes was also compared on a chronological basis, over a 53 d period, by the direct observation of the live shoot apex and developing stem internodes using a dissecting light microscope. There was a consistent tendency, after *ca* 15 d for internode lengths in the *rht3* wild-type line to be greater than those in the *Rht3* mutant (Fig. 3.9). However, when compared statistically using analysis of variance, there was a genotypic effect on internode length, ($P < 0.05$), in internodes 1, 2, 4, 7 and $P < 0.01$ on internode 3 (Statistical analysis is presented in Table A.5 of Appendix). However, the analysis showed a strong effect of time from sowing with internode length ($P < 0.001$) in internodes 1-4, which suggested that there was significant internode extension over the 53 d period of the study. Although the genotypic effect was not as significant as it becomes subsequently (Youssefian, 1986; Webb, 1987; Youssefian *et al.*, 1992b) the results do suggest that the product(s) of the *Rht3* allele may begin to initiate an effect on stem elongation around 15 d after sowing, which was about the time that the 7th leaf primordium was initiated by the shoot apex *cf.* Fig. 3.4. In support of such a suggestion, internode lengths were compared between the *rht3* wild-type and *Rht3* mutant lines in 2 different samples, considering internode extension in the periods, (a) 1-15 d and (b) 16-53 d. While there was no genotypic effect on length in any of the internodes in the 1-15 d sample, there were significant genotypic effects ($P < 0.05$) in internodes, 1, 2, 5, 6 and 7 and ($P < 0.01$) in internodes 4, 8, 9 and ($P < 0.001$) in internode 3 covering the period 16-53 d from sowing (Table 3.2).

Table 3.2 *Analysis of variance results showing levels of significance of the effect on internode length of genotype and time from sowing. Length data for each internode has been divided into 2 separate samples, (a) 1-15 d and (b) 16-53 d from sowing. */**/*** = P < 0.05/0.01/0.001 respectively. Data of Fig. 3.9.*

Internode no	(a) Source of effect		(b) Source of effect	
	Genotype	***Time***	***Genotype***	***Time***
1	n.s.	***	**	*
2	n.s.	***	*	*
3	n.s.	***	**	***
4	n.s.	***	*	**
5	n.s.	*	*	*
6	n.s.	***	n.s.	*
7	n.s.	***	*	*
8	n.s.	***	n.s.	**
9	n.s.	**	n.s.	**

Note. Statistical analysis presented in Tables A.6 & A.7 of Appendix

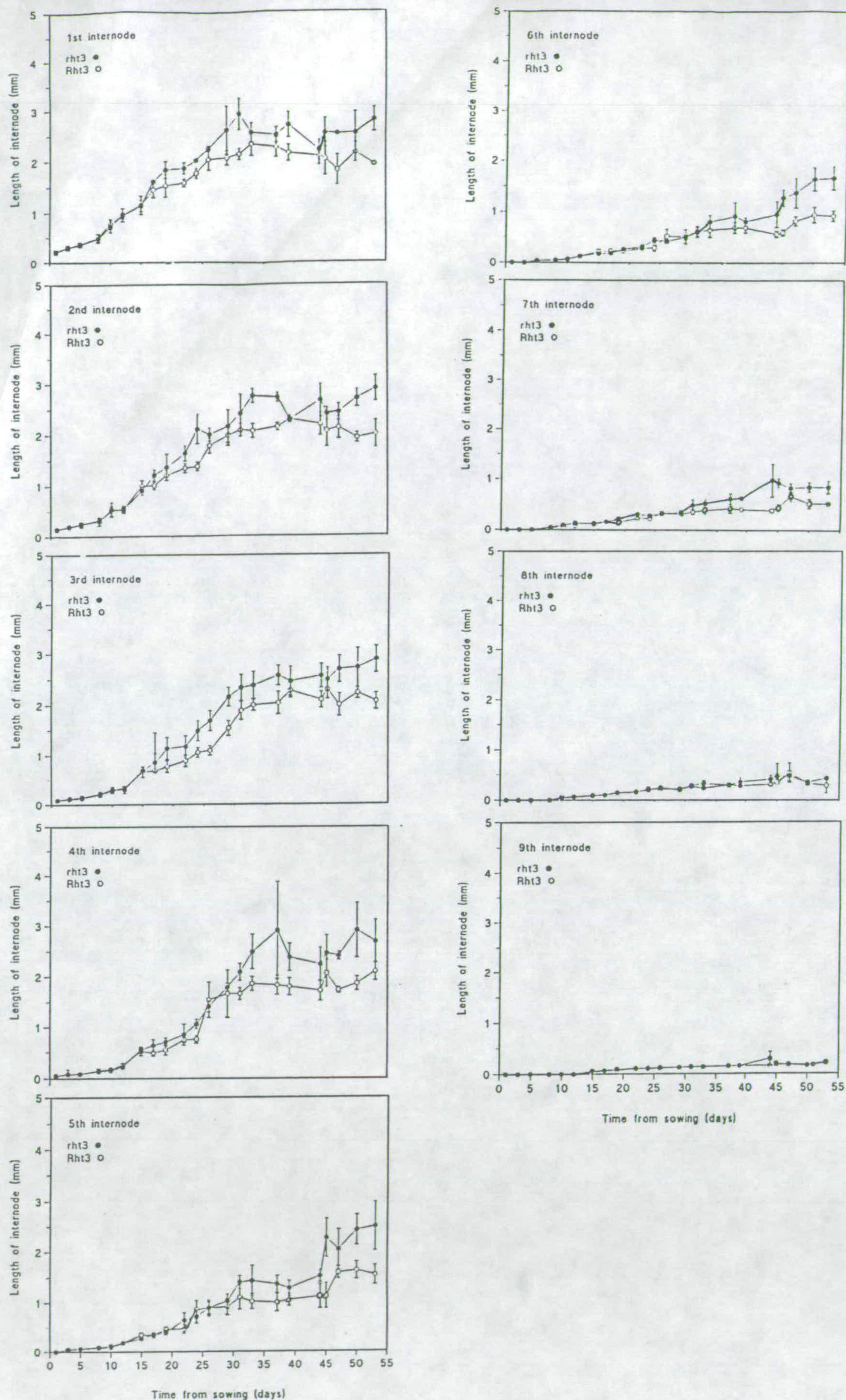


Fig. 3.9 Extension of the first nine stem internodes in the *rht3* (wild-type), (closed circles ●), and *Rht3* (mutant), (open circles ○). (± s.e., n=5) grown at 20 °C. Internode lengths were measured using a dissecting microscope following defoliation to expose the shoot apex.

3.3.4 Conclusions: lack of effect of the *Rht3* allele on apical development

There were no major effects of the *Rht3* allele on apical development and the initiation of stem internodes. In summary the *Rht3* allele:

1. had no effect on the rate of leaf primordium initiation by the shoot apex or the time taken for the shoot apex to reach the double ridge stage of development;

2. had no persistent effects on the number of cells, cellular dimensions or internode dimensions prior to d 26;

3. had no effect the morphology of the shoot apex.

3.4 DISCUSSION

In the present work the hypothesis has been investigated that the *Rht3* allele may affect early developmental events in the initiation and/or extension of stem internodes. It was proposed that cell types that may be affected by the *Rht3* allele could be those, which in the *rht3* wild-type, respond to endogenous GA by increasing in number and/or length. However, no evidence has been found to support this hypothesis. The *Rht3* allele had no significant effect on early stem internode development prior to d 15, measured in terms of cell dimensions or number. Instead, the effect of the *Rht3* allele appears to be developmentally controlled and only becomes observable *ca* 15 d after sowing.

3.4.1 Effect of the *Rht3* allele on shoot apical development and the initiation of stem internodes

Compared to apices in *rht3* wild-type line the *Rht3* allele had no obvious effects on either the morphology of the shoot apex or the rate of leaf primordium initiation (*cf.* section 3.3). The present results are in agreement with recent investigations (Brooking & Kirby, 1981; Rashid & Halloran, 1984; Fisher & Stockman, 1986; Youssefian, 1986; Youssefian *et al.*, 1992a) which similarly found no effect of the *Rht* alleles on apical morphology or the rate of leaf primordium initiation.

However, the present results are in contrast to those of Fisher (1973) and Holmes (1973) who reported apices of plants carrying a Norin 10 semi-dwarfing allele to be longer and more of an elongated cone shape than apices in wild-type cultivars. In addition, the duration of the vegetative phase was extended and the transition to floral development delayed, in shoot apices of lines carrying a semi-dwarfing allele compared to those of wild-type cultivars. It has been proposed (Youssefian *et al.*, 1992a) that the differences in apical morphology and timing of developmental events reported by Fisher (1973) and Holmes (1973) may be related to the lack of a low-temperature pre-treatment in cultivars which required vernalisation and/or that the study compared unrelated wheat varieties. The present results suggest that the latter explanation may be more likely, as grain of the winter wheat cultivar Maris Huntsman described in section 3.3 of this chapter was similarly not given a low-temperature pre-treatment, although apical morphology and the timing of developmental events were similar between the wild-type and mutant genotypes. However, the near-isogenic lines used in the present work are estimated to be homozygous at over 99 % of the loci which are unlinked to the GA-insensitive alleles (Youssefian *et al.*, 1992a).

3.4.2 When in development does the *Rht3* allele first initiate a measurable effect on stem internode extension ?

Developmental studies at the whole organ level showed that the first detectable effect of the *Rht* allele on stem elongation was after the initiation of the terminal spikelet

(Brooking & Kirby, 1981; Rashid & Halloran, 1984) during rapid stem extension (Chinoy & Nanda, 1951; Kirby & Appleyard; 1981). Youssefian (1986) and Youssefian *et al.* (1992b) using isogenic lines carrying the *Rht1*, *Rht2* and *Rht3* dwarfing alleles, considered that the genotypic effect should be present within meristematic tissue and the extension zone prior to the terminal spikelet stage of apical development, before the onset of rapid stem elongation. However, this proposal was not examined.

The first observable effect of the *Rht3* allele was to reduce the length of the 1st to 6th internodes, starting around 15 d after sowing (Fig. 3.9). At this stage in development the shoot apex has only initiated *ca* 7 out of *ca* 12 leaf primordia. Thus, the results appear to demonstrate that the genotypic effect of the *Rht3* allele on the extension of stem internodes can be observed while the shoot apex is still involved in the production of vegetative leaf primordia.

Instances were recorded in the present work where stem internodes appeared to shrink between successive developmental stages (*cf.* Fig. 3.5). It is suggested that such observations were the result of sample variation and were not caused by a reduction in internode length at the expense of an increase in internode width. Ideally it would have been better to have used a larger sample size. However, restrictions on both time and growth room facilities prevented larger samples sizes being used.

Although the effect of the *Rht3* allele was not statistically significant when tested, there were *ca* 30 % fewer cells present within the 1st and 2nd internodes of 4-leaved shoot apices of the *Rht3* mutant genotype compared with the *rht3* wild-type (Fig. 3.7). These results suggest a residual effect of the *Rht3* allele during embryogenesis. This is interesting as a similar observation and suggestion was made in a study which compared coleoptile growth characteristics in *Rht3* mutant and *rht3* wild-type lines (Flintham, 1981). The *Rht3* allele may reduce cell number within the coleoptile during embryogenesis but this initial effect is overcome by faster, early growth in the *Rht3* mutant coleoptile such that final cell number in the two lines was the same.

A similar survey of apical development over a longer period of growth was also done using the April Bearded cultivar, although not reported in this thesis. While differences existed between the two cultivars in the total number of main stem leaves formed and the time required for the apex to reach the double ridge stage of apical development, the overall results of the study were similar and supported the conclusions drawn for Maris Huntsman. The *Rht3* allele had no detectable effect on apical morphology or the rate of initiation of leaf primordia. Similarly, no specific cell types could be identified which were differentially affected in number or dimensions by the *Rht3* allele.

Thus, the *Rht3* allele appears to act in a similar manner regardless of the genetic background. While the effect of the allele is qualitatively the same, it may however be

quantitatively different.

3.4.3 Choice of a model system for the effects of the *Rht3* allele

The primary aim of the work contained within this thesis was to study the mechanism of action of the *Rht3* allele on growth. The present work has demonstrated the lack of a significant effect of the *Rht3* allele on, the rate of initiation of leaf primordia, early stem internode development or the onset of internode extension.

Expanding stem internodes are not well suited as a model system since they take 2 to 3 months to extend to a sufficient length to be used for experimental purposes. In addition, the shape of the stem internode makes it difficult to use in some experimental techniques such as stretching in an extensiometer.

A study was made, although not reported in this thesis, of the effect of the *Rht3* allele on the growth of the stem internodes, laminae and coleoptile. The *Rht3* allele had the same effect in all vegetative organs of the wheat plant, to primarily reduce the rate, but not the duration of growth compared to the *rht3* wild-type. The conclusions are consistent with the more comprehensive growth analyses of Youssefian, (1986) and Youssefian *et al.* (1992a & b) based on near-isogenic lines in both a spring and winter wheat cultivar, carrying *Rht1*, *Rht2* and *Rht3* alleles. The *Rht* alleles, or the potency of the *Rht* allele, did not affect the number of organs formed or the timing of developmental events (Youssefian *et al.*, 1992a).

A model system having a shorter growth period and being easier to use for experimental purposes than the stem internode was investigated. A leaf was chosen as the model system in which to study the effects of the *Rht3* allele in future experiments. The second leaf was chosen as the model system as it has a short growth period (Appleford & Lenton, 1991) of just 15 d and results could be compared directly with previously published work relating to other effect(s) of the *Rht* alleles on leaf growth characteristics (Keyes, 1987).

The present results have demonstrated that the *Rht3* allele does not cause a fundamental change in the characteristics of growth within the shoot apex. Instead the main effect of the *Rht3* allele appears to be one which reduces the rate of subsequent cell extension. The cellular basis of this effect of the *Rht3* allele will be investigated in the second leaf model system.

CHAPTER 4

GROWTH OF THE SECOND LEAF (L2): EFFECTS OF THE *Rht3* ALLELE AT THE CELLULAR LEVEL

Work described in Chapter 3 demonstrated that the *Rht3* allele does not affect very early events in the development of stem internodes. However, the *Rht3* allele induces similar effects on the later growth of stem internodes, leaves (Youssefian, 1986; Lenton *et al.*, 1987; Web, 1987; Kirby *et al.*, 1992; Youssefian *et al.*, 1992a & b) and the coleoptile (Flintham, 1981). While the *Rht3* markedly reduces the maximum rate of growth, it does not affect the timing of development in these organs (Youssefian *et al.*, 1992a). The second leaf (L2) has been chosen as the model system in which to study the cellular basis of these effects of the *Rht3* allele.

4.1.1 Characteristics of growth within a graminaceous leaf

Leaf growth in the *Gramineae* is confined to a short region at the base of the leaf (Beg & Wright, 1962; Boffey *et al.*, 1980; Kemp, 1980; Volnec & Nelson, 1981; Thomas, 1983a; Thomas & Stoddart, 1984; Schnyder *et al.*, 1990). The leaf is composed of three separate zones: (1) a very short basal zone (*ca* 2-5 mm) which contains the intercalary meristem and in which cells are both dividing and expanding; (2) above this a slightly longer zone (usually 2-3 cm) in which cells displaced distally from the meristem grow by expansion alone (termed the extension zone) and (3) the longest zone which stretches from the outer limit of the expansion zone to the leaf tip; cells within this third zone have ceased expansion growth and are fully mature (Beg & Wright, 1962; Davidson & Milthorpe, 1966), but they continue to accumulate dry matter (Allard & Nelson, 1991). The region of the leaf in which cells are actively growing is always enclosed within the encircling leaves and sheaths of older leaves (Dale, 1988). For this reason, the environment of the growing part of the leaf in which growth occurs is slightly different from the external conditions, because the region enclosed by the sheath maintains a higher humidity than is experienced by the mature section of the same leaf and because the region is insulated from the effects of direct light. As the leaf expands, its primary function gradually changes from that of carbon utilization and increase in size and surface area to that of net carbon production via photosynthesis (Dean & Leech, 1982; Ougham *et al.*, 1987). As cells emerge from the extension zone they are already photosynthetically competent (Wilhelm & Nelson, 1978).

A cell is considered to have grown if it has undergone an irreversible increase in volume or a permanent increase in cell wall area (Lockhart, 1965; Cleland, 1971; Green, 1976; Fry, 1988). Growth can occur by cell extension with cell division or by cell extension alone. Cell division and cell extension can be considered as distinct but superimposable processes, the rates of which can vary independently (Green, 1976). The two processes overlap, especially within the basal meristematic region. Subsequent growth is dominated by cell extension.

Growth within the graminaceous leaf is axial. Cells are displaced away from the meristem along parallel files (Sharman, 1942; McAdam *et al.*, 1989). They are displaced up, through the extension zone by the expansion of cells below them in the same file. No further cell expansion occurs once cells have reached the distal limit of the extension zone (Schnyder *et al.*, 1990). Thus, a cell file represents a linear sequence of cell age and development and one well suited to the study of biophysical and biochemical aspects of growth (Leech *at el.*, 1973; Robertson & Leatsch, 1974; Boffey *et al.*, 1979; Boffey *et al.*, 1980; Ougham *et al.*, 1987; Davies *et al.*, 1989; Schnyder & Nelson 1987, 1988 & 1989, Schnyder *et al.*, 1988 & 1990). Therefore, cell files within the wheat leaf represent a useful model system in which to determine the effects of the *Rht3* allele on both a spatial and temporal basis.

The experimental techniques and models of growth used to study growing roots can be applied to the graminaceous leaf, since they are essentially similar systems except for the relative positions of the meristem and the extension zone (Leech, 1985; Barlow, 1989). Growth in wheat leaves can be considered in terms of (i) The number of cells within the meristem (ii) the rate of cell division (iii) the final length to which cells grow within the extension zone (iv) the rate at which this is achieved (Barlow, 1989).

4.1.1.1 Analysis of the pattern of growth within the extension zone

Analysis of the cellular basis of leaf growth is complicated by the fact that cells are both moving through the extension zone and expanding at the same time (Erickson & Sax, 1956; Erickson & Silk, 1980; Gander, 1980 & 1983; Green, 1984; Silk, 1984). Local growth rates at given positions within the extension zone remain constant, while individual cells moving through the zone experience first increasing, and then decreasing rates of extension (Erickson & Silk, 1980). Understanding the complex nature of growth within the zone was solved by considering growth processes as instantaneous rates occurring in infinitesimally small regions (Erickson & Sax, 1956; Green 1984). Erickson & Sax (1956) measured many adjacent small regions through the growth zone of corn roots to calculate exact local extension rates in relation to distance from the root tip. The method analyses the relative elemental growth rate (REGR) or the local strain rate. It represents the compound rate of growth within the extension zone and has the units of mm mm^{-1} h^{-1}. REGR analysis has been successfully applied to study the spatial distribution of growth in leaves of other grasses:

Festuca (Schnyder & Nelson, 1987, 1988, 1989) and *Lolium* (Schnyder *et al.*, 1990). It has been used in this chapter, to describe and compare patterns of growth found within the extension zone of L2 in the *rht3* wild-type and *Rht3* mutant genotypes.

4.1.1.1 Effects of the *Rht* alleles on growth of wheat leaves

The *Rht1*, *Rht2* and *Rht3* alleles do not affect the number of main stem leaves or the duration of their extension (Chapter 3; Lenton *et al.*, 1987; Youssefian, 1986; Keyes *et al.*, 1989; Youssefian *et al.*, 1992a). The most obvious effect of the *Rht1*, *Rht2* and *Rht3* alleles on leaf growth is to reduce final leaf length compared to the wild-type, particularly of the lower main stem leaves (King *et al.*, 1983; Youssefian, 1987; Bush & Evans 1988; Keyes *et al.*, 1989; Hoogendoorn *et al.*, 1990; Youssefian *et al.*, 1992b). The studies of King *et al.*, (1983) and Bush & Evans (1988) also showed that the *Rht1* and *Rht2* semi-dwarfing alleles were associated with increased leaf width. In isogenic lines carrying the Norin 10 semi-dwarfing alleles, *Rht1* and *Rht2*, there was a proportional reduction in the maximum rate of elongation and epidermal cell length with increasing *Rht* dose (Keyes *et al.*, 1989). Any reduction in leaf length must depend on fewer or shorter cells, or a combination of the two factors. The basis for the effect on leaf length of the *Rht* alleles is controversial. The more potent dwarfing *Rht3* allele was recently reported to reduce both cell length and cell number (Hoogendoorn *et al.*, 1990). However, in previous studies based on *Rht1*, *Rht2* and *Rht3* the main effect of the alleles was to reduce cell length (Youssefian, 1986). In two further studies based on the same two semi-dwarfing alleles, *Rht1* and *Rht2*, the main factor associated with a reduction in the length of the first leaf sheath and blade was similarly a decrease in epidermal cell length (Keyes, *et al.*, 1989; Paolillo *et al.*, 1991). In addition, cell number within the first lamina and sheath was independent of the *Rht1* and *Rht2* alleles, whereas cell length was shown to be linearly and positively correlated with *Rht* dosage (Keyes *et al.*, 1989). So, while it cannot be dismissed that there is no effect of the *Rht* alleles on cell number, the main effect of the alleles appears to be to reduce cell length.

4.1.2 The relationship between the *Rht3* allele and endogenous gibberellins

A major pleiotropic effect of the *Rht3* allele is to confer an insensitivity to applied GA (Radley, 1970; Stoddart, 1984; Lenton *et al.*, 1987; Pinthus *et al.*, 1989; Appleford & Lenton, 1991). However, the pool size of endogenous GA_1 in basal segments of expanding leaves of the *Rht3* mutant is twenty times greater than that detected in equivalent leaf segments of the *rht3* wild-type (Lenton *et al.*, 1987; Appleford & Lenton, 1991).

If the action of the *Rht3* allele causes a specific blockage of the GA-perception and signal transduction pathway, rather than a non-specific effect on cell extension, then it should be possible to produce a phenocopy of the mutant by using an inhibitor of GA biosynthesis. Such an approach has been successful in mimicking the effect of a GA-deficiency gene,

gib-1/gib-1, in cultured tomato roots (Butcher *et al.*, 1990; Barlow *et al.*, 1991). An objective of the present study was to identify processes integral to L2 extension which, by implication, may be specifically affected by endogenous GAs. Paclobutrazol, a specific inhibitor of GA biosynthesis, (Hedden & Graebe, 1985) was used in wheat to mimic the effect of the *Rht3* allele in the wild-type. The results could then be compared with those found in the untreated mutant genotype to indicate a potential target for the product of the *Rht3* allele (Gale & Youssefian, 1985) or a possible basis for the *Rht3* effect.

4.1.2.1 Growth at low temperature and gibberellin responsiveness

The response of plant growth to low temperature is considered to be governed by the direct effect of temperature on enzyme activities; as a consequence growth rates are reduced (Eagles, 1967; Thomas & Stoddart, 1984; Stoddart *et al.*, 1988; Dale, 1982; Pollock & Eagles, 1988). Leaf surface area is often decreased while leaf thickness is correspondingly increased by prolonged exposure to low temperature (Friend *et al.*, 1962; Friend & Pomeroy, 1970; Dale, 1982; Graham & Patterson, 1982; Terry *et al.*, 1983; Korner & Larcher, 1988). In *Lolium temulentum* surface area of the fourth leaf was reduced by 45 % and fresh weight was reduced by 50 % as the growing temperature was reduced from 20 ° to 5 °C (Pollock *et al.*, 1984).

In a study involving all possible homozygous combinations of *Rht1*, *Rht2* and *Rht3* alleles temperature affected both the expression of the dwarfing alleles and their responsiveness to applied GA_3. Genotypes which were responsive to increasing temperature were also responsive to applied GA_3. Increasing potency of the dwarfing alleles, or combinations of them, was directly related to increasing unresponsiveness of shoot tissue to applied GA_3 and decreasing leaf length (Pinthus *et al.*, 1989). A suggestion whereby leaf growth may be more sensitive to GA at low temperature has been proposed by several workers (Stoddart, 1984; Stoddart & Lloyd, 1986; Pinthus *et al.*, 1989). The absolute responsiveness of the *rht3* wild-type to GA_1 was reported to be greater at 10 ° than at 20 °C (Appleford & Lenton, 1991).

The processes of growth affected by reduced temperatures have not been defined in wheat, although they are thought to affect cell wall properties directly in *Lolium* (Thomas *et al.*, 1989) and *Hordeum* (Pollock *et al.*, 1992). In the present work plants will be grown at low temperature to produce a phenocopy of the *Rht3* mutant genotype in the *rht3* wild-type. Comparison of specific growth processes at 10° and 20 °C may indicate potential processes which are affected by the products of the *Rht3* allele.

4.1.3 Research objectives from the L2 model system

The objectives of the work described within this chapter were:

1. To determine exactly what constitutes the effect of the *Rht3* allele on the growth of L2.

2. To determine when, in L2 development the *Rht3* allele becomes functional. Does it affect a specific developmental stage ? Or, is it expressed throughout the development of the second leaf ?

3. To modify the L2 system in the wild-type and mutant by the application of the plant growth regulators GA_3 and 2*S*,3*S* paclobutrazol. Perturbing the L2 system by such methods may help to identify specific processes which are absent or modified by the presence of the *Rht3* allele in the mutant genotype.

4. To investigate the effects on growth and responsiveness to applied plant growth regulators when the wild-type is grown at 10 °C and compare these with the mutant genotype at both 10 ° and 20 °C.

5. To correlate the characteristics of growth in the two genotypes at 10 ° and 20 °C with the pool size of endogenous GAs within the L2 extension zone.

4.2 MATERIALS AND METHODS

4.2.1 Growth conditions

Plant material was grown in either of two controlled environment cabinets, at 20 °C or 10 °C as described in chapter 2, Table 2.1, conditions N°s 2 and 3.

4.2.2 Sampling for leaf length, dry weight and surface area analysis

On each sampling day, 9 plants were measured (corresponding to one row per box) from each of 3 replicate boxes per genotype. Seedlings growing at 20 °C were destructively sampled daily for 10 d starting 5 d after sowing. Seedlings growing at 10 °C were sampled only every 2 d, for 16 d, beginning 10 d after sowing. The second leaf was dissected out from the first leaf and sheath as described in chapter 2 section 2.3. The length of the sheath and lamina were recorded with a mm ruler and estimated to the nearest 0.1 mm.

4.2.2.1 Surface area and dry weight determination

Total leaf surface area was measured on an AMS Optimax V image analyser taking 3 groups of 9 leaves at a time. Following determination of L2 surface area, leaf dry weights were obtained by placing the leaves in a drying oven kept at 80 °C until they remained at a constant weight.

4.2.2.2 Analysis of L2 extension, dry weight and surface-area data

Freehand curves have been drawn through the L2 extension data. The maximum rate of increase in, length, surface area and dry weight have been estimated from a linear regression line fitted to the data points between 20 to 80 % final extension as described in chapter 2 section 2.4 and Fig. 2.1. The time to reach 50 % final extension was estimated by reading directly from the freehand extension curves.

4.2.3 Measurement of the length of the basal meristem in L2

Second leaves (L2) were dissected from the encircling leaf sheath and coleoptile on the day of maximum growth rate; d 9 at 20 °C and d 16 at 10 °C. Ten successive, 1 mm long basal segments of the lamina were cut with a single-edged razor blade from a point 1 mm above the leaf base. Five replicates of each 1 mm segment were taken. Mitotic figures were visualized by the method of Ougham *et al.*, (1987). Segments were fixed overnight in 3:1 (v/v) ethanol:acetic acid, hydrolysed with 2 M HCl and stained with Feulgen solution. The preparations were viewed under an oil immersion lens and the presence, or absence of mitotic figures in each preparation recorded.

4.2.4 Measurement of the length of the extension zone of the second leaf

Each sample consisted of 9 seedlings of the same genotype in a single pot. Three plants, randomly selected, were marked daily from d 5 to d 15 in the following way. The vermiculite was removed to expose the crown of each plant. Holes were pierced, by the method of Schnyder *et al.*, (1990), with a tungsten wire (30 µm tip diameter), at 2 mm intervals through the outer coleoptile and first leaf sheath into the enclosed second leaf of each plant. Piercing began 0-1 mm, (as near as possible) from the shoot/grain junction and extended 2 mm into the exposed section of the second leaf lamina. The marking procedure was viewed though a large magnifying lens. Seedlings in which leaves were either very long or very short were rejected from the piercing procedure. An inverted (400 ml) glass beaker was placed over the seedlings to maintain high humidity before returning them to the controlled environment cabinet for a further 5 h growth period at 20 °C and 10 h growth period at 10 °C. Shoots were excised at the shoot/grain junction with a razor blade and the second leaf was dissected out as described in chapter 2 section 2.3. The distance between the centres of consecutive pin marks was measured using a binocular microscope fitted with an eye-piece graticule. Distances were measured to the nearest 0.1 mm. Measurements were ceased when at least 3 consecutive pinholes showed no increase in their initial 2 mm distance, that is they were beyond the extent of the extension zone. The total length of the extension zone was estimated by summing together all of the 2 mm segments that had extended over the 5 h growth period. Pin marks made within the basal meristematic region were not included as they were often less than 2 mm apart and did not separate over the growth period. The original lengths of the segments which extended over the experimental period were used to calculate the length of the extension zone.

Once the length of the L2 extension zone was known and the approximate length of time that the maximum length was maintained had been determined in the two genotypes, in subsequent experiments the length of the L2 extension zone was measured only during the period of maximum rate of extension of L2 when the length of the zone remained relatively stable; days 8, 9 and 10 at 20 °C, and days 15, 16 and 17 at 10 °C. Material growing at 10 °C was left for a longer growth period, of 10 h, after piercing, as the growth rate was slower.

4.2.4.1 Calculation of the relative elemental growth rate (REGR)

I would like to acknowledge the work of Ms G. Arnold of Long Ashton Research Station for generating the REGR curves, described in sections 4.2.2 and 4.2.3 of the results described in this chapter.

The length of the extension zone was calculated by combining all of the piercing data generated over the 3 experimental sampling days around the time of maximum L2 extension rate (d 8, 9 & 10 at 20 °C and d 15, 16 & 17 at 10 °C). Experimental data was analysed using a Genstat programme which calculated the REGR using the relationship of

Kemp (1980), (equation 1).

$$REGR = (\log_e l_2 - \log_e l_1) / (t_2 - t_1) \qquad \textbf{eqn 1}$$

Where l_1 = original length of segment (mm); l_2 = final length of segment (mm); t_1 = original time leaf pierced (h); t_2 = final time when leaf pin marks were measured (h)

In section 1 of the results (only), REGR values were calculated using equation 1 and a Lotus 123 spreadsheet. Values of REGR were calculated to the nearest 1 mm and a third order polynomial curve fitted to the data. Values of the maximum REGR and the day when maximum REGR was achieved were read directly from the curve.

In sections 4.2.2 and 4.2.3 of the results, values of the REGR were calculated for each 0.5 mm position along L2 within the extension zone using a Genstat statistical programme written by Ms G. Arnold of Long Ashton Research Station. The REGR data was smoothed and fitted to another logistic curve of the form used by Barlow *et al.*, (1991) (equation 2):

$$R = (BC/D)e^{-B(\ln D - M)}/(1+e^{-B(\ln D - M)})^2 \qquad \textbf{eqn 2}$$

Where R = rate of change in length ($mm\ mm^{-1}\ h^{-1}$); C = increase in segment length (mm); M = time maximum rate of extension occurs (d from sowing); B = a constant, proportional to the maximum rate of extension, and D = distance from L2 base at which REGR is measured (mm).

4.2.4.2 Calculation of the cell residence time within the L2 extension zone

Cell residence times were calculated by the method of Schnyder *et al.*, (1990). The velocity of cell displacement was calculated using equation 3

$$v_x = v_{max} \cdot L_x/L_{max} \qquad \textbf{eqn 3}$$

Where v_x= velocity of displacement ($mm\ h^{-1}$) at x mm from the leaf base, v_{max} is the leaf elongation rate ($mm\ h^{-1}$), L_x is the length of an epidermal cell at x and L_{max} is the final length of epidermal cells ($mm\ cell^{-1}$).

v_x was calculated at successive 1 mm intervals through the extension zone. The time for a segment to move through a 1 mm distance in the extension zone was equivalent to the reciprocal value of v_x i.e. $h\ mm^{-1}$. The total time for a cell to move through the whole zone was the sum of all such 1 mm time intervals, taken from the distal edge of the basal meristem to the end of the extension zone.

4.2.5 Measurement of abaxial cell lengths and widths

4.2.5.1 Preparation of epidermis

It is difficult to distinguish individual abaxial epidermal cells in the intact leaf because of the green background of spongy mesophyll cells. Hence, the epidermal cell layer must be stripped off the leaf. Two different methods were used to measure cell length. The one of choice was to scrape away the adaxial epidermis, palisade layer and mesophyll using a sharp, single-edged razor blade while the sample was submerged in water. The resulting epidermal sample was mounted in water on a glass microscope slide and viewed using a binocular microscope with low magnification X10 objective. Although this method was devised to produce an intact and clear view of the abaxial epidermis of mature tissue, it was not successful with immature, extending cells. A replica method, as described Hoogendoorn *et al.*, (1990), was used for immature tissue of the extension zone. A thin layer of clear nail varnish was painted onto the abaxial surface of the L2 segment. After 5 min the varnish was removed, virtually intact, by overlaying a strip of Sellotape. The entire varnish section could then be carefully peeled off the segment as the Sellotape was removed. The Sellotape, with the epidermal impression was stuck to a glass microscope slide and viewed under low power on a binocular microscope.

4.2.5.2 Measurement of cell lengths and widths

Two different abaxial cell lengths were measured. In section 4.3.1 intercostal cell lengths were measured, defined as cells in files which did not contain stomata, as the distance from one cross wall to the next cross wall in the same file. These will be referred to as intercostal cells (*cf.* Fig. 4.1). However, intercostal cell lengths became very long and difficult to measure when the *rht3* wild-type was treated with GA_3. Consequently, the shorter interstomatal cell lengths were measured in sections 4.2.2 and 4.2.3 of the results. Interstomatal cells have been defined as cells between stomata within the same cell file. This distance between stomatal cells most often contained 1 cell, if there was more than 1 cell, the measurement was ignored. The distance from the end of one stomatal guard cell to the start of the next stomatal guard cell in the same file was counted as the interstomatal distance (*cf.* Fig. 4.1). Cell widths were measured at their mid-point. 25 cells per leaf, in 5 different files, were measured for each sample. The data was recorded using VIDS II software `2-dot programme' by viewing the projected image of the cell through Vickers light microscope linked to an AMS image analyser and digitised drawing tablet attached to an Apple computer

Cell lengths and widths were recorded every 2 mm for the first 20 mm from the leaf base, then every 5 mm until 50 mm, and then if the leaf was long enough, every 10 mm from

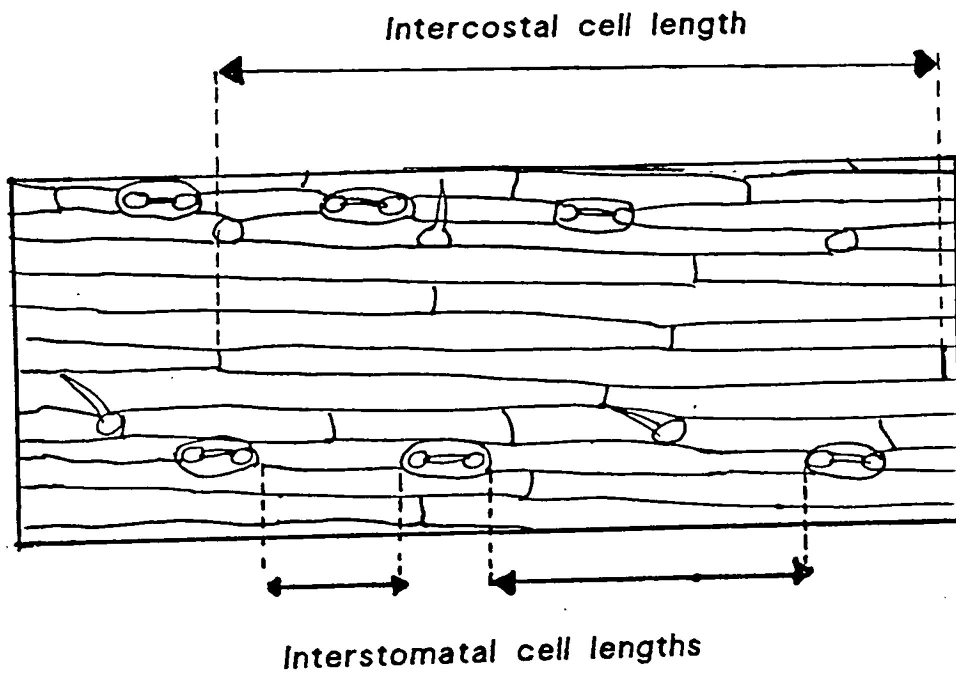


Fig. 4.1 Measurement of interstomatal (cells within files which contain stomata) and intercostal (cells within cell files which do not contain stomata) cell lengths on the abaxial epidermal surface of L2 from an epidermal impression.

EX BIBL. UNIV. EDINBURGEN

50 to 100 mm from the leaf base. Cell dimensions were recorded in 5 adjacent cell files on 3 separate leaves.

4.2.5.3 Calculation of total cell number per stomatal epidermal cell file

Final L2 length was divided by the final mean interstomatal or intercostal cell length. The total number of stomata per L2 length was approximately the same in the two genotypes. Hence, the omission of the stomata themselves in the calculation of cell number derived from interstomatal cell lengths meant that the calculated cell number can only be a relative estimate between the two lines and not an absolute value. The total cell number was then re-calculated ± one standard deviation.

4.2.6 Statistical tests of significance

Two way analysis of variance was performed on REGR curves in sections 4.2.2 and 4.2.3 using a Genstat statistical package by Ms G. Arnold of Long Ashton Research Station and in section 4.2.1 using two-way analysis of variance using the Minitab statistical package. Comparison of cell dimensions, cell numbers and cell residence times between the *rht3* wild-type and *Rht3* mutant were made using Student's t test (Clarke, 1980).

4.2.7 Measurement of the endogenous levels of gibberellin

Gibberellins GA_{19}, $_{20}$, $_{29}$, $_{1}$, $_{8}$ and $_{3}$ were measured using GC-MS, by a sandwich student, Sean Richardson who was working in the laboratory at the same time, using the method of Appleford & Lenton (1991). Leaf segments used corresponded to the length of the L2 extension zone in the two genotypes (Table 4.1)

Table 4.1 *Details of segments used to determine the endogenous GA status of L2 in the rht3 (wild-type) and Rht3 (mutant) lines. Leaves sampled on d 9 at 20 °C and d 18 at 10 °C.*

Genotype/ temperature	Length range of second leaves (mm)	Segment length (mm)	Number of segments used	Fresh wt. of segments (g)
rht3 20 °C	120-140	28	423	6.3
Rht3	80-90	18	356	5.3
rht3 10 °C	85-95	20	360	4.2
Rht3	75-85	18	472	5.6

4.3 RESULTS

4.3.1 Characterisation of the L2 model system

4.3.1.1 Growth pattern of L2

The following sequence of development in L2 of *Triticum aestivum* L. *cv* Maris Huntsman, *rht3* (wild-type) grown in long days (16 h light/8 h dark, 265μmol $m^{-2}s^{-1}$) at 20 °C, is based on the observations of over 100 plants. The second leaf (L2) is approximately 60 μm long within the embryo of the grain, 15 d later, when growing at 20 °C, it is fully expanded (Fig 4.2). The first 6 d of growth correspond to the initial phase of growth of L2 and represent the first part of the main extension phase of the first leaf (L1). The total length of L2 at this time does not exceed *ca* 50 mm. By d 6 the tip of L2 begins to emerge from within the hollow tube formed by the encircling L1, which has reached half its final length and is increasing in length at its maximum rate. By d 8 the growth rate of L1 has begun to decline. The lamina has almost reached maximum length and growth has been transferred to the extension of the first leaf sheath. L2 is now increasing in length at its maximum rate and growth is entirely extension of the lamina. This high rate of growth is sustained for a period of 3-4 d. Up to *ca* d 11 the sheath of L2 remains less than 5 mm in length below the ligule. Only when extension of the lamina is nearly complete is growth transferred to the sheath. The maximum rate of sheath extension occurs *ca* d 12 and 13. By d 15, L2, lamina and sheath, are typically fully extended (Fig. 4.2). Based on these observations L2 was sampled around d 9 when it was increasing in length at its maximum rate.

4.3.1.1.1 Comparison of growth parameters of L2 in the *rht3* (wild-type) and the *Rht3* (mutant) lines

Second leaf extension data has been analysed as described in section 4.2.2.2 and chapter 2 section 2.4.

In both genotypes L2 reached 50 % final length on *ca* d 9 (Fig 4.3a & Table 4.2). The *Rht3* allele caused a 41 % reduction in the maximum elongation rate which resulted in a 44 % reduction in final L2 length compared to the *rht3*, wild-type line (Fig. 4.3a & Table 4.2). The duration of L2 growth was not affected by the *Rht3* allele, full length of L2 being achieved on d 14/15 in both genotypes (Fig. 4.3).

In both genotypes, L2 achieved 50 % maximum surface area by d 10-11, 1.5 d after reaching 50 % maximum leaf length. The *Rht3* allele caused a 36 % reduction in the maximum rate of lamina surface area expansion resulting in a 40 % reduction in final surface area compared with the *rht3* wild-type (Table 4.2 & Fig. 4.3b). Since increase in surface area is a product of length and unrolled leaf width it appears that although unrolling is delayed

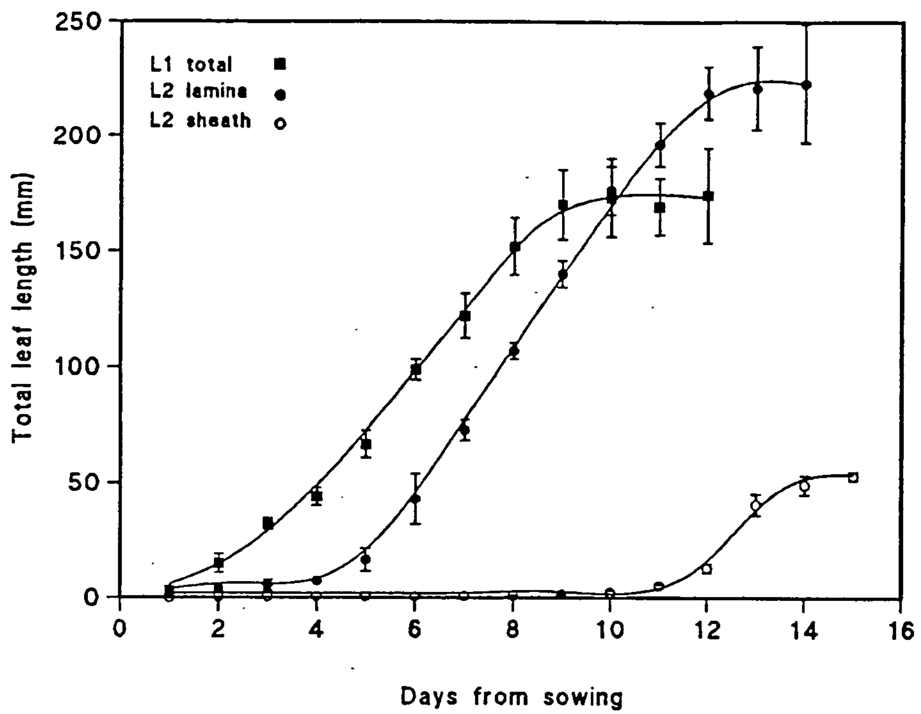


Fig. 4.2 Pattern of growth of the first and second leaf, of the *rht3* (wild-type) at 20 °C showing the sequential emergence of L2 (lamina and sheath) once the first leaf (L1) has reached approximately 50 % of its final length. Freehand curves have been fitted to leaf extension data. (n=9).

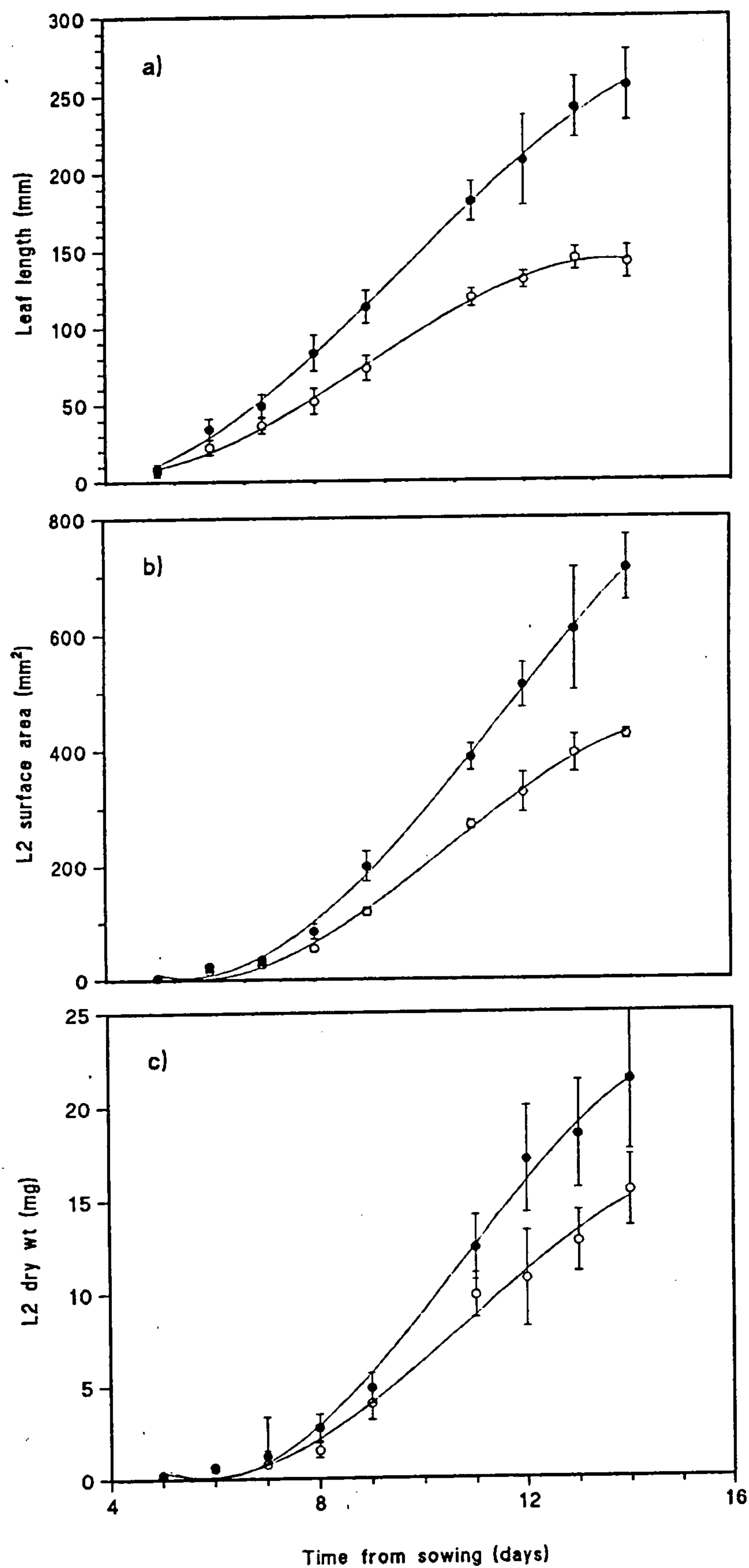


Fig. 4.3 Genotypic comparison L2 development in *rht3* (wild-type ●) and *Rht3* (mutant, ○) lines grown at 20 °C, (± s.e., n=18) showing increase in; (a) leaf length, (b) surface area and (c) dry mass with time. Freehand curves have been fitted to L2 extension data.

compared with linear extension there is no apparent genotypic difference in the unrolling pattern in the *rht3* wild-type and *Rht3* mutant lines.

In both genotypes, L2 reached 50 % final dry mass on d 10-11. The *Rht3* allele reduced the maximum rate of L2 dry mass accumulation by 33 % which resulted in a reduction in final L2 dry mass of 28 % (Table 4.2 & Fig. 4.3c). The calculated final value of specific leaf area, the area per unit dry mass was not significantly different between the two genotypes; 32.12 x 10^3 and 27.61 x 10^3 mm^2 g^{-1} dry mass in the *rht3* wild-type and *Rht3* mutant lines respectively.

The data shown in Table 4.2 are the means from 1 experiment. While the absolute values obtained for the measured parameters varied between individual replicate experiments, their relative difference between the two genotypes remained the same. The differences between experiments seemed to be related to sowing depth affecting emergence time.

Table 4.2 *Parameters (± s.e.) describing the growth of L2 in the rht3 (wild-type) and the Rht3 (mutant) lines grown at 20 °C. Data derived from freehand curves fitted to extension data and a linear regression line fitted to the period of 20 to 80 % maximum extension (cf. chapter 2 section 2.4). (n=18) Data of Fig. 4.3.*

Growth parameter	Genotype	Time (d) to 50 % final	Final value	Maximum rate
Length	*rht3*	9.3	256.1 (22.1)mm	31.9 mm d^{-1}
	Rht3	8.9	144.1 (7.3)mm***	18.9 mm d^{-1}
Area	*rht3*	10.7	712 (56) mm^2	104 mm^2 d^{-1}
	Rht3	10.5	425 (37) mm^2***	66 mm^2 d^{-1}
Dry mass	*rht3*	10.8	21.3 (3.7) mg	3.38 mg d^{-1}
	Rht3	10.4	15.4 (1.9) mg***	2.27 mg d^{-1}

*** = difference significant at $P < 0.001$

The *Rht3* allele does not affect the time course of development of L2. The primary effect of the allele is to reduce the maximum rate of growth - in length, surface area and dry mass. As the duration of growth is the same in the two lines, the *Rht3* allele significantly reduced the final length, surface area and dry mass of L2 by *ca* 40-50 % in the *Rht3* mutant leaves compared to those in the *rht3* wild-type.

4.3.1.1.2 Length of L2 basal meristem

The length of the L2 meristem was measured to determine whether it was affected, in a similar way to final L2 length, by the *Rht3* allele. The *Rht3* allele reduced the length of the meristem by *ca* 30 %, compared to the *rht3* wild-type (Table 4.3).

Table 4.3 *Mean length of the L2 meristematic region (± s.e.) in the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C and sampled on d 9 (n = 13).*

Genotype	Mean length of the meristematic region from the leaf base (mm)
rht3	5.3 (0.7)
Rht3	3.7 (0.5)***

*** = difference significant $P < 0.001$

Quantitative measurements were not made in the two lines which would have permitted the mitotic index within the basal meristems to be calculated. However, within the basal 1-3 mm of L2 in the *Rht3* mutant, a higher proportion of cells appeared to exhibit mitotic figures than in the *rht3* wild-type. This could suggest that the *Rht3* allele may have an additional effect in reducing the length of the cell cycle.

4.3.1.2 Cellular and temporal pattern of L2 growth

4.3.1.2.1 Estimation of the length of the L2 extension zone

The development of the extension zone of L2 was investigated using a piercing procedure as described by Schnyder *et al.*, (1990) (*cf.* section 4.2.4). Seedlings were pierced over a period corresponding to the main extension phase of L2, from d 5 until d 14 (Fig. 4.4). The maximum length of the extension zone was the length of that part of the leaf over which points separated over the 5 h growth period. This length remained stable, within 3 or 4 mm, during the main period of lamina extension, around d 8, 9 and 10 (Fig. 4.4). There were high standard errors associated with both the early and late data points. During the early days of sampling, L2 was not always sufficiently long to be pierced by the wire. In the later data, the developmental status of the L2 sheath greatly influenced the results. The exact day on which sheath extension began was found to vary between d 10 and 13 and was unpredictable between individual plants. However, the pattern of development of the L2 extension zone, and its relative stability over a 3 or 4 d period was similar in both the *Rht3* mutant and *rht3* wild-type lines. Based on the results shown in Fig. 4.4 subsequent work on the L2 extension zone, growing at 20 °C, used plants sampled only on days 8, 9 and 10.

4.3.1.2.2 Relative elemental growth rate through L2 extension zone

Extension of L2 was analysed in greater detail by calculating the relative elemental growth rate (REGR) using the eqn 1 (*cf.* section 4.2.4.1). The REGR increased rapidly 3-4 mm from the leaf base (corresponding to the meristem) to reach a maximum at 8-11 mm in both genotypes (Fig. 4.5). There was however, no significant difference in the maximum

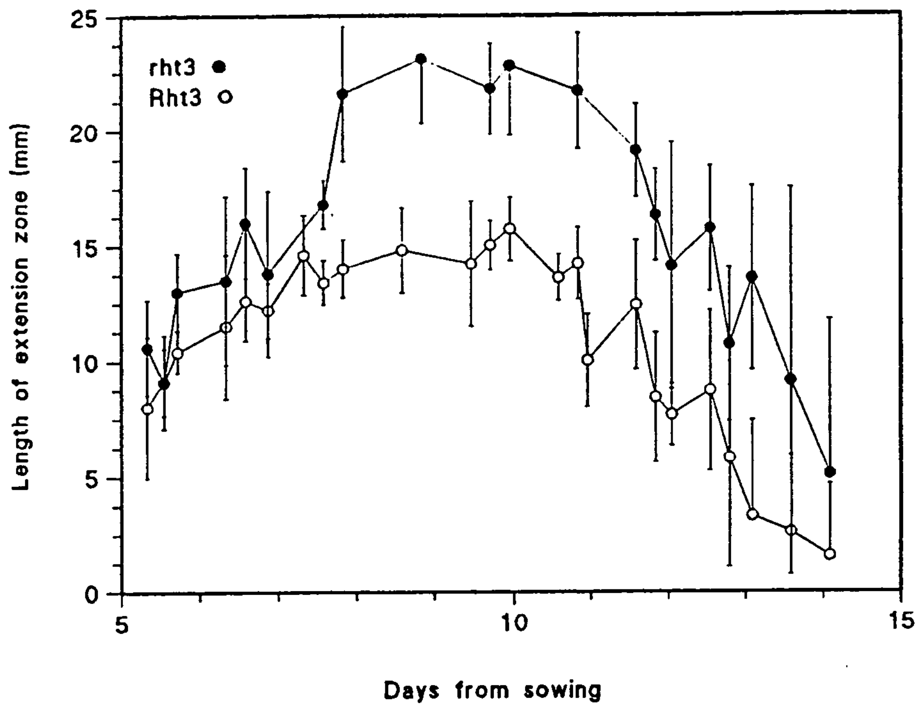


Fig. 4.4 Development of the L2 extension zone (± s.e., n=9) throughout the main extension period of L2 within the *rht3* (wild-type) and *Rht3* (mutant) lines grown at 20 °C.

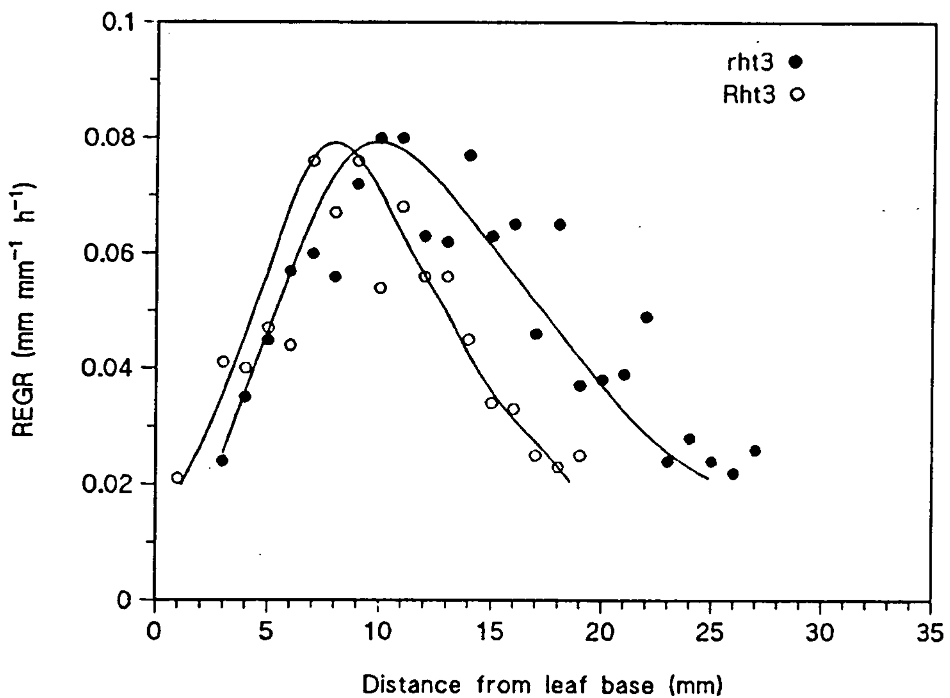


Fig. 4.5 Spatial distribution of REGR within the extension zone of L2 of the *rht3* (wild-type) and the *Rht3* (mutant) lines grown at 20 °C sampled around d 9, the period of maximum L2 extension growth. Third order polynomial curves fitted to data points as described in section 4.2.4.1. Error bars have been omitted for clarity.

REGR achieved within the zone between the *rht3* wild-type and *Rht3* mutant lines (Fig. 4.5, Table 4.4). The main difference between the two genotypes was in the length of the region over which any cell extension occurred (above an arbitrary figure of REGR > 0.02); this was *ca* 36 % shorter in the *Rht3* mutant than the *rht3* wild-type line. The distance corresponded to the length of the L2 extension zone (Table 4.4). The extension zone lengths shown in Table 4.4 were calculated from the pooled data, over a 3 d sampling period of the original length of all of the 2 mm segments which had extended during the 5 h growth period. The curves shown in Fig. 4.5 have been drawn using a fitted curve, in this example a 3rd order polynomial curve, based on the final lengths of the 2 mm segments, which tends to overestimate the length of the extension zone. In fact it was difficult using either method, to determine the exact distance from the leaf base that the value of REGR had declined to zero. There tended to be a very gradual decline of REGR around the tail end of the curve; in addition, variation between individual plants was high around the distal end of the extension zone.

Table 4.4 *Parameters (± s.e.) describing the relative elemental growth rate (REGR) within the extension zone of the rht3 (wild-type) and the Rht3 (mutant) lines grown at 20 °C and sampled around d 9. REGR parameters were calculated from 3rd order polynomial curves fitted to data obtained using the piercing technique. The length of the extension zone was estimated by pooling together the marking results of one complete experiment and calculating the mean length of all 2 mm segments which had increased in length over the 5 h growth period. (n = 19). Data of Fig. 4.5.*

Genotype	Position of maximum REGR (mm from base)	Maximum REGR ($mm\ mm^{-1}\ h^{-1}$)	Extension zone length (mm)
rht3	10.2	0.078	19.9 (3.2)
Rht3	8.1	0.079	12.7 (1.5)***

*** = difference significant $P < 0.001$.

Therefore, the only apparent effect of the *Rht3* allele in the mutant genotype was to reduce the length of the extension zone itself. The length of the L2 extension zone at 20 °C has been estimated in 3 different experiments. The average zone length was found to vary between each experiment by only 3 mm in the *rht3* wild-type and 4 mm in the *Rht3* mutant.

The magnitude of the difference in values of REGR between the two genotypes, at increasing distances from the leaf base was compared using student's t test. Although the REGR curve relating to the *Rht3* mutant line appeared to be shifted to the left of that of the *rht3* wild-type in the region 2-10 mm from the leaf base (Fig. 4.5), the difference when tested was not found to be significant (Table 4.5). Around the position of the maximum REGR there was no significant difference in REGR between the two genotypes. However, at a distance *ca* 14 mm from the leaf base REGR values were significantly higher in the extension zone of the

rht3 wild-type than at equivalent positions within the extension zone of the *Rht3* mutant line (Table 4.5).

Table 4.5 *Comparison of REGR values and significance level (Sig), at increasing distances from the leaf base in the L2 extension zone of the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C and sampled on d 9. (15 d.f). Data of Fig. 4.5.*

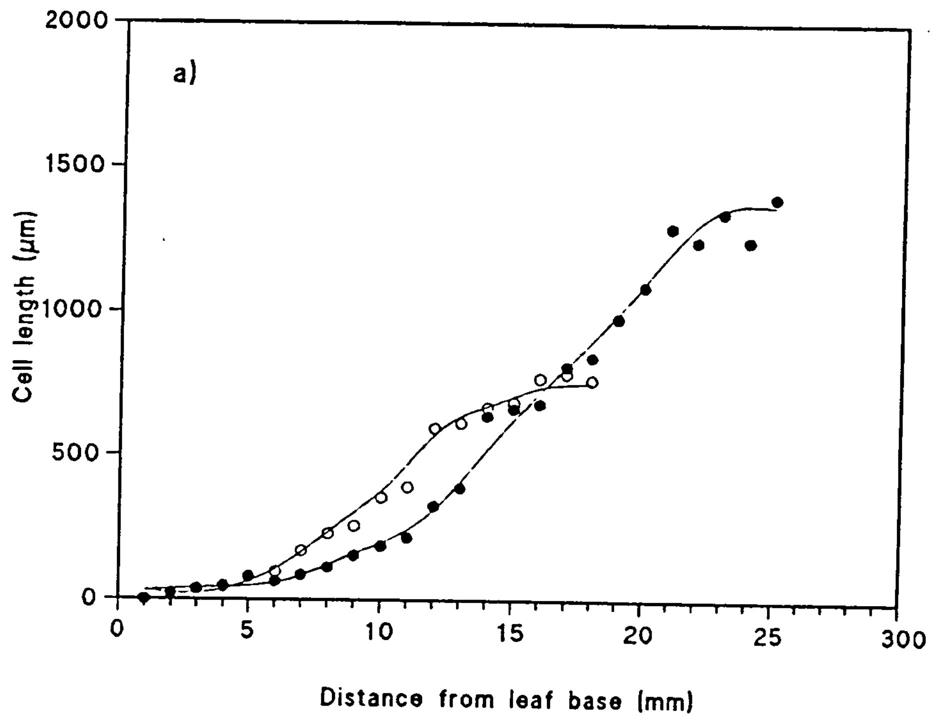
Genotype	REGR mm mm^{-1} h^{-1} Distance (mm) from base of L2						
	4	6	8	10	12	14	16
rht3	0.0313	0.0512	0.0547	0.0830	0.0682	0.0667	0.0665
Rht3	0.0400	0.0533	0.0647	0.0700	0.0578	0.0338	0.0280
Sig	n.s.	n.s.	n.s.	n.s.	n.s.	***	***

*** = difference significant at $P < 0.001$

4.3.1.2.3 Epidermal cell elongation rates

Epidermal cell length increased in both genotypes as cells moved through the extension zone (Fig. 4.6a). The onset of rapid cell elongation (defined as when epidermal cell length was > 20 % final length, *cf.* Fig. 2.1 chapter 2., section 2.4), began from a distance *ca* 5 mm nearer the leaf base in the *Rht3* mutant than in the *rht3* wild-type. However, cell extension ceased *ca* 8 mm nearer the leaf base in the *Rht3* mutant than in the *rht3* wild-type line (Fig. 4.6a & Table 4.6). The results support previous observations that the length of both the basal meristem, after which rapid cell extension began, and the extension zone, after which cell elongation ceased, were shorter in the *Rht3* mutant line than in the *rht3* wild-type.

If the increase in epidermal cell length was plotted as a function of the time taken to move from the base of L2, through the basal meristem to the distal border of the extension zone, a clear effect of the *Rht3* allele could be seen (Fig. 4.6b & Table 4.6). Cells in the *Rht3* mutant genotype have *ca* 54 h shorter initial period (for definition of initial period *cf.* Fig. 2.1) prior to the onset of the maximum rate of epidermal extension compared to cells in the *rht3* wild-type. However, cells in the *rht3* wild-type line increase in length at *ca* twice the rate of those in the mutant during the period of most rapid cell extension, from 20-80 % final length (for definition *cf.* Fig. 2.1), (Fig. 4.6b & Table 4.6). Therefore, the period through which cells in L2 of both genotypes are extending at their respective maximum rates, is in fact similar, *ca* 13 and 10 h in the *rht3* wild-type and *Rht3* mutant respectively (Table 4.6). Cells move



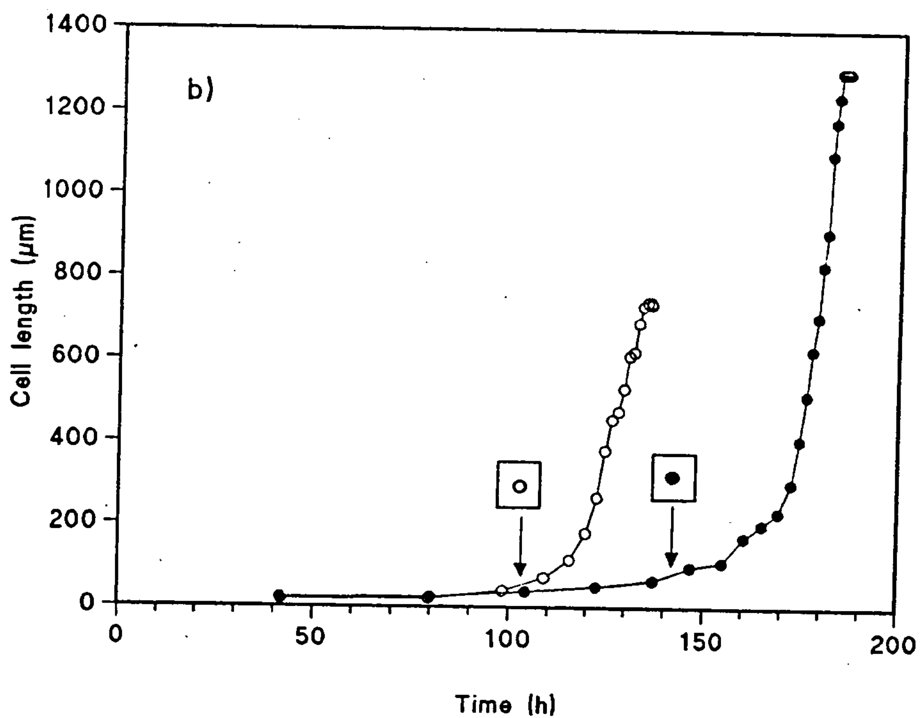


Fig. 4.6 Epidermal cell elongation within the L2 extension zone of the *rht3* (wild-type) (●) and the *Rht3* (mutant) (○) lines grown at 20 °C and sampled around d 9, the period of maximum L2 extension. (a) increase in epidermal cell length with distance from the leaf base; (b) increase in epidermal cell length expressed on a time basis, calculated as described in section 4.2.4.2. Arrows mark the distal limit of the meristem border.

only relatively slowly through the meristem and within the initial region of the extension zone. Cells had only reached 50 % final length after *ca* 175 and 125 h in the *rht3* wild-type and *Rht3* mutant lines respectively (Fig. 4.6b). However, the time for a cell to grow from 50 % to complete its final length at the distal boundary of the extension zone was only *ca* 10 h in both the *rht3* wild-type and *Rht3* mutant lines. Cell extension did not cease exactly level with the calculated extension zone border. On a time basis, the 'position' of the extension zone distal limit was *ca* 183 and 130 h in the *rht3* wild-type and *Rht3* mutant lines respectively. Therefore, almost all longitudinal cell growth occurred within a (final) 24 h period.

Table 4.6 *Estimated parameters of cell extension on a linear axis, increase in epidermal cell length with distance from leaf base; and on a time axis, increase in cell length as a function of time required to extend to that length, through the basal meristem and extension zone. Data of Fig. 4.6a & b. Maximum rates of increase estimated from linear regression line fitted to curve between 20 and 80 % final cell length as described in section 2.4 & Fig. 2.1*

Parameter	Genotype	Duration of phase		Maximum rate
		Initial	Extension	
Cell extension (length)	*rht3*	11 mm	9 mm	92.2 $\mu m\ mm^{-1}$
	Rht3	6 mm	6 mm	73.4 $\mu m\ mm^{-1}$
Cell extension (time)	*rht3*	170 h	13 h	66.8 $\mu m\ h^{-1}$
	Rht3	116 h	10 h	34.1 $\mu m\ h^{-1}$

The results shown in Fig 4.6b suggest that an additional effect of the *Rht3* allele may be to reduce the length of the cell cycle such that cells in the *Rht3* mutant line exit the meristematic region and enter the extension zone faster than they do in the *rht3* wild-type.

4.3.1.2.4 Residence time of cells in the extension zone of the wild-type and mutant

Calculation of the REGR based on the displacement of pin holes over a set period of time, allowed positions along the leaf axis to be converted to a time scale. As the length of the basal meristem was known, the time for cells in both genotypes to cross the extension zone could be calculated. (Note: data shown in Table 4.7 refers to residence time within the extension zone only, not, as shown in Fig. 4.6b through the meristem as well). However, the *Rht3* allele mainly appears to affect how the total residence time is divided between the different regions of the zone. Total cell residence time within the extension zone can be split into two parts; (i) time to travel from the edge of the meristem to the position of maximum REGR, and (ii) time from maximum REGR to the distal limit of the extension zone. The *Rht3* allele appears to primarily reduce (ii), the length of time a cell takes to move from the

position of maximum REGR to the outer edge of the extension zone (Table 4.7).

Table 4.7 *Calculation of a time basis for cell residence time (± s.e.) within the L2 extension zone. Data derived from cell length measurements and leaf elongation rates using eqn 3 cf. section 4.2.4.2. Plants of rht3 (wild-type) and Rht3 (mutant) lines were grown at 20 °C (n=7).*

Genotype	Time for a cell to move (h)		
	Through total L2 extension zone	From meristem to position of maximum REGR	From maximum REGR to outer edge of extn zone
rht3	45.2 (4.6)	31.7 (3.0)	13.5 (1.6)
Rht3	31.7 (3.3)	25.9 (3.9)	5.8 (0.4)***

*** = difference significant at $P < 0.001$

The result clearly identifies the effect of the *Rht3* allele as being to reduce the time that a cell takes to extend from 50 to 100 % of its final length, (Table 4.7), after the position of maximum REGR and therefore suggests that a major effect of the *Rht3* allele may be to reduce longitudinal cell extension.

4.3.1.3 Effect of the *Rht3* allele on epidermal cell dimensions and number

There was a significant ($P < 0.001$) reduction in intercostal epidermal cell length in the *Rht3* mutant line compared to the *rht3* wild-type (Table 4.8), while there was no obvious difference in the calculated total number of epidermal cells in L2 of the two lines (Table 4.8). The results show that the primary effect of the *Rht3* allele was to reduce cell extension. No significant differences were found in either cell width or area per unit mass of L2 in the two genotypes (Table 4.8).

Table 4.8 *Effect of the Rht3 allele on cell number, cellular dimensions, and specific leaf area (± s.e.) in mature L2 epidermis. Cell measured were located on the abaxial epidermal surface within intercostal cell files in the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C (n=23).*

Genotype	Total intercostal cell number per file^{-1}	Cell dimension length (µm)	width (µm)	specific leaf area (DM) (mm^2 mg^{-1})
rht3	208 (18)	1380 (131.5)	33.0 (0.7)	30.96
Rht3	210 (21)	740 (83.7)***	34.8 (2.1)	30.89

*** = difference significant from the *Rht3* wild-type at $P < 0.001$

Compared to the *rht3* wild-type, the principal effect of the *Rht3* allele was to

significantly reduce cell length but not total cell number per file. The present results are in contrast to those of Hoogendoorn *et al.*, (1990), who reported that in Maris Huntsman the *Rht3* allele reduced cell number. The present results are, however, in keeping with the observations on lines of Maris Huntsman carrying the *Rht1* and *Rht2* alleles in which a linear relationship was shown between increasing *Rht1* and *Rht2* gene dose and reduced epidermal cell length (Keyes *et al.*, 1989).

4.3.1.4 Conclusions; the influence of the Rht3 allele on growth of L2

The effect of the *Rht3* mutant allele on the growth of L2 can be summarised as the following;

1. a reduction in both the absolute rate of L2 extension and final L2 length;
2. the absence of an effect on the duration of L2 extension;
3. the absence of effect on the maximum REGR reached within the extension zone, or the distance from the leaf base at which this was achieved;
4. a reduction in values of REGR subsequent to the position of maximum REGR and a reduction in the length of the L2 extension zone;
5. a reduction in the length of time a cell spends within the meristem and therefore perhaps an increase in the rate of cell production;
6. a reduction in the length of time a cell takes to reach 20 % final length and the time to move from the position of maximum REGR to the outer edge of the extension zone;
7. a reduction in epidermal cell length but not total cell number per abaxial intercostal cell file.

Section 4.3.1 has provided a characterisation of growth in the L2 model system of the *rht3* wild-type and *Rht3* mutant lines. The L2 model system will subsequently be modified by the application of PGRs, in an attempt to further investigate processes of growth which may be specifically affected by the *Rht3* allele.

4.3.2 The effect of applied growth regulators on L2 morphology and growth characteristics

In this section of results growth of L2 which has been characterised in section 4.3.1 will be modified by the application of growth regulators.

Second leaves of *rht3* wild-type plants treated with 10 µM 2*S*,3*S* paclobutrazol resembled those of the untreated *Rht3* mutant line (Plate 4.1 & 4.2). Treated leaves of the *rht3* wild-type were shorter, darker green, and more deeply ridged than those of the untreated control plants. The opposite morphological characteristics were observed in *rht3* wild-type leaves treated with 10 µM GA_3; they were longer, lighter green and less deeply ridged. In addition, application of GA_3 in combination with 2*S*,3*S* paclobutrazol reversed the morphological effects of 2*S*,3*S* paclobutrazol on leaves of the *rht3* wild-type line (*cf.* section 4.3.2.1.1, Plate 4.3). In comparison, in L2 of the *Rht3* mutant line there was no effect of applied GA_3 and only a limited reduction in L2 length in response to applied 2*S*,3*S* paclobutrazol (Plate 4.2).

In the *rht3* wild-type, 2*S*,3*S* paclobutrazol caused a 49 % reduction in the maximum growth rate which was associated with reduction of 52 % in L2 final length compared to the untreated control (Table 4.9; Fig. 4.7). Applied GA_3 had the opposite effects; the maximum growth rate was increased by 35 % and L2 final length was increased by 27 % compared to the untreated control (Table 4.9 & Fig. 4.7). In addition, 2*S*,3*S* paclobutrazol tended to reduce the duration of L2 extension. Applied GA_3 had the opposite effect and tended to increase the duration of L2 growth (Table 4.9). However, in both this experiment and a further similar experiment, the duration of the extension period was consistently shortest with 2*S*,3*S* paclobutrazol, longer in the control and longest with GA_3.

Table 4.9 *Parameters (± s.e.) describing L2 growth characteristics of the rht3 (wild-type) line treated with 5 µM GA_3 and 5 µM 2S,3S paclobutrazol (Pac) as a root drench. Plants were grown at 20 °C. Parameters were calculated from freehand curves and linear regression as described in chapter 2, section 2.4 (n=30). Data of Fig. 4.7).*

Treatment	Maximum growth rate (mm h^{-1})	Calculated final L2 length (mm)	L2 50 % final length (d)
Untreated	0.84	225.0 (15.0)	11.2
GA_3	1.29	307.1 (15.9)***	11.7
Pac	0.43	108.4 (7.3)***	9.3

*** = difference significant at $P < 0.001$ compared to untreated control

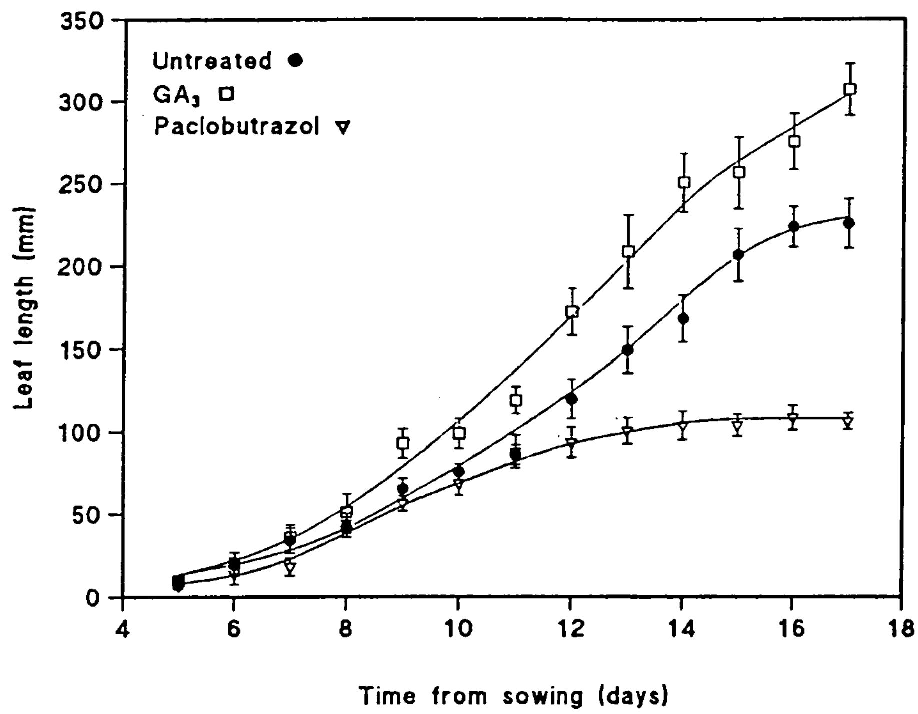


Fig. 4.7 The effect of 10 µM GA_3 and 2*S*,3*S* paclobutrazol on L2 extension in the *rht3* (wild-type) line (± s.e., n=18) grown at 20 °C.

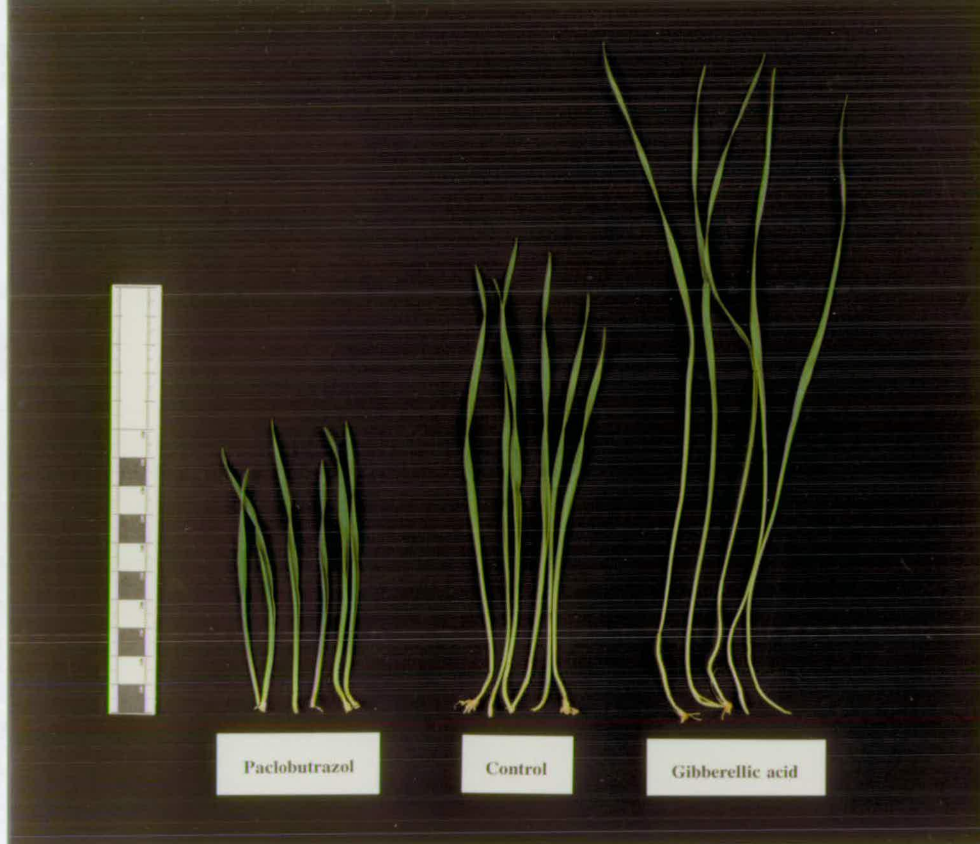


Plate 4.1 Second leaves of the *rht3* (wild-type) line of the untreated control, treated with either 10 µM GA_3 or 10 µM 2*S*,3*S* paclobutrazol. Leaves were grown at 20 °C and photographed 9 d after sowing.

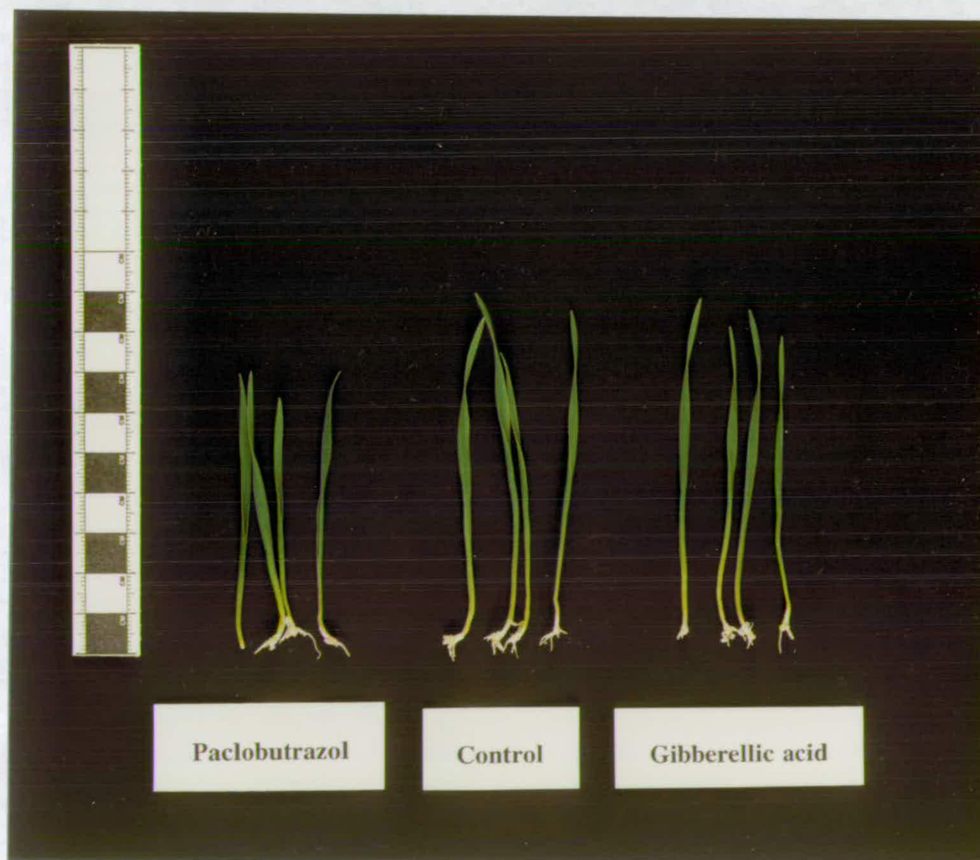


Plate 4.2 Second leaves of the *Rht3* (mutant) line of the untreated control, treated with either 10 µM GA_3 or 10 µM 2*S*,3*S* paclobutrazol. Leaves grown at 20 °C and photographed 9 d after sowing.

4.3.2.1 Effect of applied gibberellin and paclobutrazol on the cellular and temporal development of L2

4.3.2.1.1 How do gibberellin and paclobutrazol affect the length of the extension zone ?

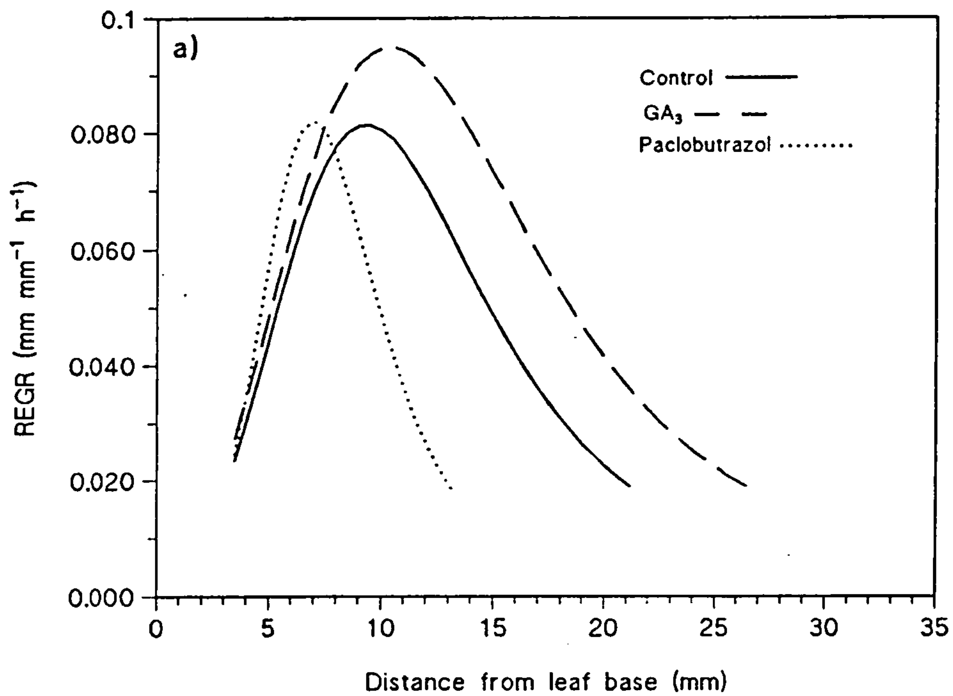
The main effect of the applied growth regulators was to change the length of the L2 extension zone. In the *rht3* wild-type, compared to the untreated control, GA_3 increased the extension zone length by 17 %, while 2*S*,3*S* paclobutrazol reduced it by 48 % (Table 4.10). The length of the extension zone of the *rht3* wild-type treated with 2*S*,3*S* paclobutrazol was similar to that of the untreated *Rht3* mutant. Although application of 2*S*,3*S* paclobutrazol to the *Rht3* mutant line slightly reduced the size of the extension zone, there was no response to applied GA_3 (Table 4.10). The applied chemicals had no significant effect on the value for maximum REGR in either genotype although the distance from the leaf base at which maximum REGR was reached, was affected; GA_3 increased it significantly in the *rht3* wild-type and 2*S*,3*S* paclobutrazol decreased it, in both genotypes (Fig. 4.8a & b, Table 4.10). Application of 10 μM 2*S*,3*S* paclobutrazol to the *rht3* wild-type reduced final L2 length to less than that of the untreated *Rht3* mutant. However, leaves treated with 2*S*,3*S* paclobutrazol of both genotypes had similar final lengths (Table 4.10). By contrast applied GA_3 caused a 22 % increase in final leaf length in the *rht3* wild-type whereas the *Rht3* mutant was unresponsive.

The maximum point of the REGR curve was difficult to estimate accurately as the data points around this region showed a high degree of scatter. While the maximum REGR appeared higher in leaves of the *rht3* wild-type treated with GA_3, compared to leaves treated with 2*S*,3*S* paclobutrazol or the untreated control in Fig. 4.8a, this effect was not reproduced when the experiment was repeated.

Table 4.10 *Effect on the REGR $\geq$ 0.02 mm $mm^{-1}h^{-1}$, final length and extension zone length (± s.e.) of L2 in the rht3 (wild-type) and the Rht3 (mutant) lines treated with 10 μM GA_3 and 10 μM 2S,3S paclobutrazol (Pac) as a root drench. Plants were grown at 20 °C and sampled around d 9. REGR parameters were calculated from fitted curves described in eqns 1 & 2, section 4.2.4.1. The length of the extension zone was calculated from the mean length of the sum of all the 2 mm segments which extended over the 5 h growth period. Data of Fig. 4.8a & b (n=37).*

Genotype	Treatment	Extension zone length (mm)	Maximum REGR ($mm.mm^{-1}h^{-1}$)	Position of maximum REGR (mm)	L2 final length (mm)
rht3	Untreated	21.7	0.081 (0.0023)	9.3 (0.2)	225.0 (15.0)
	GA_3	26.1***	0.095 (0.0055)	10.3 (0.3)***	307.1 (15.9)***
	Pac	11.3***	0.082 (0.0029)	6.9 (0.1)***	108.4 (7.3)***
Rht3	Untreated	12.8	0.081 (0.0030)	8.3 (0.1)	147.3 (5.3)
	GA_3	13.1	0.083 (0.0031)	8.4 (0.1)	145.5 (3.6)
	Pac	10.2**	0.083 (0.0033)	7.1 (0.1)***	112.4 (7.3)**

/* = difference significant compared to untreated control at $P < 0.01/0.001$



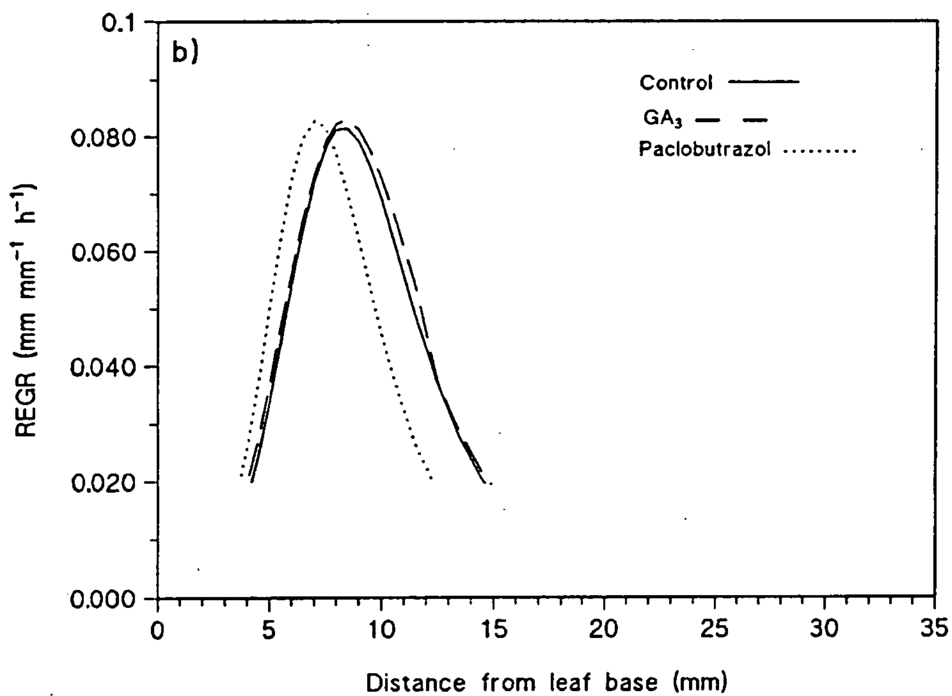


Fig. 4.8 Spatial distribution of REGR ($\geq$ 0.02 mm mm^{-1} h^{-1}) within the extension zone of L2 in (a) *rht3* (wild-type) and (b) *Rht3* (mutant) lines sampled around d 9, the period of maximum extension growth in L2 grown at 20 °C of the untreated control or treated with 10 µM GA_3 or 2*S*,3*S* paclobutrazol. REGR curves have been smoothed using equation 2 described section 4.2.4.1.

Comparison of REGR in the untreated and treated *rht3* wild-type at increasing distance from the leaf base showed that REGR in leaves treated with 2*S*,3*S* paclobutrazol were similar to *ca* 7 mm from the leaf base but decreased significantly ($P < 0.001$) in positions distal to 10 mm from the leaf base when compared to the untreated control. Treatment of the *rht3* wild-type with applied GA_3 gave some evidence that the value of REGR was greater ($P < 0.05$) than the untreated control around 6-8 mm from the leaf base. There was no significant difference in REGR in treated and untreated leaves 10 mm from the leaf base. However at 12 mm and at more distal positions through the extension zone the higher REGR values of leaves treated with GA_3, compared to the untreated controls was highly significant ($P < 0.001$).

The ability of GA_3 to reverse the inhibitory effect of 2*S*,3*S* paclobutrazol was examined in the *rht3* wild-type line, which was GA-responsive. In this experiment the concentration of the applied growth regulators was 5 µM both individually and in the combination treatment.

As shown previously, the main effect of GA_3 and 2*S*,3*S* paclobutrazol was to alter the length of the extension zone without affecting the maximum REGR (Table 4.11 & Fig. 4.9). The concentration of applied GA_3 completely overcame the inhibitory effects of 2*S*,3*S* paclobutrazol on the length of the extension zone (Table 4.11) and on L2 morphology (Plate 4.3). The value of REGR in the GA_3-treated plants and in the combination treatment of GA_3 + 2*S*,3*S* paclobutrazol, within the L2 extension zone were superimposable (Fig. 4.9).

The results suggest that a phenocopy of the *Rht3* allele can be produced in the *rht3* wild-type by inhibiting the biosynthesis of GA with 2*S*,3*S* paclobutrazol. Thus, inhibiting GA production appeared to have the same consequences developmentally as blocking GA action with the *Rht3* allele. Applied GA_3 and 2*S*,3*S* paclobutrazol, and, by implication endogenous GA status, appear to define the upper and lower limits of the length of the extension zone in the *rht3* wild-type line. An important feature of the *Rht3* allele is to restrict the length of the extension zone in L2, such that it is unresponsive to applied, and probably endogenous GA.

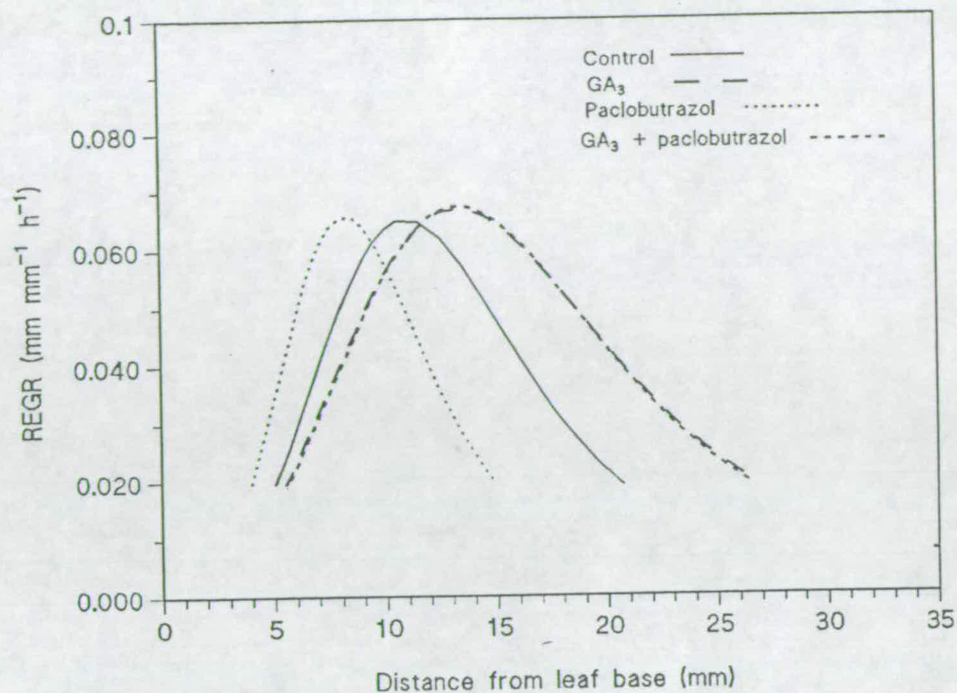


Fig. 4.9 Spatial distribution of REGR (≥ 0.02 mm mm^{-1} h^{-1}), within the extension zone of L2 in the *rht3* (wild-type) line sampled on d 9, the period of maximum L2 extension growing at 20 °C; of the untreated control and treated with 5 µM GA_3, 2*S*,3*S* paclobutrazol and 5 µM GA_3 + 2*S*,3*S* paclobutrazol. REGR curves were smoothed using equation 2 described in section 4.2.4.1.

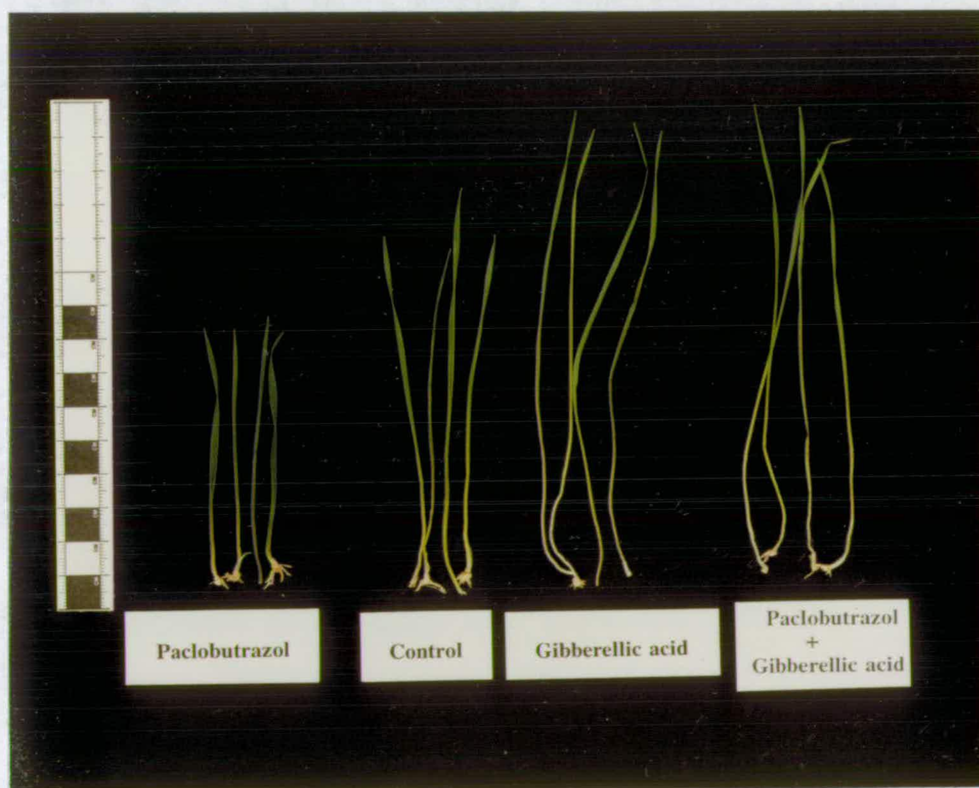


Plate 4.3 Second leaves of the *rht3* (wild-type) line in the untreated control, treated with 5 µM GA_3, 5µM 2*S*,3*S* paclobutrazol or a combination treatment of GA_3 + 2*S*,3*S* paclobutrazol. Leaves grown at 20 °C and photographed 9 d after sowing.

Table 4.11 *Effect on position and maximum REGR ≥ 0.02 mm mm^{-1}h^{-1}, estimated from curves fitted using eqn 1 & 2 described in section 4.2.4.1. The extension zone length (± s.e.) has been calculated as the sum of all the original 2 mm segments which extended over the 5 h growth period, in the rht3 (wild-type) line treated with 5 µM GA_3, 2S,3S paclobutrazol (Pac), and GA_3 + 2S,3S paclobutrazol (GA_3+Pac). Plants were grown at 20 °C and sampled around d 9. Data of Fig. 4.9 (n=36).*

Treatment	Extension zone length (mm)	Maximum REGR (mm mm^{-1} h^{-1})	Position of maximum REGR (mm)
Untreated	22.32	0.065 (0.0018)	10.98 (0.16)
GA_3	29.59***	0.067 (0.0023)	13.29 (0.26)***
Pac	11.96***	0.066 (0.0043)	8.30 (0.26)***
GA_3+Pac	28.84***	0.067 (0.0017)	13.30 (0.19)***

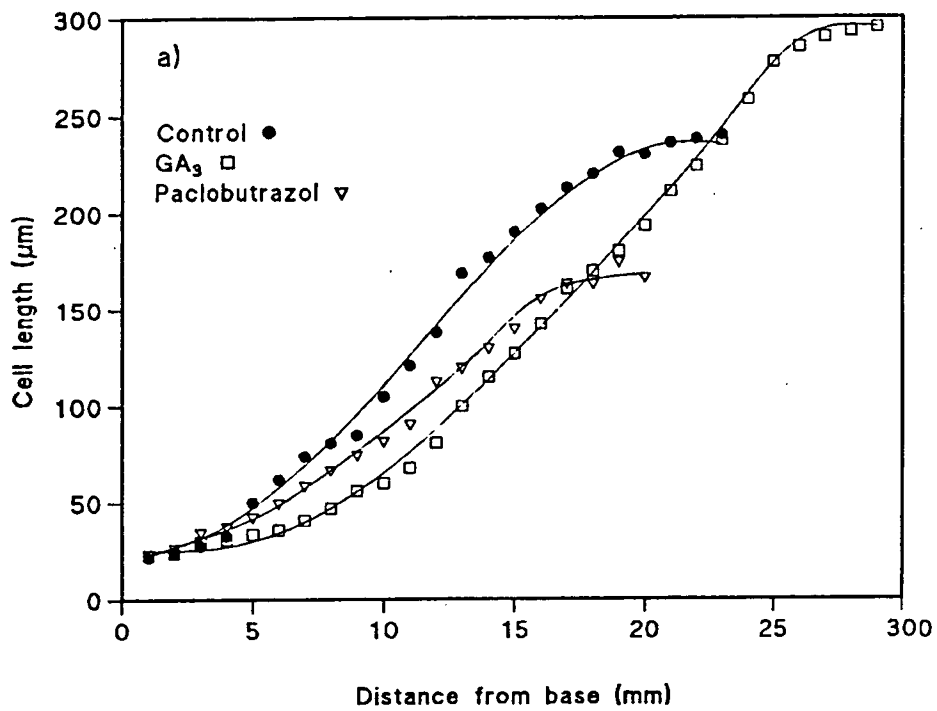
*** = difference significant from untreated control P < 0.001

4.3.2.1.2 Effect of gibberellin and paclobutrazol on cell length and residence time within L2 meristem and extension zone

Treatment with 5 µM GA_3 and 2*S*,3*S* paclobutrazol increased and decreased respectively final interstomatal cell length (Fig. 4.10a). The increase in cell length could also be represented on a time axis using eqn 3 (*cf.* section 4.2.4.2). Final cell length was achieved closest to the leaf base in 2*S*,3*S* paclobutrazol treated leaves, further away in the control and furthest from the leaf base in GA_3 treated leaves (Fig. 4.10a). The results were consistent with GA_3 and 2*S*,3*S* paclobutrazol increasing and decreasing respectively, the length of the L2 extension zone and perhaps also GA_3 increasing the length of the basal L2 meristem.

The time for a cell to reach 50 % final size was *ca* 60 h in the untreated control and 2*S*,3*S* paclobutrazol treated leaves and *ca* 70 h in the GA_3 treated leaves (Fig. 4.10b). However, the time taken for a cell to grow from 50 % to full extension was only *ca* 12 h in the untreated and 2*S*,3*S* paclobutrazol treated leaves and *ca* 10 h in the GA_3 treated leaves. The time 'position' of the outer extension zone border was *ca* 71, 79 and 75 h in the control, GA_3 and 2*S*,3*S* paclobutrazol treated leaves respectively. Thus, cell extension from 50 to 100 % final length is a short period of time (< 15 h) immediately prior to reaching the outer edge of the extension zone.

Comparison of the effect of applied GA_3 and 2*S*,3*S* paclobutrazol on cell residence times within the L2 extension zone could not be calculated as the length of the basal meristem in treated leaves was not measured. However, the cell length data presented in Fig. 4.10a & b suggested that, compared to the untreated control, 2*S*,3*S* paclobutrazol may reduce and GA_3 may increase, the length of the L2 basal meristem in the *rht3* wild-type.



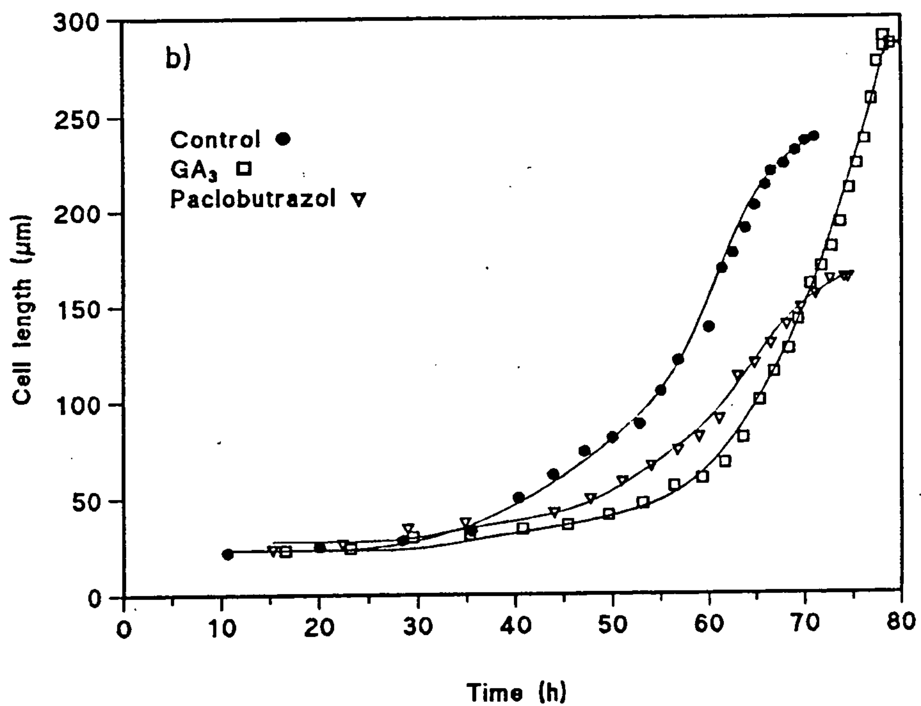


Fig. 4.10 Increase in interstomatal epidermal cell length in the *rht3* (wild-type) line sampled around d 9, the period of maximum extension growth of L2 grown at 20 °C, with increasing distance from the leaf base (a) and (b) with increasing cell length expressed as a function of time, calculated as described in section 4.2.4.2. Plants were untreated (control) or treated with 5 μM GA_3 or 5 μM 2*S*,3*S* paclobutrazol.

The results presented in Fig. 4.10a & b, support the suggestion that *2S,3S* paclobutrazol may promote processes which prevent continued cell extension while applied GA_3 functions in the opposite manner to affect processes which promote continued cell extension.

4.3.2.2 Effect of paclobutrazol and gibberellin on cell dimensions and cell numbers

The *Rht3* allele reduced final L2 length and cell length compared to the *rht3* wild-type without an effect on cell number (Table 4.12) Applied *2S,3S* paclobutrazol significantly reduced final L2 length and cell length ($P < 0.001$) in the *rht3* wild-type. While *2S,3S* paclobutrazol significantly reduced final L2 length in the *Rht3* mutant ($P < 0.01$), it had no effect on cell length (Table 4.12). However, in both genotypes *2S,3S* paclobutrazol tended to reduce cell number. In the *rht3* wild-type, GA_3 significantly increased L2 length ($P < 0.001$) and interstomatal cell length ($P < 0.05$) and tended to reduce cell width (Table 4.12). However, the effects of GA_3 on epidermal cell dimensions were perhaps somewhat obscured by high standard errors associated with measurement of epidermal cell dimensions. Cell width was not significantly affected by either the *Rht3* allele, applied *2S,3S* paclobutrazol or GA_3. The similarity in cellular dimensions of the *Rht3* mutant and *rht3* wild-type treated with *2S,3S* paclobutrazol suggests that the application of the inhibitor may initiate cellular responses similar to those produced by the *Rht3* allele but, in addition, the inhibitor may decrease cell number.

Table 4.12 *Effect of 10 µM GA_3 and 2S,3S paclobutrazol (Pac) on final, abaxial, interstomatal, epidermal cell dimensions (± s.e.). Cells lengths were measured between stomata within individual files of cells on the abaxial surface of L2 in the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C and sampled around d 9 (n=19).*

Geno- -type	Treatment	Final L2 length (mm)	Epidermal cell dimensions length (µm)	width (µm)	Interstomatal cell number
rht3	Untreated	264.8 (15.0)	240.3 (28.6)	23.6 (1.1)	1103 (174)
	GA_3	337.6 (19.8)***	290.6 (53.2)*	18.7 (2.5)	1164 (237)
	Pac	109.8 (1.9)***	134.8 (37.8)**	25.4 (2.0)	814 (189)
Rht3	Untreated	139.9 (1.3)	121.0 (19.3)	25.3 (3.1)	1156 (232)
	GA_3	145.5 (3.6)	136.5 (15.4)	25.8 (3.0)	1065 (167)
	Pac	112.4 (7.3)***	120.9 (9.4)	25.3 (1.2)	929 (144)

*/** /*** = difference significant at $P < 0.05/0.01/0.001$ compared to the untreated control.

Thus, the effect of both GA_3 and *2S,3S* paclobutrazol on the final length of L2 is mediated more through an effect on cell length than on cell number. Although *2S,3S* paclobutrazol also tended to decrease cell number, this was not an effect of the *Rht3*

allele. It is also interesting to note that although applied GA_3 may extend the length of the basal L2 meristem (Fig. 4.10a & b) it does not appear to markedly affect final cell number. This could suggest that GA_3 may increase the length of the cell cycle.

4.3.2.3 Effect of gibberellin and paclobutrazol on mass per unit length of L2

Application of GA_3 increased, and 2*S*,3*S* paclobutrazol significantly decreased, cell length in the *rht3* wild-type. The results suggested that GA may be affecting the orientation of cell expansion.

Compared to the untreated control, application of 2*S*,3*S* paclobutrazol to the *rht3* wild-type increased the dry matter per unit length of L2 by 23 %, while GA_3 acted in the opposite direction and reduced it by 25 % (Fig. 4.11). No significant differences in the dry matter per unit length of leaf in response to applied GA_3 or 2*S*,3*S* paclobutrazol were observed in the *Rht3* mutant line.

The effects on the mass per unit length of L2 of both GA_3 and 2*S*,3*S* paclobutrazol in the *rht3* wild-type are probably related in part to the actual numbers of cells present within a unit length of leaf. Thus, in a unit length of lamina of the *rht3* wild-type treated with GA_3 there would be fewer but longer cells compared to the same unit length of lamina treated with 2*S*,3*S* paclobutrazol. However, while mass per unit length in the *rht3* wild-type was reduced by the application of the growth regulators, by *ca* 20 %, compared to the untreated control, final cell length was reduced by a larger proportion of *ca* 30-40 %. No significant effects on either cell length or amount of leaf dry matter per unit length of leaf were found in the lamina of the mutant genotype treated with either GA_3 or 2*S*,3*S* paclobutrazol, compared to the untreated control. This was expected as the mutant is insensitive to applied GA and shows only a very limited responsiveness to 2*S*,3*S* paclobutrazol. It would therefore appear that the effect of the applied PGRs may be to reorientate growth but they predominantly affect growth in the longitudinal axis.

4.3.2.4 Conclusions

The effects of PGRs on the growth characteristics of L2 in the *rht3* wild-type and *Rht3* mutant lines can be summarised:

1. Application of 2*S*,3*S* paclobutrazol to the *rht3* wild-type produced a phenotype similar to the untreated mutant and reduced the rate of growth of L2 to that of the untreated *Rht3* mutant. This effect of 2*S*,3*S* paclobutrazol in the wild-type could be completely reversed by the application of GA_3.

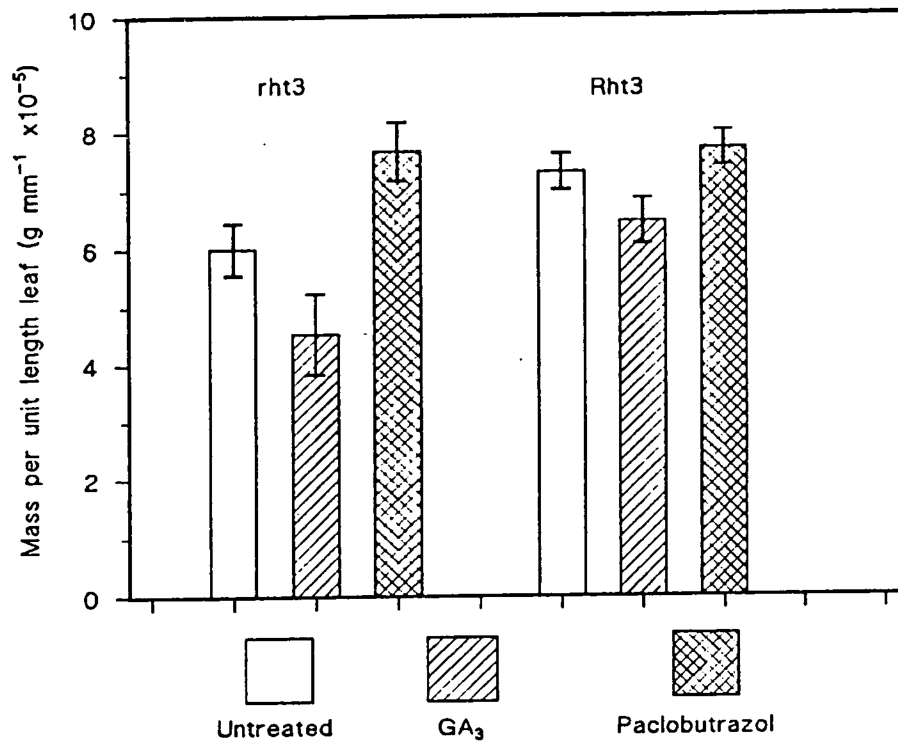


Fig. 4.11 Effect on the dry mass per unit length of L2 in the *rht3* (wild-type) and *Rht3* (mutant) lines (± s.e., n=9), following treatment with 5 μM GA_3 and 5 μM 2*S*,3*S* paclobutrazol. L2 was sampled around d 9, the period of maximum extension growth at 20 °C.

2. Application of GA_3 alone, to the *rht3* wild-type both increased the rate of growth and produced a longer, paler green leaf. In comparison, there were no effects of the applied PGRs on the maximum growth rate or phenotype of the *Rht3* mutant.

3. Application of PGRs did not affect the maximum REGR achieved within the L2 extension zone of either genotype. However, GA_3 tended to increase, and 2*S*,3*S* paclobutrazol to decrease, the distance from the leaf base at which this was reached. In the *Rht3* mutant REGR curves for treated leaves resembled those for the untreated control.

4. In the *rht3* wild-type, GA_3 significantly increased and 2*S*,3*S* paclobutrazol significantly decreased the length of the extension zone. Application of 2*S*,3*S* paclobutrazol caused only a slight reduction in the length of the zone in the *Rht3* mutant genotype.

5. 2*S*,3*S* paclobutrazol may reduce cell number in both genotypes whereas this was not an effect of the *Rht3* allele in the mutant genotype.

6. The primary effect of PGRs in the wild-type, as with the *Rht3* allele itself, was to affect cell lengths but not particularly cell numbers.

Application of 2*S*,3*S* paclobutrazol and GA_3 to the *rht3* wild-type has demonstrated that the *Rht3* allele and endogenous GA probably affect the same mechanisms through which cell length is controlled.

4.3.3 Effect of low temperature; modification of L2 growth

The L2 model system which has been previously characterised (section 4.3.1) and modified by the application of PGRs (section 4.3.2) will be further investigated by its response to growth at low temperature.

In the *rht3* wild-type, growth at 10 °C compared to 20 °C reduced leaf length and surface area by 23 % and 40 % respectively, and increased leaf dry weight by 27 % (Tables 4.2, 4.13 & 4.14). In the *Rht3* mutant line, low temperature had a negligible effect on leaf length while it decreased leaf surface area and increased leaf dry mass by 17 % and 40 % respectively (Table 4.2, 4.13 & 4.14). Thus, while growth at 10 °C had significantly different effects on leaf length and surface area between the two genotypes, it had a lesser effect on L2 dry mass (Table 4.14).

4.3.3.1 Genotypic comparison between the *rht3* (wild-type) and the *Rht3* (mutant) lines grown at 10 °C

Second leaf growth in the two lines was analysed as described in 4.2.2.2 and chapter 2, section 2.4.

Grown at 10 °C, the morphology of L2 in the *rht3* wild-type resembled that of the *Rht3* mutant; leaves were shorter, darker green and more deeply ridged. There was no significant change in the morphology of L2 in the *Rht3* mutant line when grown at the reduced temperature.

In both genotypes L2 reached 50 % maximum extension around d 18-19. The *Rht3* allele caused a 23 % reduction in the maximum elongation rate which resulted in a 19 % reduction in final L2 length compared with the *rht3* wild-type line (Table 4.13 & Fig. 4.12a).

In both genotypes L2 reached 50 % maximum surface area by d 20 (Table 4.13). The *Rht3* allele caused a 14 % reduction in the maximum rate of increase in L2 surface area which resulted in a 8 % reduction in final L2 surface area compared with the *rht3* wild-type line (Table 4.13 & Fig. 4.12b).

In both genotypes L2 reached 50 % final dry mass on *ca* d 21-23. The *Rht3* allele reduced the maximum rate of L2 dry mass accumulation by 30 % which resulted in a reduction in final L2 dry mass of 21 % compared to the *rht3* wild-type line (Table 4.13 & Fig. 4.12c).

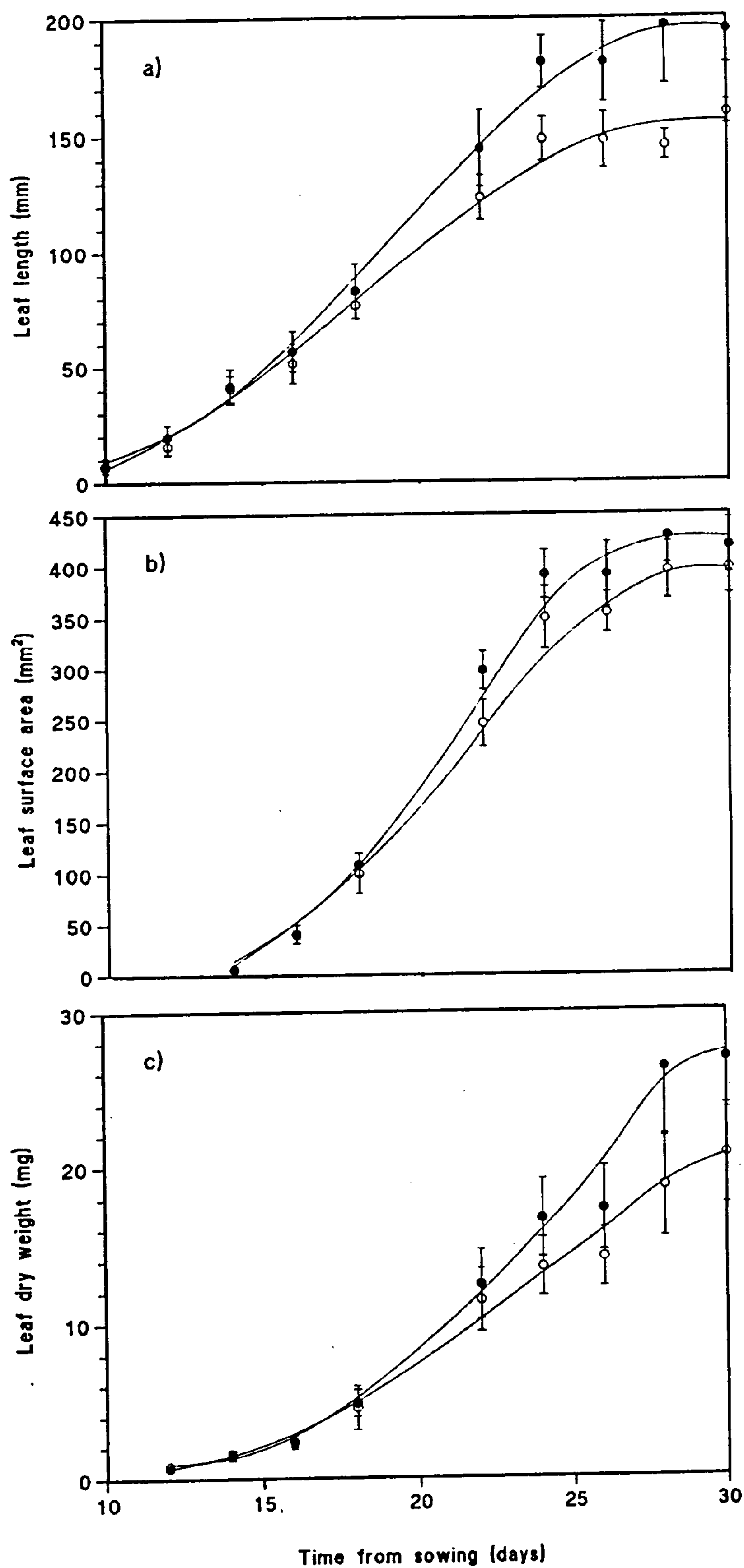


Fig. 4.12 Genotypic comparison of the *rht3* (wild-type, ●) and the *Rht3* (mutant, O) lines grown at 10 °C (± s.e., n=18). Increase in; (a) total L2 length, (b) L2 surface area and (c) L2 dry weight with time. Freehand curves have been fitted to L2 extension data.

Table 4.13 *Parameters (± s.e.) describing the growth of L2 in the rht3 (wild-type) and the Rht3 (mutant) lines grown at 10 °C. Data derived from freehand curves fitted to extension data and a linear regression line over the period of 20 to 80 % maximum extension, as described in section 4.2.2.2 and section 2.4, chapter 2. (n=18). Data of Fig. 4.12.*

Growth parameter	Genotype	Time (d) to 50 % final	Final value	Maximum rate of increase
Length	*rht3*	18.6	196.5 (11.4)mm	15.5 mm d^{-1}
	Rht3	18.2	159.4 (4.9)mm***	12.0 mm d^{-1}
Area	*rht3*	20.0	428.1 (26.7) mm^2	44.5 mm^2 d^{-1}
	Rht3	20.1	394.5 (19.5) mm^2***	38.1 mm^2 d^{-1}
Dry mass	*rht3*	22.5	27.2 (4.5) mg	1.99 mg d^{-1}
	Rht3	21.3	21.6 (3.2) mg*	1.39 mg d^{-1}

*** / * = difference significant at $P < 0.001$ / 0.05

Table 4.14 *Comparison of rht3 and Rht3 ratios of final L2 length, surface area and dry mass of L2 grown at 10 and 20 °C. Data of Table 4.2 and 4.13.*

Temperature (°C)	Ratios of temperature effect at 10 ° : 20 °C		
	L2 length	L2 surface area	L2 dry mass
rht3	0.76	0.60	1.27
Rht3	1.10	0.83	1.40

Clearly, reducing the growing temperature from 20 ° to 10 °C initiated a more marked reduction in leaf length and surface area in the *rht3* line compared to the *Rht3* mutant (Table 4.14). As a consequence, the pattern of L2 growth in the two lines is more similar when plants are grown at 10 ° than at 20 °C.

4.3.3.1.1 Length of second leaf basal meristem

In section 4.3.1.1.2 it was shown that at 20 °C the *Rht3* allele reduced the length of the L2 basal meristem when compared to the *rht3* wild-type. At 10 °C, the *Rht3* allele reduced the length of the L2 basal meristem by 31 % compared to the *rht3* line at 10 °C (Table 4.15). However basal meristem length was reduced by a similar proportion in both the *rht3* wild-type and *Rht3* mutant lines as shown by the ratio of meristem lengths at the two temperatures (Table 4.15).

Table 4.15 *Mean length of the L2 meristematic region (± s.e.) in the rht3 (wild-type) and Rht3 (mutant) growing at 10 °C sampled on d 18 (n=6), and comparison with the ratio of mean meristem length at 10 ° & 20 °C. Data of Table 4.3 & 4.15.*

Genotype	Length of the meristem region from the leaf base (mm)	Ratio of meristem length 10 ° :20 °C
rht3	4.8 (0.4)	0.90
Rht3	3.3 (0.5)**	0.89

** = difference significant $p < 0.01$

The results suggest that low temperature reduces the length of the L2 basal meristem, which acts in addition to the effect of the *Rht3* allele.

4.3.3.2 How does low temperature affect the cellular and temporal pattern of growth along L2 ?

Extension of L2 growing at 10 °C was analysed further by calculating the relative elemental growth rate (REGR) using eqn 1 described in section 4.2.4.1. The REGR increased rapidly to reach a maximum 9-10 mm from the leaf base in both genotypes (Fig. 4.13). There was no significant difference in the maximum REGR achieved within the L2 extension of the *rht3* wild-type and *Rht3* mutant lines (Fig. 4.13, Table 4.16). The length of the L2 extension zone has been calculated from the original positions of the pin holes and not from Fig. 4.13 (*cf.* Table 4.14). Although the length of the extension zone was reduced by 33 % in the *rht3* wild-type compared to its length at 20 °C, it was still 16 % longer than the length of the zone in the *Rht3* mutant (Table 4.16).

Table 4.16 *Parameters (± s.e.) describing the relative elemental growth rate (REGR) ≥ 0.005 within the extension zone of the rht3 (wild-type) and the Rht3 (mutant) lines grown at 10 °C and sampled around d 18. The position of maximum REGR and maximum rate have been calculated from curves fitted to REGR data described in section 4.2.4.1. The length of the extension zone was the mean length of all of the original 2 mm segments which extended over the 10 h growth period. Results represent means derived from one experiment. (n=36). Data of Fig. 4.13. The lower section of table shows the ratio of the effect of temperature on the measured REGR and extension zone parameters. Data of table 4.4 and 4.16.*

Genotype	Position of maximum REGR (mm from base)	Maximum REGR ($mm\ mm^{-1}\ h^{-1}$)	Extension zone length (mm)
rht3	9.9 (0.18)	0.027 (0.0011)	14.8
Rht3	9.0 (0.17)	0.029 (0.0013)	12.5***
Ratios of temperature effect at 10 ° : 20 °C			
rht3	0.97	0.34	0.74
Rht3	1.04	0.36	0.98

*** = difference significant $P < 0.001$

The results suggest that low temperature affects at least two aspects of L2 growth; the length of the extension zone and the maximum REGR achieved within the zone. However, the *Rht3* allele affects only one of these two responses - a reduction in the length of the extension zone, as shown by the ratios of the effect of temperature (Table 4.16). In both genotypes the reduction in the temperature from 20 ° to 10 °C caused a 67 % reduction in the maximum REGR. In the *rht3* wild-type this was coupled with a 25 % reduction in the length of the extension zone (Tables 4.4 & 4.16).

The results suggest that the *Rht3* mutant could be more accurately described as one which exhibits insensitivity to increased, rather than decreased temperatures. If the temperature is increased to 20 °C, the maximum REGR is increased, but not length of the extension zone.

4.3.3.2.1 Does growth at 10 °C affect the cell residence time within the extension zone ?

It was suggested (section 4.3.1.2.4, Table 4.7) that a characteristic effect of the *Rht3* allele at 20 °C was to reduce the length of time that a cell takes to move from the position of maximum REGR to the distal limit of the extension zone.

Growth of L2 at 10 °C reduced the total cell residence time compared to 20 °C in the *rht3* wild-type by *ca* 25 % (Tables 4.7 & 4.17). However, the total cell residence time of a cell in the *Rht3* mutant line was only reduced by *ca* 5 % (Table 4.17 & 4.7). There were no significant differences in cell residence times between the *rht3* wild-type or the *Rht3* mutant

lines at 10 °C, either in the total time, or the time spent in different regions within the zone (Table 4.17).

Table 4.17 *Effect of low temperature on cell residence times (± s.e.) calculated using eqn 3 described in section 4.2.4.2 within L2 extension zone of the rht3 (wild-type) and Rht3 (mutant) lines grown at 10 °C. The lower section of table shows the ratio of the effect of temperature on the calculated cell residence times at 10 ° and 20 °C. Data of Table 4.7 and 4.17.*

Genotype	Time (h) for a cell to move Through total L2 extension zone	From meristem to position of maximum REGR	From maximum REGR to outer edge of zone
rht3	33.7 (4.1)	19.4 (4.0)	14.3 (4.0)
Rht3	30.1 (1.6)	16.6 (1.6)	13.5 (1.0)
Ratios of temperature effect at 10 ° : 20 °C			
rht3	0.74	0.61	1.06
Rht3	0.95	0.64	2.32

The decrease in total residence time was more marked in the *rht3* wild-type than in the *Rht3* mutant, as shown by the ratios in Table 4.17. However, growth at 10 °C, had a more obvious effect on the *Rht3* mutant than the *rht3* wild-type in terms of how total cell residence time was partitioned. The length of time a cell spends travelling from the meristem border to the position of maximum REGR was decreased at 10 °C by *ca* 36-39 % in both genotypes. However, in the *Rht3* mutant, the time for a cell to travel from maximum REGR to the outer edge of the extension zone was increased by *ca* 130 % at 10 ° compared to 20 °C (Table 4.17), while cell residence time through the same zone was not affected in the *rht3* wild-type at 10 °C. Hence, at 10 °C cell residence times within (all) regions of the L2 extension zone in the *Rht3* mutant were more similar to those in the *rht3* wild-type line, and especially through the zone from maximum REGR to the distal border of the extension zone. These effects could suggest that the *Rht3* mutant line is unresponsive to an increase in the growing temperature to 20 °C.

Cells of the *Rht3* mutant genotype spend *ca* twice the length of time within the region of the L2 extension zone which has been identified (section 4.3.1.2.4 & Fig. 4.6), as being the one in which cells increased from *ca* 50 to 100 % final length. Therefore, cells of the *Rht3* mutant line have a longer period of time in which to extend at 10 ° compared to 20 °C. However, cell lengths within the *Rht3* mutant are still significantly shorter than in the *rht3* line (Table 4.18). This would appear to demonstrate that the effect of the *Rht3* allele in preventing continued cell extension may be even more pronounced at 10 ° than at 20 °C.

4.3.3.3 Effect of growth at low temperature on epidermal cell dimensions and number

The effect of low temperature on epidermal cell dimension and specific leaf area was also investigated (Table 4.18).

Table 4.18 *Effect of growth at 10 °C on cell number, cellular dimensions and specific leaf area (± s.e.) in mature L2 epidermis. Interstomatal cells were measured on the abaxial epidermal surface in the rht3 (wild-type) and Rht3 (mutant) lines. (n=21). Lower section of table shows ratios of temperature effects on measured parameters. Original interstomatal cell dimension and number data given in footnote to this table.*

Genotype	Total cell number per file	Cell dimensions length (µm)	width (µm)	specific leaf area (mm^2 mg^{-1} DM)
rht3	1353 (542)	154.9 (41.7)	22.2 (1.6)	20.13
Rht3	1151 (451)	136.8 (10.4)*	25.5 (2.3)	21.05
Ratios of temperature effect at 10 ° : 20 °C				
Temp (°C)				
10	1.10	0.70[a]	0.94[a]	0.65
20	1.00	1.12[a]	1.00[a]	0.68

*/** = difference significant at P > 0.05/0.01

[a] = based on comparison of equivalent interstomatal cell lengths at 20 °C; cell length 218 ±45 & 121 ±19 µm in the *rht3* wild-type and *Rht3* mutant respectively and cell width 23.6 ±2.4 & 25.3 ± 3.1 µm in the *rht3* wild-type and *Rht3* mutant respectively. Based on these cell lengths total interstomatal cell number per file in the *rht3* wild-type and *Rht3* mutant were 1214 & 1156 respectively

Although epidermal cell lengths in the *rht3* wild-type at 10 °C were reduced by *ca* 29 % compared to their values at 20 °C, they were still significantly longer than those of the *Rht3* mutant line (Table 4.18). Growth at 10 °C did not affect the total number of cells within an epidermal file, in either genotype. This suggests that low temperature like the *Rht3* allele and applied PGRs, predominantly affect the process of cell extension and not cell division (sections 4.3.1 and 4.3.2). The major difference between the two lines and their response to growth at low temperature was that, in the *rht3* wild-type cell length was markedly reduced whereas it was not in the *Rht3* mutant. The leaf area per unit mass was very similar in both the *rht3* wild-type and the *Rht3* mutant at 10 °C. There was a 30 % reduction in the area per unit mass of L2 observed in both genotypes at 10 °C compared to 20 °C as shown by the ratios described in Table 4.18. The results could suggest that at 10 °C more assimilate may be stored in the cell walls or conversely, at 20 °C, L2 may contain more air spaces. However, an estimation of the proportion of the total dry matter which was present in the cell walls of the two genotypes was not made. Overall, growth at low temperature reduced the specific leaf area in both genotypes in a way which was independent of the *Rht3* allele.

4.3.3.4 Can the application of gibberellin reverse the effect of low temperature in the wild-type ?

Growth of L2 in the *rht3* wild-type at 10 °C produced leaves which were morphologically similar to those in the *Rht3* mutant line. If application of GA_3 to the *rht3* wild-type grown at 10 °C could reverse the effect of low temperature on the length of the extension zone and final leaf length, then it could provide evidence that the processes of L2 growth affected by the *Rht3* allele are those through which GA may increase cell length in the *rht3* wild-type. Leaves of the *rht3* wild-type treated with GA_3 were morphologically similar at the reduced temperature of 10 °C to L2 treated with GA_3 growing at 20 °C. However, in the *rht3* wild-type line grown at 10 °C, untreated control leaves and those treated with 2*S*,3*S* paclobutrazol resembled leaves of the *Rht3* mutant line.

No significant differences were observed in the maximum REGR achieved within the L2 extension zone of GA-treated or untreated control plants grown at 10 °C (Table 4.19 & Fig. 4.14). The REGR values were comparable to those found in the untreated *rht3* wild-type and *Rht3* mutant (*cf.* Table 4.16). However, the distance from the leaf base at which the maximum REGR was reached was significantly different between the treatments; 2*S*,3*S* paclobutrazol was the shortest followed by the control and finally GA_3 (Fig. 4.14). The same pattern of significant effects of the applied growth regulators on the length of the extension zone was found as at 20 °C (Table 4.10), GA_3 significantly increased the zone length while 2*S*,3*S* paclobutrazol significantly reduced it (Table 4.19). The critical result was that while the length of the extension zone was significantly reduced in the untreated control at 10 °C, treatment of the *rht3* wild-type with GA_3 and 2*S*,3*S* paclobutrazol did not affect the distal and proximal zone's length respectively (Fig. 4.15 & 4.19). Therefore, the application of both 2*S*,3*S* paclobutrazol and GA_3 seem to be able to override the effect of low temperature in the control of cell extension.

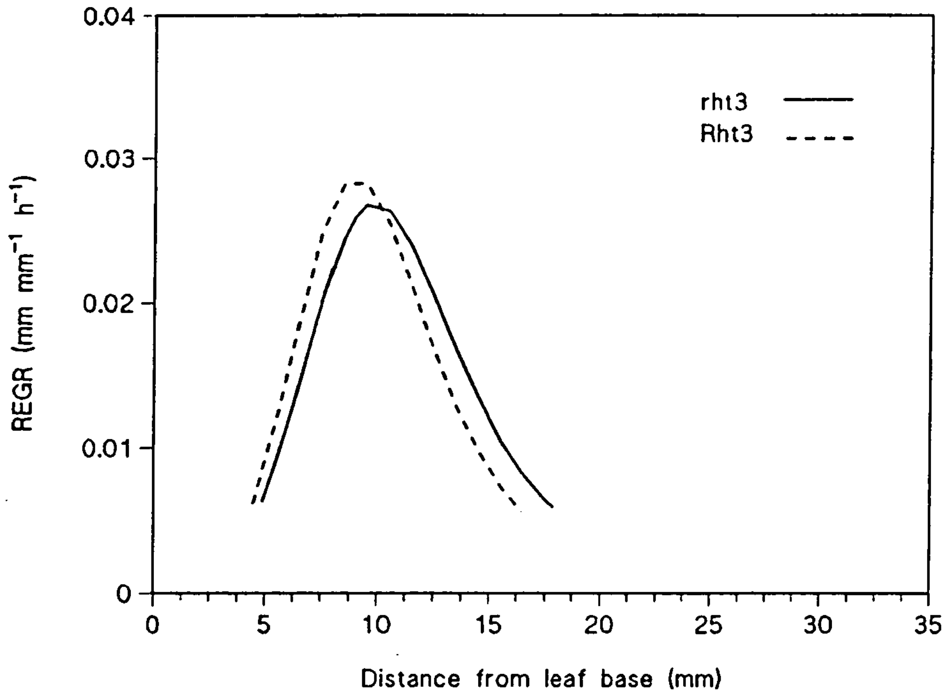


Fig. 4.13 Spatial distribution of growth, REGR ≥ 0.005 mm $mm^{-1}h^{-1}$, within the L2 extension zone of the *rht3* (wild-type) and the *Rht3* (mutant) lines grown at 10 °C and sampled around d 18, the period of maximum L2 extension at this temperature. REGR curves were smoothed using the equation 2 described in section 4.2.4.1.

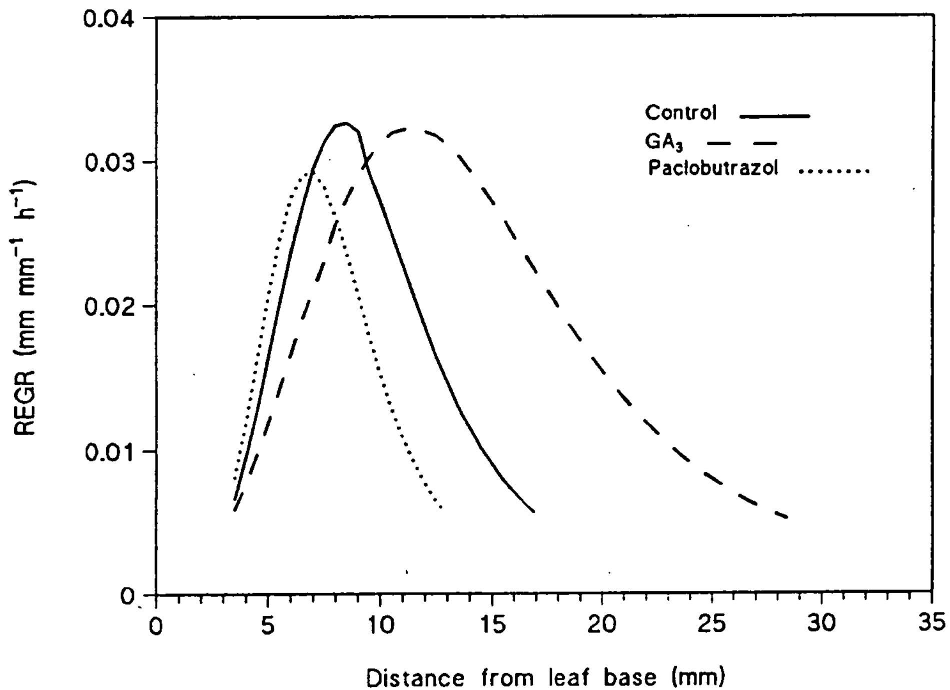


Fig. 4.14 Spatial distribution of growth, REGR within the L2 extension zone of the *rht3* (wild-type) line grown at 10 °C treated with 10 µM GA_3 and 10 µM 2*S*,3*S* paclobutrazol. Leaves were sampled around d 16 the period of maximum L2 extension. REGR curves were smoothed using equation 2 described in section 4.2.4.1

Table 4.19 *Effect of treatment with 10 μM GA_3 and paclobutrazol (Pac) on final L2 length (n=9), maximum REGR (± s.e) and position within the L2 that this is achieved (n=37) in the rht3 (wild-type) line grown at 10 °C and sampled around d 16. Length of extension zone calculated by summation of all of the 2 mm segments which extended over the 10 h growth period. Data of Fig. 4.14. Lower section of table shows the ratio of temperature effect on the measured parameters, data of Table 4.10 & 4.19.*

Treatment Temp (°C)	Maximum REGR ($mm\ mm^{-1}\ h^{-1}$)	Position of maximum REGR (mm)	Length of L2 extension zone (mm)	Final L2 length (mm)
Untreated	0.032 (0.0023)	9.30 (0.18)	14.97	143.0 (8.3)
GA_3	0.032 (0.0035)	10.34 (0.27)**	27.63***	281.9 (7.6)
Pac	0.029 (0.0015)	6.81 (0.15)**	10.22***	76.3 (3.9)
Ratio of temperature effect at 10 ° : 20 °C				
Untreated	0.39	1.00	0.68	0.63
GA_3	0.34	1.00	1.05	0.92
Pac	0.35	0.98	0.90	0.70

/* = difference significant at $P < 0.01/0.001$ compared to untreated control.

Although applied GA_3 reversed the effects of low temperature and 2*S*,3*S* paclobutrazol on final L2 length and the length of extension zone, it did not alter the maximum REGR achieved within the L2 extension zone compared to the untreated control (Table 4.19). The maximum REGR was reduced by *ca* 66 %, as shown by the ratios (Table 4.19), in all treatments compared to the same treatment at 20 °C.

As expected, the application of 2*S*,3*S* paclobutrazol at 10 °C had very little effect on the pattern of L2 growth observed in the *rht3* wild-type. The results suggest that GA_3 and 2*S*,3*S* paclobutrazol affect the size of the L2 extension zone independently of temperature.

Application of 2*S*,3*S* paclobutrazol to the *rht3* wild-type at 10 °C reduced L2 length more than the *Rht3* allele itself at 20 °C (Table 4.19 & 4.13). The length of L2 in the *rht3* wild-type treated with 2*S*,3*S* paclobutrazol at 10 °C was *ca* 35 % shorter than that with the same treatment at 20 °C. The reduction in L2 final length of *rht3* wild-type treated with 2*S*,3*S* paclobutrazol was more severe than the reduction in L2 length associated with the *Rht3* allele, at either temperature (Tables 4.2 & 4.19).

Applied GA_3 and 2*S*,3*S* paclobutrazol appear to determine the distal and proximal limits of the L2 extension zone in *rht3* wild-type grown at both 10 ° and 20 °C. The results suggest that the effects of temperature on the length of L2 extension zone length may be mediated through changes in the levels of endogenous GA.

4.3.3.5 Measurement of the concentration of endogenous GA

While working at Long Ashton Research Station concurrent work of a sandwich student examining GA status in extension zones of L2 grown under the same conditions as material for the developmental studies was in progress. I was, however, involved in the preparation of the leaf tissue and defining the length of the L2 segments which were used. This unpublished data (Table 4.20), (Richardson, Appleford & Lenton) is included to aid discussion of the relationship between GA status and responsiveness. Overall the results showed good agreement with the previously published data (Appleford & Lenton, 1991) from lower leaf segments of plants growing at lower light intensities .

The main feature from the present results is that the concentration of active GAs, GA_1 and GA_3 was the same in the L2 extension zone of the *rht3* wild-type plants grown at both 10 ° and 20 °C, despite a 57 % reduction in the rate of L2 elongation at 10 °C. However, the level of GA_1 was reduced by 58 % and the level of GA_{19} increased by 72 % in the *Rht3* mutant, despite no change in the rate of leaf elongation as the temperature was reduced from 20 ° to 10 °C. As shown previously, GA_1 and GA_3 accumulate in the extension zone of the GA-insensitive *Rht3* mutant (Appleford & Lenton, 1991; Table 4.20).

Table 4.20 *Concentration of GAs measured within the extension zone of L2 grown at 10 ° and 20 °C. Leaves sampled on d 9 grown at 20 °C and on d 18 grown at 10 °C. Characteristics of samples analysed are described in Table 4.1 (section 4.2.6).*

Genotype	Temp (°C)	Concentration of GA (ng g^{-1} fresh wt) GA_{19}	GA_{20}	GA_{29}	GA_1	GA_8	GA_3
rht3	20	7.1	1.0	0.4	2.1	12.1	0.9
rht3	10	9.2	0.4	0.4	2.0	8.8	0.9
Rht3	20	1.9	1.8	0.5	24.4	11.3	4.6
Rht3	10	6.7	0.8	0.3	10.3	11.6	3.1

(Unpublished results, presented with permission, of Richardson, Lenton and Appleford)

The results suggest that the reduction in maximum REGR in both the *rht3* wild-type and the *Rht3* mutant lines associated with growth at 10 °C, while they may be correlated with the concentration of GAs they do not appear to be directly related to it. Growth at 10 °C reduced the maximum REGR in the *rht3* wild-type while the concentration of GAs remained unchanged. The reduction in length of the extension zone of the *rht3* wild-type at 10 °C, compared to 20 °C, is clearly not due to a reduction either in the concentration or content of endogenous GA_1 or GA_3 (Table 4.20), yet it retains the capacity to respond fully to saturating doses of applied GA (Fig. 4.14). Further GA analysis, in conjunction with cell length data has

suggested that expressed on a cellular, (per cell), basis the concentration of active $GA_1 + GA_3$ in the *rht3* wild-type is higher within the L2 segment taken 1-2 cm from the leaf base at 20 ° compared to the same L2 segment at 10 °C. This could suggest that the cellular compartmentation of active GAs is an important factor in the mechanisms through which active GAs may regulate cell extension.

4.3.3.6 Conclusions

The effect of growth at 10 °C on L2 growth can be summarised;

1. The pattern of L2 growth was more similar between the *rht3* wild-type and *Rht3* mutant at 10 ° than at 20 °C.

2. Low temperature produced a mimic of the *Rht3* mutant line in the *rht3* wild-type, the maximum rate of L2 extension being more severely reduced in the *rht3* wild-type than in the *Rht3* mutant. Second leaves of the *rht3* wild-type were shorter, darker green, and resembled leaves of the *Rht3* mutant at both 10 ° and 20 °C.

3. The maximum REGR reached within the extension zone was the same in both genotypes, although it was reduced *ca* 65 % compared to growth at 20 °C. The position within the extension zone at which this was reached was similar both between the two lines and at both temperatures.

4. Total cell residence time within the extension zone was decreased in both genotypes compared to 20 °C. However, how the total time was partitioned did appear to be influenced by the *Rht3* allele, with relatively more time being spent in the region from maximum REGR to the outer limit of the extension zone.

5. Compared to growth at 20 °C, cell length was still significantly longer in the *rht3* wild-type than the *Rht3* mutant line. There was no obvious effect of low temperature on total cell number.

6. Application of 2*S*,3*S* paclobutrazol and GA_3 to the *rht3* wild-type had the same effect on L2 final length and the extension zone length as they did at 20 °C. However, final L2 length and the length of the extension zone in the untreated *rht3* control was significantly reduced compared to that at 20 °C.

7. The concentration of endogenous GA was the same in the *rht3* wild-type at 10 °C as at 20 °C despite a 27 % reduction in leaf length and a 67 % reduction in maximum REGR at 10 °C. In the *Rht3* mutant the concentration of GA_1 at 10 °C was halved compared to that measured at 20 °C but this was not correlated with any significant reduction in the rate of L2 growth.

4.4 DISCUSSION

4.4.1 Possible basis for the effect of the *Rht3* allele on the growth of L2

The results presented in this chapter have described how the *Rht3* allele, applied PGRs and low temperature can modify growth in the L2 model system. All treatments produced a common effect, to alter the length of the L2 extension zone (Fig. 4.15).

The extension zone is the only region of the leaf in which mean cell length increases (Beg & Wright, 1962; Boffey *et al.*, 1980; Kemp, 1980; Thomas, 1983a; Schnyder *et al.*, 1990). The zone is also the site of temperature perception, (Watts, 1974; Peacock, 1975; Thomas & Stoddart, 1984; Stoddart *et al.*, 1986) and a sink, (possibly also a source), for endogenous GAs (Simpson, 1958; Wheeler, 1973; Dale, 1976; Appleford & Lenton, 1991). It is therefore logical to conclude that the zone represents a likely target site within the leaf for factors, capable of influencing processes of L2 growth, such as the products of the *Rht3* allele, plant growth regulators and temperature.

The primary effect of the *Rht3* allele within L2 is to reduce the length of the extension zone. Hence, at the cellular level, the *Rht3* allele appears to restrict the potential of a cell for continued extension growth in the longitudinal axis of the leaf compared to an equivalent cell in the *rht3* wild-type.

4.4.2 Effects on cell numbers

In the present work the *Rht3* allele did not markedly reduce total epidermal cell numbers. This was in contrast to a study based on the *Rht1*, *Rht2* and *Rht3* alleles in both the Maris Huntsman and April Bearded cultivars (Hoogendoorn *et al.*, 1990). While a 15 % reduction in organ length associated with the *Rht1* and *Rht2* alleles was virtually all accounted for by a reduction in cell length, a 50 % reduction in organ length associated with the *Rht3* allele resulted from reduction in both cell number and cell length. A different study on near-isogenic lines carrying the *Rht1* and *Rht2* semi-dwarfing alleles showed that although there was a linear relationship between increasing *Rht* gene dosage and reduced epidermal cell length there was no obvious effect of the *Rht* alleles on cell number (Keyes *et al.*, 1989). It is possible that while the *Rht* alleles may always reduce cell length, their effect on cell number may be variable. The conditions in which the plants are grown may affect cell numbers more than the *Rht* alleles.

In the present experiments the *Rht3* allele had no effect on epidermal final cell number at either 10 ° or 20 °C. The reduction in L2 length associated with the mutant allele at 10 °C was, therefore, also related to a reduction in final cell length. Other studies in wheat have similarly found no effect (Dale, 1982), or very little effect of temperature (Friend & Pomeroy,

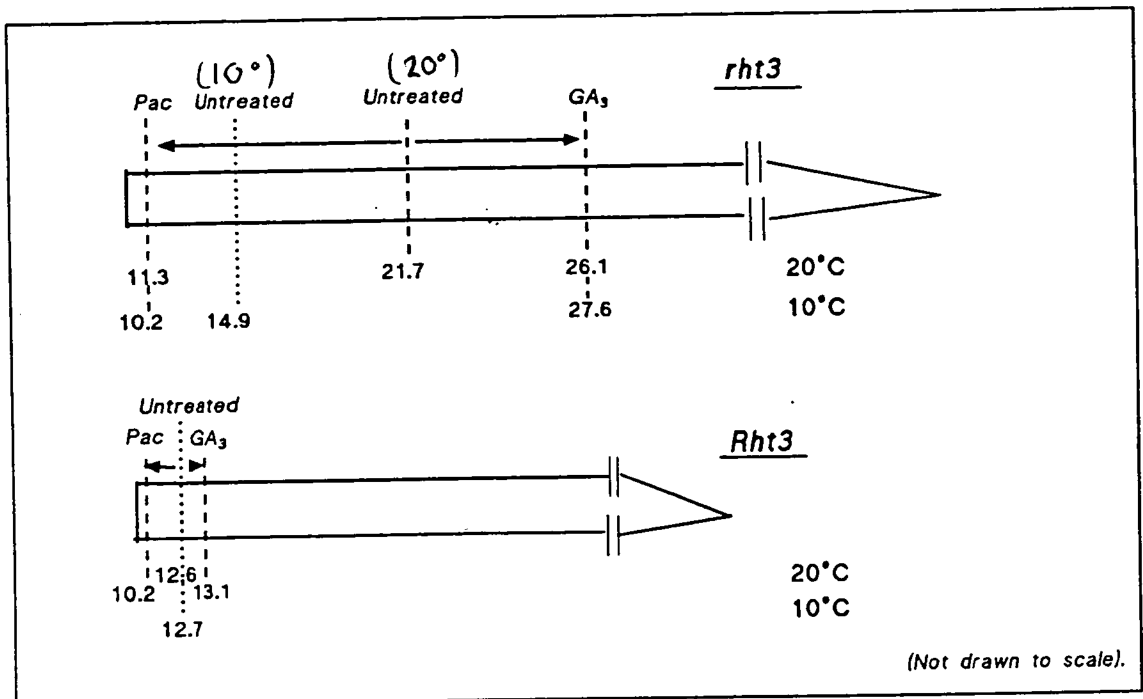


Fig. 4.15 Diagrammatic representation of the effect of applied plant growth regulators, 10 µM GA_3 or *2S,3S* paclobutrazol on the length of L2 extension zone (mm) in, the *rht3* (wild-type) and *Rht3* (mutant) lines sampled around d 9 at 20 °C and d 16 at 10 °C. The arrows represent the alteration in the length of the extension zone possible in response to applied growth regulators and temperature from untreated control position at 20 °C.

1970) on cell number.

4.4.2.1 Measurement of epidermal cell length as a means of calculating cell number

In the present study cell number was determined from measurements of both cell length and L2 length. However, the choice of a suitable cell type with which to compare the *rht3* and *Rht3* lines was more problematic than may have been anticipated. All cell length measurements were associated with high standard errors. Adjoining cells in the same file could be twice the length of each other. The existence of a very sharp meristem border could account for such large differences in cell length. One cell may have undergone an extra cell division as it left the meristem compared to the cell above it in the same file (Webster, 1980). High errors associated with epidermal cell measurements are not unusual. Beg & Wright (1962) found that cell length was so variable in leaves of *Phalaris arundinacea* that it was impossible to tell when growth had ceased.

Two different epidermal cell types were measured in the present study. This illustrated a further complicating factor, that the *Rht3* allele may act more severely and at a slightly different stage of L2 development on different cell types. The onset of rapid intercostal cell extension appeared to be delayed more in the *rht3* wild-type than in the *Rht3* mutant. The onset of rapid extension of these cells was also delayed compared to interstomatal cells on the same leaf surface (Fig 4.6 & Fig 4.10). However the genotypic effect of the *Rht3* allele in reducing final epidermal cell length compared to the wild-type was the same in both cell types. An additional problem was that intercostal cell lengths were *ca* 80 % longer than interstomatal cell lengths. The extreme length of intercostal cells, particularly when the *rht3* wild-type was treated with GA_3, made them difficult to measure accurately as they would extend beyond one field of view of the microscope. Hence, cell length measurements in section 4.3.2 and 4.3.3 of the results (the effect of applied growth regulators and low temperature) were made on the shorter interstomatal cell lengths. While neither the *Rht3* allele, 2*S*,3*S* paclobutrazol, nor GA_3 appeared to obviously reduce stomatal length or frequency, this did present a further complicating factor as final cell number was calculated by dividing final L2 length by the mean interstomatal cell length, thereby not accounting for the stomata themselves. However, the overall conclusion, that the *Rht3* allele significantly reduced cell length, while having no effect on cell numbers, in both intercostal and interstomatal cells was the same irrespective of whether the stomata themselves were accounted for.

In the present study the length of the basal meristem was estimated as that distance from the leaf base at which no further mitotic figures were detected. Because of the heterogeneous cell types in the leaf, the method may yield a meristem length which relates to the cell type which remains meristematic longest. In the wheat root epidermal cells remained meristematic longer than cortical cells (Hejnowicz, 1959). In leaves of *Phaseolus vulgaris*

cell extension stopped earlier in the palisade layer than the epidermis (Verbellan & De Greef, 1979).

However, in summary, all of the present results, based on epidermal cells, indicated the *Rht3* allele severely reduced cell length, while having no obvious effect on final cell numbers.

4.4.2.2 Effects of growth regulators, the *Rht3* allele and low temperature on final cell number

In the present work, the *Rht3* allele, applied 2*S*,3*S* paclobutrazol and growth at 10 °C all reduced final cell length compared to the *rht3* wild-type at 20 °C. Application of 2*S*,3*S* paclobutrazol however, did have a small, but not significant, effect in reducing epidermal cell numbers in both genotypes. Such an effect of paclobutrazol has been reported previously. 2*S*,3*S* paclobutrazol inhibited cell division in celery cell culture (Haughan *et al.*, 1988). Paolillo *et al.* (1991) also suggested that ancymidol, a GA-biosynthesis inhibitor with the same site of action as paclobutrazol (Hedden & Graebe, 1985), may inhibit cell division in isogenic lines of wheat, unlike the *Rht* alleles, which had no effect on cell division.

Application of GA_3 did not affect total cell numbers, in the *rht3* wild-type at either temperature, or in the *Rht3* mutant at 20 °C. However, while most reports agree that applied GA promotes cell elongation, its effect on cell division is different in different systems; cell division was prematurely halted following application of GA_3 to the intercalary meristem of *Avena* internodes which reduced cell numbers in the pith and epidermis (Kaufman, 1965). On the other hand, applied GA_3 stimulated cell division in the sub-apical meristem of *Hyocyamus niger* and *Samolus parviflorum* (Sachs *et al.*, 1959) and had no effect on cell division in wheat coleoptiles (Rose & Adamson, 1969) or lettuce hypocotyls (Jones & Moll, 1983).

4.4.3 Control of the length of the extension zone

4.4.3.1 Measurement of the length of the extension zone

Leaf elongation in grasses is confined to an extension zone, which is always enclosed by older encircling leaf sheaths (Esau, 1977; Dale, 1988; Schnyder *et al.*, 1988). Any assessment of the pattern of growth within the extension zone therefore requires the use of techniques which damage the leaf (Schnyder *et al.*, 1990). Either marking leaves with ink (Volnec & Nelson, 1981), or piercing holes (Davidson & Milthorpe, 1966; Kemp, 1980; Schnyder *et al.*, 1987), reduces the rate of leaf elongation. In the present experiments, piercing of growing wheat leaves reduced the leaf elongation rate, in both genotypes, by *ca* 25-30 %. In *Festuca* the combined effect of piercing, at 3 mm intervals, and handling, reduced the leaf elongation rate by 24-41 % (Schnyder *et al.*, 1987). However, piercing

reduced the absolute rates of elongation similarly, in all of the 3 mm segments, throughout the growing zone. Therefore, piercing holes within the extension zone did not affect the spatial distribution of growth, or the calculated distance over which any cell elongation occurred.

In the present work the length of the L2 extension zone at 20 °C, was 19.9 and 12.7 mm in the *rht3* wild-type and *Rht3* mutant lines respectively. The extension zone of the first leaf in wheat was estimated to be *ca* 30 mm but this was based on mesophyll cell length measurements (Ellis *et al.*, 1983).

The length of the extension zone in near-isogenic lines developed at 0, 2 or 4 levels of dwarfing genes was 19.6, 18.4 and 16.5 mm respectively (Paolillo *et al.*, 1991). Using widely spaced needle holes the length of the extension zone in leaves of *Dactylis* was *ca* 35 mm (Davidson & Milthorpe, 1966). While it may be expected that different species would exhibit extension zones of varying length, accurate measurements of the length, and distribution of growth within the extension zone are difficult to obtain. In the present study the length of the L2 extension zone also appeared to be related to the level of irradiance in which the wheat seedlings were grown. Higher levels of irradiance were associated with an increase in the length of the L2 extension zone in both genotypes, but to a greater degree in the *rht* wild-type line.

A problem with marking techniques is that a compromise has to be made between (i) using a sufficiently long growth period after marking for holes to move apart a measurable distance from each other and (ii) using as short a growth period as possible so that the zone's length is not overestimated because holes made in the distal region of the zone become displaced upwards out of the zone, by the extension of cells below them in the same file (Silk, 1984). Such a problem was illustrated by the 40 % variation in the estimated extension zone length in leaves of wheat when holes were pierced only every 10 mm and leaves then left for a 24 h growth period (Kemp, 1980). In the present work, holes were pierced at 2 mm intervals as a compromise distance between minimising wounding effects and making accurate measurements of subsequent hole separation. In addition, seedlings were left only for a 5 or 10 h growth period at 20 ° or 10 °C respectively. A growth period less than 5 h meant that holes made in the leaf would not separate very far from each other, which would have had a disproportionally large effect on the accuracy of hole displacement measurements. However, a short growth period made the position of the distal boundary of the extension zone difficult to locate, particularly in the slower growing *Rht3* mutant line, or *rht3* plants which had been treated with 2*S*,3*S* paclobutrazol. Despite such considerations similar values for the length of the L2 extension zone were found consistently in repeated experiments. Thus, the estimated lengths of the zone in both genotypes, treated and untreated, were probably representative of their true values.

4.4.3.2 Influence of plant growth regulators on the length of the extension zone in the *rht3* wild-type line

The present work has suggested that changes in the level of endogenous GA may regulate the length of the extension zone in the *rht3* wild-type line. Thus, the insensitivity to GA of the *Rht3* mutant line may explain why the length of the extension zone is reduced compared to that in the *rht3* wild-type line.

At both 10° and 20 °C, compared to the untreated control, application of 2*S*,3*S* paclobutrazol and GA_3 significantly decreased, and increased respectively, the length of the extension zone in the *rht3* wild-type. Neither treatment caused any significant alteration in the length of the extension zone of the *Rht3* mutant line (Fig. 4.15).

The similarity in the length of the extension zone in the untreated *Rht3* mutant and the *rht3* wild-type treated with 2*S*,3*S* paclobutrazol suggests that the *Rht3* allele, and the GA biosynthesis inhibitor may set the same proximal limit to the zone's length. Thus, the high levels of GA_1 which are present in the *Rht3* line cannot be utilized to increase the length of the extension zone. In the *rht3* wild-type, applied GA_3 can set a distal limit to the extension zone significantly above than that of the untreated control (Fig. 4.15), suggesting that in the *rht3* wild-type endogenous GA does not completely saturate the mechanism through which the extension zone length is controlled. In the present work, it was assumed that the applied PGRs did however, saturate the mechanism through which the length of the zone is controlled and that the maximum and minimum possible length respectively of the extension zone at both 10 ° and 20 °C in the *rht3* wild-type, was *ca* 27 mm set by GA_3 and *ca* 10 mm set by 2*S*,3*S* paclobutrazol. Reducing the concentration of applied GA_3 and 2*S*,3*S* paclobutrazol from 10 to 5 μM, produced no equivalent reduction in the length of the extension zone in the *rht3* wild-type at 20 ° or 20 °C.

The results are consistent with the suggestion that the extension zone length may be related to tissue sensitivity to GA. Such an idea has recently been proposed in a study based on isogenic lines carrying the *Rht1* and *Rht2* semi-dwarfing alleles and their growth response to applied GA_3 and the GA-biosynthesis inhibitor, ancymidol (Paolillo *et al.*, 1991). Application of GA_3 increased, and ancymidol decreased, the length of the extension zone in GA-sensitive lines. Increasing *Rht* dosage was linearly related to an increasing unresponsiveness to the applied PGRs and the possible variation in the extension zones' length.

4.4.3.3 Influence of low temperature on the growth of the L2 extension zone

The primary effect of a reduction in the growing temperature from 20 ° to 10 °C was a 65 % reduction in the maximum value of REGR in the extension zone of both genotypes. In the *rht3* wild-type this was also associated with a reduction in the length of the extension zone, which could be completely reversed by applied GA_3, which restored the extension zone

length to the same length as it was at 20 °C when also treated with GA_3. Thus, the absolute responsiveness of the extension zone to applied GA_3 was greater at 10 ° than at 20 °C. There was very little effect on the length of the extension zone in the *Rht3* mutant (Fig. 4.15).

The present results are consistent with the suggestion that GA may be a more effective regulator of low than high temperature growth (Stoddart & Lloyd, 1986; Pinthus *et al.*, 1989; Appleford & Lenton, 1991) and that the *Rht3* allele may be considered as conferring high temperature insensitivity as well as GA-insensitivity. The *slender* mutant of barley represents the opposite extreme to the *Rht3* mutant and is an overgrowth mutant in which there is constitutive expression of GA-regulated genes (Chandler, 1988; Lanahan & Ho, 1988), so that tissues appear to be saturated in GAs and do not respond to 2S,3S paclobutrazol (Croker *et al.*, 1990). The *slender* mutant also has an altered temperature/growth relationship (Stoddart & Lloyd, 1986; Pollock *et al.*, 1992); while the growth in the wild-type stopped around 5 °C, as in other grasses (Stoddart, 1986), *slender* continued to grow down to -5 °C at which point damage occurred to the extension zone (Stoddart & Lloyd, 1986).

4.4.4 Extent of meristem activity

At both 20 ° and 10 °C the *Rht3* allele reduced the length of the L2 basal meristem (Tables 4.3 & 4.15). Although not directly measured in the present work, applied GA_3 and 2*S*,3*S* paclobutrazol also appeared to affect the length of the meristem. Calculation of cell residence times within both the meristem and extension zone of L2 suggested that compared to the untreated control, the onset of cell extension (*cf.* Fig. 2.1, Chapter 2) was delayed when L2 was treated with GA_3 and was earlier when treated with 2*S*,3*S* paclobutrazol (Fig. 4.10b). This would be consistent with GA_3 increasing, and 2*S*,3*S* paclobutrazol decreasing, meristem length in the wheat leaf as they do in the *gib-1* tomato mutant (Barlow *et al.*, 1991). The present results may also support the involvement of GA in the maintenance of intercalary meristem activity as suggested by Phinney (1984), and Metraux (1988).

In order to account for final cell number being the same in all treatments, while the length of the basal meristem is altered, by the *Rht3* allele, growth at 10 °C and perhaps by the application of PGRs, it seems necessary to assume that the duration of the cell cycle may be affected. Thus, in treatments in which the length of the meristem is reduced, cell residence times within the meristem may be less and this implies that the cell cycle may be shorter in these cases. There was no evidence to suggest that meristematic activity continued longer in leaf ontogeny in the *Rht3* mutant than in the *rht3* wild-type line. Although detailed measurements of the duration of cell cycle were not made in the present work this would be an interesting area for future research.

In summary, the *Rht3* allele, applied PGRs and low temperature all principally affected the process of cell extension and not cell division. The mechanisms through which

this eventual effect on cell length is achieved are however unclear, and may involve effects of the treatments on both the length of the L2 basal meristem and the duration of the cell cycle.

4.4.5 Final conclusions

Effects initiated in L2 of the *rht3* wild-type at 20 °C by the application of growth regulators and growth at 10 °C can be compared with the effects of the *Rht3* allele on L2 growth (Table 4.22). Thus, L2 of the *rht3* wild-type treated with 2*S*,3*S* paclobutrazol or grown at 10 °C shows many characteristics similar to those of L2 in the *Rht3* mutant. In addition, growing the *rht3* wild-type at 10 °C may produce a more faithful phenocopy of the *Rht3* allele than that produced by inhibiting GA-biosynthesis using 2*S*,3*S* paclobutrazol. The effect of the *Rht3* allele may be therefore more complex than simply blocking GA biosynthesis.

Table 4.22 *Characteristics of L2 growth and development which are affected relative to the untreated rht3 (wild-type) at 20 °C by: the Rht3 (mutant) allele, growth at low temperature, or the application of growth regulators, pac = (2S,3S paclobutrazol). (+) = affected, (0) = unaffected (?) = unknown, not measured.*

L2 growth characteristic	Treatment which may affect growth				
		rht3			
	Rht3	10 °C	GA_3	Pac	GA_3 + Pac
Phenotype	+	+	+	+	+
Final L2 length	+	+	+	+	+
Absolute growth rate	+	+	+	+	+
Cell length	+	+	+	+	+
Cell width	±	±	+	+	+
Cell number	0	0	0	±	0
Duration of L2 growth	0	+	+	+	+
Length of meristem	+	+	?	?	?
Extension zone length	+	+	+	+	+
Maximum REGR	0	+	0	0	0
Position of max. REGR	0	±	+	+	+
Cell elongation rate	+	+	+	+	?
Total residence time[a]	±	+	+	+	+
Specific residence time[b]	+	0	0	+	0
Specific dry weight[c]	0	0	+	+	+
Endogenous GA[d]	+	0	?	+	?

[a] = the length of time a cell spends in both regions of the extension zone, [b] = the length of time a cell spends travelling from the position of max REGR to the outer edge of the extension zone, [c] = dry weight of L2 expressed g mm^{-2}, [d] = level of active GA_1

The overall effect of the *Rht3* allele appears to be one which involves prematurely ageing the cell and reducing the final length that a cell can achieve. It is further suggested that GA is involved in the maintenance of cell extension and that the effects of the *Rht3* allele on the growth of L2 are a direct consequence of its insensitivity to endogenous GA, so that GA-controlled cell extension does not occur.

The next logical step in the elucidation of the mechanism of action of the *Rht3* allele would be an investigation of the biophysical parameters which control the process of cell extension in the two different genotypes.

CHAPTER 5

THE BIOPHYSICAL CONTROL OF CELL EXTENSION

5.1 INTRODUCTION

In wheat, the effect of the *Rht3* allele is to reduce final organ length of stem internodes, leaves and coleoptile compared to the *rht3* wild-type line (Gale & Youssefian, 1985; Lenton *et al.*, 1987). The reduced organ length associated with the *Rht3* allele appears to be manifest principally through a reduction in epidermal cell length and to a much lesser extent through a reduction in cell number (Chapter 4; Nilson *et al.*, 1957; Stoddart, 1984; Keyes, *et al.*, 1989). The reduction in cell length resulted mainly from a reduced rate of cell extension occurring earlier in the *Rht3* mutant than the *rht3* line (*cf.* Fig. 4.5). This would be consistent with *Rht3* allele promoting processes which stop a cell growing. The work described in this chapter seeks to compare factors thought to control cell extension in the *rht3* wild-type and *Rht3* mutant lines. The proposed hypothesis which was to be tested in this chapter was that the product(s) of the *Rht3* allele may specifically affect factor(s) influential in controlling cell extension. The second leaf (L2) system as described in Chapter 4, has been used again as a model system to investigate the effects of the *Rht3* allele. The biophysical parameters which are considered to be important in the control of cell expansion have been included in equations first described by Lockhart (1965).

5.1.1 Equations describing the process of cell extension

The expansion of a plant cell can be regarded as a physical process in which the uptake of water and the extensibility of the cell wall lead to an irreversible increase in cell volume (Cleland, 1971; Preston, 1974; Tomos, 1985; Ray, 1987; Fry, 1988).

Lockhart (1965) summarized a growth equation which has recently been discussed in detail by Cosgrove (1986), in which the steady-state cell expansion is achieved when the rate of water uptake (water transport) equals the rate of wall yield (metabolically controlled) hence the rate of increase of cell volume (dV/dt) can be expressed by equation 1 (Ray *et al.*, 1972; Cosgrove, 1986; Barlow, 1986):

$$1/V.\, dV/dt = m\, L_p/(m + L_p).\, (\sigma\, \pi_{i\text{-}e} - P) \qquad \textit{eqn (1).}$$

where $1/V. dv/dt$ = instantaneous rate of increase in cell volume ($cm^3 s^{-1}$), L_p = hydraulic conductivity ($MPa^{-1} h^{-1}$), V = cell volume (cm^3), σ = reflection coefficient (dimensionless), π_{i-e} = difference in osmotic pressure between the cell and the external medium (MPa) and P = turgor pressure (MPa).

However, if water transport does not limit growth as has been found in excised hypocotyl and coleoptile segments (Taiz, 1984) then equation 1 can be simplified to equation 2 (Ray *et al.*, 1972; Tyree & Jarvis, 1982; Barlow, 1986). The model described by equation 2 considers the rheological properties of the cell wall and describes a linear relationship between irreversible cell extension and cell turgor (P), once a minimum turgor or yield threshold (Y) has been exceeded:

$$1/V.dv/dt = m (P - Y) \qquad \textit{eqn (2)}$$

where dv/dt = instantaneous rate of increase in cell volume ($cm^3 s^{-1}$), m = wall extensibility ($MPa^{-1} h^{-1}$), P = turgor pressure within the cell (MPa) and Y = the yield threshold (MPa).

Thus, one or more of the biophysical parameters described in eqn (2) must be affected whenever the rate of cell expansion changes.

The total wall extensibility, m, is often considered to incorporate two components (Cleland, 1958 & 1971): the elastic extensibility (E_{ex}), which is reversible and the plastic extensibility (P_{ex}), which is irreversible. The latter has therefore been considered to represent the process of cell extension or growth and has been termed the viscoelastic property of the cell wall. P_{ex} is equivalent to the irreversible plastic extension which was correlated with the auxin-stimulated growth rates described by Heyn (1933).

The relationship described in eqn 2 has been used successfully to study the control of growth in several plant species: *Nitella*, (Green *et al.*, 1971), *Avena*, (Cleland, 1959), *Pisum* (Greacon & Oh, 1972), soybean (Bunce, 1977) and wheat roots (Pritchard *et al.*, 1990).

The entry of water into the cell is a function of the water potential and the relative resistance to water movement defined by L_p, the hydraulic conductivity (Tomos, 1985). Water moves into the cell down the water potential gradient. In growth it is the wall loosening which provides the lowering of the water potential and therefore the influx of water (Tomos, 1985). The biophysical parameters described in eqn (2) are in practice difficult to measure in the intact plant. A major problem in leaves of the *Gramineae* is the unknown relative importance of the different pathways of water movement, apoplastic or symplastic, within plant tissues (Molz & Ferrier, 1982; Boyer, 1985; Dale, 1988; Tomos & Wyn Jones, 1988).

For the purposes of the work to be presented in this chapter it has been assumed that

the hydraulic conductivity of tissue within the extension zone of L2 is not rate limiting. Thus, the rate of cell extension within the leaf tissue can be adequately defined by the biophysical parameters included in equation 2 (Ray *et al.*, 1972; Tyree & Jarvis, 1982).

5.1.1.1 Mechanism of cell expansion

Cosgrove (1987) proposed a series of events to describe the process of cell extension: (1) a load bearing bond within the cell wall is broken, (2) the plastic elements present within the wall extend causing a reduction in turgor pressure, (3) a water potential gradient is set up and water enters the cell via osmosis to re-establish the equilibrium and (4) this is accompanied by an influx (or synthesis) of new solutes to compensate for the dilution effects of the water entering the protoplast. The protoplast's osmotic pressure is maintained by biosynthesis of cell metabolites and by the active uptake of solutes. Thus, continued energy for wall loosening and turgor homoeostasis is dependent upon the metabolic activity of the protoplast to provide the energy required to drive cell expansion (Ray & Reusnik, 1962; Fry, 1988).

The model described by Cosgrove (1987) presents several points at which the process of cell extension could potentially be controlled by the plant: (i) the initial wall loosening event itself, (ii) the supply of water to enter the expanding cell and (iii) the movement and nature of solutes that enter the cell (Tomos, 1985).

To permit continued cell extension, the ability of the wall to extend has to be continually restored. The process has been termed wall loosening and must integrate the breaking of load-bearing bonds and the intussusception of new wall material (Cleland, 1971).

The process requires a stimulus, termed wall loosening factor (WLF)) to initiate the wall loosening event. Protons (H^+) (Rayle & Cleland, 1980) and calcium ions (Ca^{2+}) (Cleland, 1986) have been proposed to fulfil this role. Whether factors such as H^+ or Ca^{2+} ions stimulate wall loosening directly, or whether their effect is mediated through a signal transduction pathway involving factors such as an increase in enzyme activity is uncertain and, at present, difficult to prove (Taiz, 1984).

5.1.1.2 Measurement of the parameters described in the Lockhart equation

The interpretation of parameters described within the Lockhart equation (2) is subject to certain constraints. The parameters do not always act independently. The effective turgor, (P-Y), has been found in several systems to be metabolically controlled (Green et al., 1971; Bunce, 1977; Tyree & Jarvis, 1982; Davies & Van Volkenburgh, 1983; Tomos *et al.*, 1989; Okamoto *et al.*, 1990). In wheat, excision of the root was itself found to affect subsequent properties of the cell wall (Pritchard *et al.*, 1988). Additionally, measurements of the parameters have often been taken from isolated tissue segments rather than from intact plants. It is debatable how representative or accurate such results, made on isolated tissue segments,

may be in describing the control of cell expansion which may occur within the intact plant.

The cell wall extensibility in the work to be described has been measured with an adaptation of the Instron technique (Cleland, 1967; Van Volkenburgh *et al.*, 1983). The basic principle of operation is to apply a constant rate of increase in strain to the sample while the induced stress is measured simultaneously. However, the major limitation of such an approach is that stress is induced in the sample only in a uni-axial direction. *In vivo*, stress on the cell wall is produced multi-axially by the turgor pressure (Tomos, 1985). Kamiya *et al.*, (1963) found that uni-axially applied stress resulted in 3 to 4-fold more extension than the equivalent stress induced as a result of turgor pressure. Furthermore, the tissue segment is often mechanically conditioned, by boiling in methanol for several minutes to remove the effect of internal cell stresses and the activity of inhibitory enzyme activity (Cleland, 1967). In the work to be described in this chapter, measurements have been made on both live and conditioned L2 material.

The turgor pressure term described in equation (2) of the Lockhart equation, is difficult to measure directly unless a pressure probe is used, as first described by Green (1968) to measure turgor pressure in *Nitella* cells. Unfortunately, such equipment was not available for the present study. Instead, the osmotic pressure was measured; this depends on the total solutes within the cell sap (Lawlor & Leech, 1985). In practice this can be a good approximation of the turgor pressure which exists within the cell (Green, 1968). However, the turgor pressure can be estimated indirectly if the water potential (Ψ) is known using the relationship described in equation 3.

$$\Psi = \pi - P \qquad \textit{eqn (3)}$$

Where; Ψ = water potential (MPa); P = turgor pressure (MPa) and π = osmotic pressure (MPa).

Measurement of P_{ex}, one of the two rheological properties of the cell wall, P_{ex} and Y (Tomos *et al.*, 1984) has been described above. However, Y, the threshold, or critical turgor below which cell wall extension ceases, is in practice also difficult to measure (Taiz, 1984). In *Nitella*, Y was shown to be subject to complex metabolic control (Green *et al.*, 1971). In addition, Y may vary in response to illumination (Taylor & Davies, 1986) or tissue age (Tyree & Jarvis, 1982; Pritchard *et al.*, 1991).

The idea for the present work was to measure the π, m and Y parameters of the Lockhart equation (eqn 2), and to compare how cell enlargement was controlled, both between the two genotypes and under different conditions.

5.1.2 Effect of low temperature on cell extension

The effect of the *Rht3* allele is expressed less efficiently when the mutant and wild-type lines are grown at low temperature (Chapter 3, Stoddart & Lloyd, 1986; Pinthus *et*

al., 1989; Appleford & Lenton, 1991). By investigating the effect of low temperature growth on the biophysical parameters considered to regulate cell extension, possible indicators of the mode of action of the *Rht3* allele may be identified.

Maintenance of sufficient turgor to drive cell extension at the low temperature does not appear to be a major factor limiting the reduced rate of growth associated with low temperature (Woodward & Friend, 1988; Thomas *et al.*, 1989). The available evidence suggests that the major growth-limiting factor at low temperature is the rheological properties of the cell wall, incorporated in m, described in equation 2 (Thomas *et al.*, 1989; Pritchard *et al.*, 1990). However, due to the complexity of the living plant system there is very little direct evidence to link a reduction in cell wall plasticity with the lower temperature (Pollock & Eagles, 1988).

5.1.3 Effect of plant growth regulators on cell extension

In the majority of plant systems examined the effect of plant growth regulators (PGRs) is to affect predominantly the extensibility of the cell wall (Cleland, 1986).

Cell wall extensibility (m) is always increased in response to auxin treatment (Cleland, 1967; Yamamoto *et al.*, 1970; Cosgrove, 1985; Kutschera & Schopfer, 1986). Applied gibberellin (Adam *et al.*, 1975; Keyes *et al.*, 1989) and cytokinin (Thomas *et al.*, 1981; Brock & Cleland, 1985), have also been shown to increase m. Work based on the *Rht1* and *Rht2* semi-dwarfing alleles in wheat, showed that the application of GA_3 initiated a significant increase in P_{ex} in the *rht* wild-type lines, while having no effect on the properties of the wall in the *Rht1* and *Rht2* lines (Keyes, 1987; Keyes *et al.*, 1989). Conversely, when an inhibitor of GA biosynthesis, ancymidol, was applied to the wild-type, P_{ex} was reduced. There was no significant effect of either growth regulator in the mutant genotypes (Keyes, 1987; Keyes *et al.*, 1990). Application of abscisic acid (ABA) was found to have the opposite effect and reduce P_{ex} in bean leaves (Van Volkenburgh & Davies, 1983a), and maize coleoptiles (Kutschera & Schopfer 1986).

The maintenance of cell turgor was found to be unaffected by treatment with PGRs in several different systems (Cosgrove & Cleland, 1983b; Stuart & Jones, 1977; Zack & Loy, 1984).

However, the effect of PGRs on cell turgor is not clearly defined. If PGRs increased turgor without increasing m or Y an increase in the growth rate would occur. An alternative situation could be that PGR's may promote the uptake of solutes to allow continued cell extension. It has been found difficult to establish whether solute uptake is a direct or indirect effect of the PGR application (Cleland, 1986).

The investigation of factors thought to control the process of cell extension is one in which measurements are derived from a complex, interactive system. Therefore, measuring

individually the factors described in the Lockhart (eqn 2), when in the intact leaf they may interact, means that the effect of one factor alone cannot be accurately described as controlling cell extension.

5.1.4 The effect of the *Rht3* allele on cell extension

The objectives of the work to be described in this chapter are;

1. to identify which of the biophysical parameters considered to govern the rate of cell extension is specifically controlled by the *Rht3* allele.

2. to establish whether the application of plant growth regulators and/or low temperature modifies the relationship between the effect of the *Rht3* allele and the parameters assumed to control the rate of cell extension.

5.2 MATERIALS AND METHODS

5.2.1 Growth conditions

Plants used in the Edinburgh extensiometer and for the wall yield threshold were grown under condition N° 1 (Chapter 2, Table 2.1). Material used in the Aberystwyth extensiometer was grown in conditions N° 4 at 20 °C and N° 5 at 10 °C (Table 2.1). Material used to measure osmotic pressures was grown in conditions N° 2 at 20 °C and N° 3 at 10 °C (Table 2.1).

5.2.2 Measurement of the plastic extensibility (P_{ex}) of the cell wall

Two different extensiometers were used to measure P_{ex} within basal segments of L2. The extensiometers differed in several respects: (1) their detailed design, (2) the experimental procedure adopted to calculate the value of P_{ex}, (3) the condition of the tissue sample stretched in the device and (4), the length of the tissue sample which was positioned between the upper and lower clamp (see below). However, both extensiometers were constructed as described Van Volkenburgh *et al.*, (1983), and employed the same principle of applying a steadily increasing rate of strain to the sample while simultaneously measuring the stress induced. For convenience, the two extensiometers have been referred to according to their geographical location, Edinburgh or Aberystwyth.

5.2.2.1 Sampling procedure for tissue in determination of cell wall extensibility

Plant material grown at 20 °C was sampled 9 d after sowing. The rolled-up L2 was carefully dissected out from the first leaf sheath as described in section 2.3 Chapter 2. A 1 cm long basal segment of L2 was cut from a point 2 mm above the shoot/grain junction using a sharp single-edged razor blade. Material growing at 10 °C was collected in the same way, except that it was sampled 16 d after sowing.

5.2.2.2 Edinburgh extensiometer

The Edinburgh extensiometer was the instrument described by Milligan (1986). Only previously conditioned L2 material grown at 20 °C was measured in this device. The extensiometer had a fixed distance of 5 mm between the upper and lower clamps into which the L2 segment was positioned. A force equivalent to a 12 g load was applied to the L2 segment by moving the lower clamp downwards at a rate of 1.5 mm min^{-1}. The corresponding increase in length of the segment and the stress induced was simultaneously plotted on an xy-axis chart recorder. The load was then released, by reversing the motor, which returned the lower clamp and the segment length to their original positions. The stretching procedure was then repeated on the same L2 segment. The resulting load extension curve was again recorded on an xy-axis chart recorder. P_{ex} was determined by calculating the

difference in the extensibility between the two separate stretches. A tangent drawn to the curve on the first stretch was equivalent to the total extensibility ($P_{ex} + E_{ex}$). A tangent drawn to the extension curve on the 2nd stretch was equivalent to E_{ex} alone. Therefore, the value of P_{ex} was determined by calculating the difference in the extensibility shown by the two curves. The values were modified by correction factors (see below) and P_{ex} was expressed as % extensibility per 10g applied load.

5.2.2.3 Conditioning of tissue segments prior to use in the extensiometer

All tissue was conditioned by boiling in MeOH for 2 min (Cleland, 1967) prior to stretching in the Edinburgh extensiometer. Segments conditioned in this way could be stored in cold MeOH for a period of up to 3 months prior to use. L2 segments were rehydrated immediately prior to stretching by wetting them with distilled water after they had been inserted between the upper and lower clamp of the extensiometer.

5.2.2.4 Aberystwyth extensiometer

The Aberystwyth extensiometer was the instrument described by Stoddart *et al.*, (1986), except that in the present experiments a cooling collar was not fitted to the tissue sample. P_{ex} was measured in live L2 material grown at either 10 °C or 20 °C. Unlike the Edinburgh device, the Aberystwyth extensiometer permitted longer tissue segments to be inserted between the upper and lower clamps. Therefore, in addition to a 1 cm segment, as used in the Edinburgh extensiometer, a segment containing the entire L2 extension zone was stretched; at 20 °C this was 30 and 20 mm in the *rht3* wild-type and *Rht3* mutant lines respectively and at 10 °C this was 15 mm in both genotypes.

Second leaf (L2) tissue was dissected out from the encircling first leaf and sheath as described in section 2.3 Chapter 2 and used immediately. The leaf sample, still rolled up as in the plant, was attached to the upper and lower clamps by the application of a small drop of Superglue at either end of the segment. The glue was allowed to dry for a period of *ca* 3 min prior to the segment being stretched. A total load of 10g was applied to the sample in a single load/relaxation cycle. The induced stress was recorded on an xy-axis chart recorder. The area under the hysteretic curve drawn on the recorder corresponded to the energy lost as heat during the work cycle and was an indication of the plasticity of the cell walls. The xy plotter was first calibrated in both the x and y directions (Fig. 5.1a & b). Therefore, an increase in length, or linear variable displacement transducer (LVDT) displacement of 0.028 mm in the sample between the upper and lower clamps was equivalent to a deflection of 1 mm along the x axis (Fig 5.1a) and a displacement of 78 mm along the y axis was equivalent to the application of a 10 g load to the sample between the upper and lower clamps (Fig 5.1b). P_{ex} was determined by calculating the irreversible increase in segment length on the x-axis which resulted from the application of a 10 g equivalent force load to the leaf sample (Fig. 5.2) and

the corrected value (*cf.* 5.2.2.5) was finally expressed as % extensibility per 10g applied load.

5.2.2.5 Correction factors for the plastic extensibility

In order to compare L2 segments of equivalent cross-sectional area and dry mass between the two different genotypes. Thus, values of P_{ex} in both extensiometers were subsequently modified by the application of correction factors as described by Cleland (1967) (Table 5.1).

The radius of the rolled up L2 segments was measured to the nearest 0.1 mm using a dissecting light microscope fitted with an eye-piece graticule. The cut surface of a 1 mm section was viewed from above. The cross-sectional area was determined as πr^2. The dry weight of tissue segments was recorded after they had been stretched in the extensiometer. Segments were placed in a drying oven and maintained at 80 °C until they remained a constant weight. The correction factors, shown in Table 5.1, were applied by multiplying the value of P_{ex} per 10g applied load by the reciprocal of both the dry weight and the surface area.

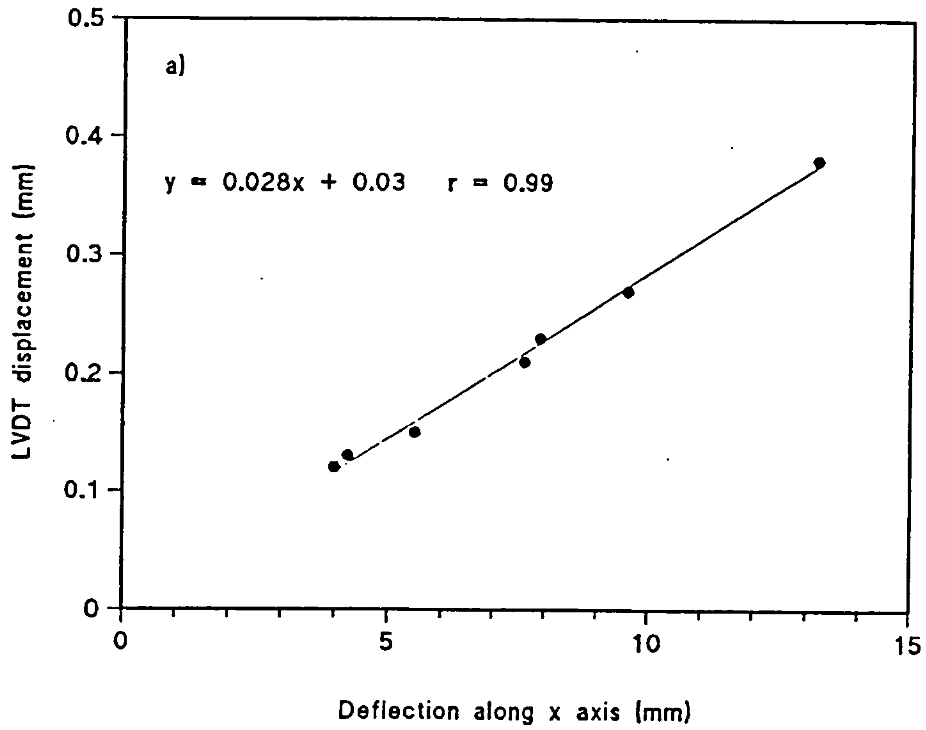
Table 5.1 *Correction factors (± s.e.) of L2 segment sectional surface area and dry weight used to calculate P_{ex} in the rht3 (wild-type) and Rht3 (mutant) at 10 ° and 20 °C in both the Edinburgh and Aberystwyth extensiometers (n=9).*

Temp (° C)	Correction factor	*rht3*	*Rht3*
20	L2 sectional area (mm^2)	0.56 (0.013)	0.77 (0.011)
10		0.98 (0.008)	1.00 (0.011)
20	L2 segment dry weight (mg)	0.95 (0.16)	1.02 (0.19)
10		1.32 (0.17)	1.35 (0.18)

5.2.3 Estimation of osmotic pressure

Two successive 1 cm segments per leaf were cut using a sharp single-edged razor blade from a point 2 mm above the shoot/grain junction. The segments were frozen immediately in liquid nitrogen. Each sample taken 0-1 or 1-2 cm from the leaf base of each genotype contained many segments of L2 to a total of *ca* 0.25 g fresh wt. Samples were wrapped in aluminium foil and allowed to thaw at room temperature. The tissue sample was then inserted into the barrel of a disposable 1 ml syringe. Cell sap was expelled from the leaf tissue by pushing down the plunger of the syringe; sap was collected in an open Eppendorf tube placed over the syringe tip.

The osmotic pressure of the expelled sap was measured in 3 replicate samples in a 8 μl sample volume on a Wescor 5100c osmometer. Osmotic pressures were converted from mmol kg^{-1} to MPa using Van't Hof's formula;



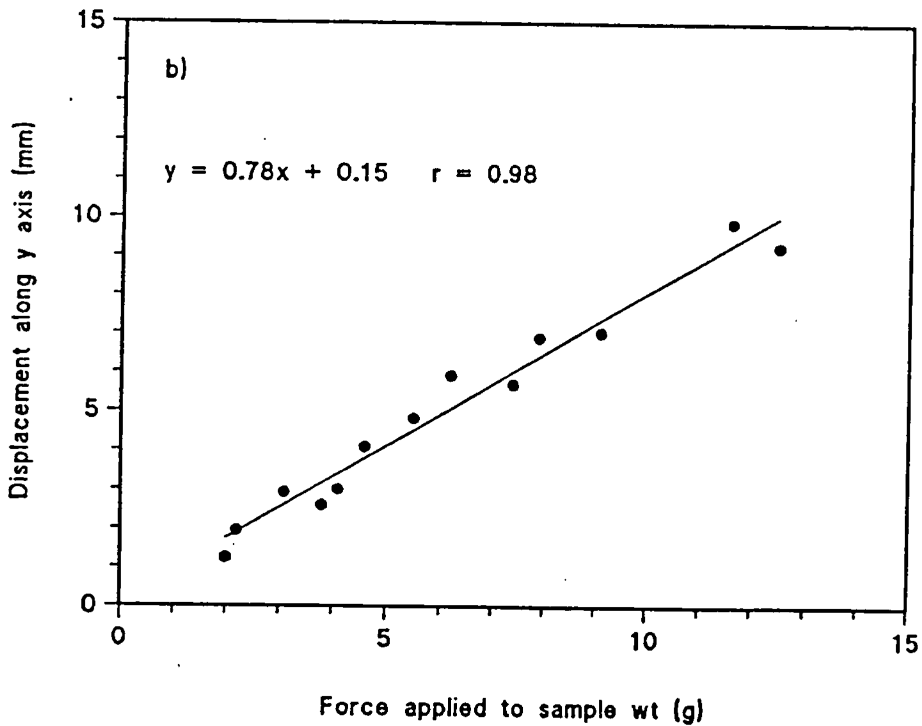
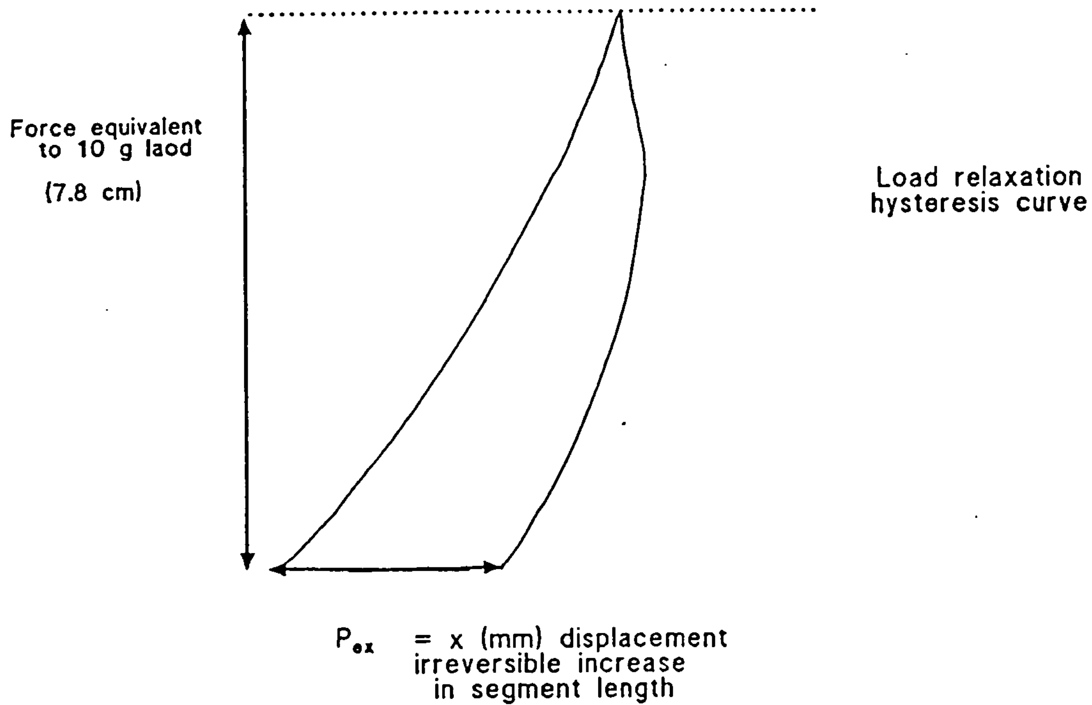


Fig. 5.1 Calibration curves for the Aberystwyth extensiometer, (a) in the x direction, increase in segment length (LVDT displacement) with change in x (mm) and (b) in the y direction, increase in segment length per applied force load (g). Linear regression lines have been fitted to calibration data.



Calculation of P_{ex}

x (mm) x LVDT displacement factor (0.028) = y

y x 1/surface area correction factor x 1/dry weight correction factor = Z

Therefore, Z = the cell wall extensibility per 10g applied load.

% increase in length = Z/original segment length x 100

Fig. 5.2 Sample calculation of P_{ex} from a load relaxation hysteresis curve, measured on the Aberystwyth extensiometer.

$\pi s = RT[m^3 \text{ MPa mol}^{-1}] \times (\text{mmol Kg})$

e.g. At 20 °C $RT = 0.00244 \text{ m}^3 \text{ MPa mol}^{-1}$

5.2.4 Estimation of the wall yield threshold

The yield threshold was measured by the method of Cleland (1959). Two successive 1 cm segments per L2 were cut from a point 2 mm above the shoot/grain junction. Six different solutions of polyethylene glycol (PEG) 4000 were made, ranging in osmotic pressure from 0 to 1.2 MPa. Seven L2 segments, from the same position within L2, and of the same genotype, were added to 10 ml of each solution or 10 ml of distilled water, in a 5 cm diameter glass petri dish. The segments were incubated for 6 h at room temperature on a slowly rotating table. Following the incubation period, segments were removed from the PEG solution, or distilled water, and gently blotted dry on soft tissue. The final length of the segments was estimated to the nearest 0.1 mm using a dissecting microscope fitted with an eye-piece graticule.

The yield threshold, Y, was determined by calculating the turgor pressure, P, of the L2 segments in each of the different PEG solutions. It was assumed that the tissue water potential (Ψ) was the same value as the osmotic pressure of the external bathing solution. The turgor pressure of the L2 segments was calculated using equation 3 described in section 5.1.1.2. L2 osmotic pressure was measured in an osmometer as described in section 5.2.3. L2 segment turgor pressure in each PEG solution and distilled water was then plotted against final L2 segment length. Y was determined from the value of P where the plateau section of the curve intersected the linear section, that is, once the turgor pressure was in excess of Y which first permitted cell extension (*cf.* Fig. 5.4). Thus, the break point in the lines represented the balance turgor pressure, above which cell expansion occurs, if it was assumed that the osmotic concentration of the cell contents did not change during the 6 h period of incubation (Cleland, 1959).

5.3 RESULTS

5.3.1 Measurement of the cell wall extensibility

Segments of L2 taken beyond the end of the extension zone, in both genotypes, were totally inextensible. The tissue displayed no plastic deformation, measured on either the Edinburgh or the Aberystwyth extensiometer. However, tissue segments taken within the L2 extension zone in both the *rht3* wild-type and *Rht3* mutant lines were less rigid and did show some plastic deformation.

5.3.1.1 Cell wall extensibility of L2 material grown at 20 °C

In conditioned 1 cm segments of L2 tissue stretched in the Edinburgh extensiometer, the *Rht3* allele reduced P_{ex} by 77 % compared to the *rht3* wild-type (Table 5.2). While the absolute values of P_{ex}, measured in 1 cm long segments, differed between the two extensiometers, the *Rht3* allele reduced P_{ex} by a similar relative amount, 65 %, when a 1 cm 'live' segment was stretched in the Aberystwyth extensiometer (Table 5.2).

The Aberystwyth extensiometer also allowed P_{ex} of the whole L2 extension zone to be measured. The calculated values of P_{ex} through the entire length of the extension zone were lower than those recorded in the 1 cm basal segment of L2 (Table 5.2). This was the direct effect of expressing P_{ex} as a % increase in length, rather than as an absolute increase in length. Thus, the values of P_{ex} shown in Table 5.2 were reduced, 61 % in the *rht3* wild-type and 43 % in *Rht3* mutant in the whole extension zone segments, compared to P_{ex} measured in 1 cm basal segments (Table 5.2). However, the absolute increase in length of both segments, of either genotype as a result of a 10 g applied load was very similar. Therefore, the *Rht3* allele significantly reduced P_{ex} of the cell wall irrespective of the proportion of the L2 extension zone which was stretched in the extensiometer. The results demonstrate that P_{ex} could be considered as an intrinsic property of the cell walls as suggested by Silk & Erickson (1979), and is not particularly a function of the number of cross walls present in the segment being stretched.

The values of P_{ex} were significantly greater for L2 material of both genotypes grown at 20 °C in the Aberystwyth compared to the Edinburgh extensiometer. Proportionally P_{ex} of both genotypes was increased by a similar amount (2.9 and 4.4-fold in the *rht3* and *Rht3* lines respectively). It was difficult to know which of several interacting variables could be responsible for the large discrepancy in P_{ex} determined on the two different extensiometers as different growth conditions, treatment of L2 tissue and extensiometers were being compared. However, at 20 °C, in all cases P_{ex} of the *Rht3* mutant was significantly less than that of the *rht3* wild-type.

5.2.1.2 Cell wall extensibility of L2 material grown at 10 °C

When plants were grown at 10 °C, there was no significant difference in P_{ex} measured in the *rht3* and *Rht3* lines in a 1 cm long basal L2 segment (Table 5.2). Compared to the P_{ex} measured at 20 °C in the equivalent 1 cm segment, P_{ex} was reduced by 83 % and 56 % in the *rht3* wild-type and *Rht3* mutant lines respectively (Table 5.2 & 5.3). Therefore, a 10 °C reduction in the growing temperature reduced P_{ex} of both genotypes, but the effect was more marked in the *rht3* wild-type than in the *Rht3* mutant.

The values of P_{ex} measured at 20 °C in live segments containing the whole L2 extension zone were decreased by 28 and 18 % in the *rht3* wild-type and *Rht3* mutant respectively compared to the P_{ex} of a 1 cm live segment also measured at 20 °C (Table 5.2). This discrepancy in the measured P_{ex} was the result of expressing the extensibility as a % increase in segment length, as opposed to just the extensibility per 10 g applied load.

It could be concluded that reducing the growing temperature to 10 °C reduced P_{ex} in the *rht3* wild-type line in a way which was comparable with the effect on P_{ex} of the *Rht3* allele itself at 20 °C (Tables 5.2 & 5.3). The principal result was that a reduction in the growing temperature from 20 ° to 10 °C produced a reduction in P_{ex} of the *rht3* wild-type to a value similar to that in the *Rht3* mutant at the same temperature.

Table 5.2 *Comparison of the cell wall extensibility (P_{ex}) (s.e., n=9), in L2 of the rht3 (wild-type) and Rht3 (mutant) grown at 10 ° and 20 °C, sampled on d 16 and d 9 respectively. P_{ex} of killed 1 cm segments measured in Edinburgh extensiometer. Live 1 cm and whole extension zone (30 mm and 20 mm in rht3 and Rht3 lines respectively at 20 °C and 20 mm in both genotypes when grown 10 °C) were measured in the Aberystwyth extensiometer. All results have been corrected for differences in segment dry weight and cross-sectional area by factors shown in Table 5.1.*

	P_{ex} % extensibility per 10 g load			
	20 °C		10 °C	
Sample	*rht3*	*Rht3*	*rht3*	*Rht3*
Conditioned 1 cm segment	3.97 (1.80)	0.92 (0.64)***	-	-
Live 1 cm segment	11.56 (1.79)	4.04 (0.72)***	1.94 (0.87)	1.79 (0.74)
Live whole L2 extension zone	4.49 (0.93)	2.32 (0.38)***	1.40 (0.42)	1.46 (0.44)

*** = difference significant at P < 0.001 compared to *rht3* wild-type at the same temperature.

Note; statistical analysis performed on actual Pex results rather than percentage results.

Table 5.3 *Comparison of the effect of temperature on P_{ex} of the two genotypes as shown by the ratio of P_{ex} 10:20 °C measured in different length live segments of L2. Data of Table 5.2*

Sample	*rht3*	*Rht3*
Live 1 cm	0.16	0.44
Whole extension zone	0.31	0.62

P_{ex} of conditioned L2 segment not measured at both 10° and 20 °C

The results shown in Table 5.2 also suggest that killing the tissue prior to recording P_{ex} may destroy factors which are important in determining P_{ex}. However, due to the other variables between the two sets of data such a conclusion must be interpreted with caution. The principal conclusion is that at 20 °C, the *Rht3* allele significantly reduced P_{ex} of the cell walls within the L2 extension zone (Table 5.2 & 5.3). Such a marked reduction in P_{ex} is likely to be a major contributory factor in the reduction in final cell length associated with the *Rht3* allele at this temperature. However, when plants were grown at 10 °C, P_{ex} was not significantly different between the two genotypes (Table 5.2 & 5.3). This observation supports the results of Chapter 4 that the effect of the *Rht3* allele can be mimicked in the *rht3* line by growing plants at 10 °C.

5.3.2 Measurement of cell turgor pressure

5.3.2.1 Measurement of the osmotic pressure of expressed cell sap

Measurement of (π), the osmotic pressure in the L2 system, has been assumed to give an approximate estimate of maximum P, the turgor pressure term which is described in the Lockhart equation (eqn 2, section 5.1.1). The osmotic pressure of expressed cell sap was measured in two successive 1 cm segments of L2 taken through the extension zone of the *rht3* wild-type and *Rht3* mutant lines grown at 20° and 10 °C.

At 20 °C, in the most basal L2 segment, 0-1 cm, there was a significant ($P < 0.05$) reduction in π in the *Rht3* mutant genotype compared to the *rht3* wild-type line (Table 5.4). However, the difference in π was not significantly different between the two genotypes in the next successive segment of L2, taken 1-2 cm from the leaf base (Table 5.4) but there were large standard errors associated with π, particularly in the *Rht3* mutant line in this segment. The osmotic pressure of expressed cell sap has been measured in the two genotypes growing at 20 °C in several different experiments. While the *Rht3* allele consistently tended to reduce the osmotic pressure of expressed cell sap compared to the *rht3* wild-type line, the magnitude of its effect varied between individual experiments. The data shown in Table 5.4 is derived from one representative experiment.

Growth of L2 at 10 °C was associated with an increase in π in both L2 segments and in both genotypes compared to the values of π obtained at 20 °C (Table 5.4). The effect on π of both genotype and a reduction in the growing temperature was tested using analysis of variance (Table 5.5). While temperature had a very significant effect on π ($P < 0.001$) there was no significant effect of genotype or interaction of genotype with temperature (Table 5.5). Therefore, at 10 °C compared to 20 °C the value of π tended to be more similar both between the two genotypes and in both of the L2 segments tested (Table 5.4). The increase in π observed at 10 °C was therefore considered to be an effect induced by low temperature and not one which was particularly associated with the *Rht3* allele (Table 5.5).

Table 5.4 *Osmotic pressure (± s.e.) of expressed cell sap from either of two successive 1 cm basal segments of L2 of the rht3 (wild-type) and the Rht3 (mutant) lines. L2 sampled either on d 9 or d 16 when grown at 10 ° or 20 °C respectively. (n=3).*

Genotype	Temp. (°C)	Osmotic pressure (MPa)	
		0-1cm	1-2cm
rht3	20	0.862 (0.008)	0.863 (0.010)
Rht3	20	0.789 (0.027)*	0.797 (0.022)
rht3	10	0.961 (0.045)	0.981 (0.018)
Rht3	10	0.965 (0.069)	0.992 (0.050)

* = difference significant at $P < 0.05$ compared to the equivalent L2 segment of the *rht3* wild-type growing at the same temperature

Table 5.5 *Two way analysis of variance to compare effects of genotype and temperature on osmotic pressure in L2 in the segment taken 0-1 cm from the L2 base in the rht3 (wild-type) and Rht3 (mutant) lines sampled on d 9 & 16 at 20 & 10 °C respectively. DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, Sig = level of significance,.*

Source of variance	DF	SS	MS	F	Sig
Genotype	1	0.00460	0.00460	2.44	n.s.
Temperature	1	0.06063	0.06063	32.25	***
Interaction	1	0.00550	0.00550	2.93	n.s.
Error	8	0.01505	0.00188		
Total	11	0.08579			

The results show that the *Rht3* allele reduced π compared to the *rht3* line in the basal segment of the L2 extension zone (Table 5.4). Additional measurements of π in more distal regions of the L2 lamina growing at 20 °C suggested that *Rht3* allele continued to reduce π compared to the *rht3* wild-type as the tissue matured (results not shown).

Overall, the results suggest that the *Rht3* allele reduces the turgor pressure within cells of the L2 extension zone when compared to the *rht3* wild-type line. There was a significant

effect of low temperature which increased π in both genotypes. This effect of the *Rht3* allele may be partly responsible for the reduced rate of cell extension associated with the *Rht3* mutant genotype. The present results also demonstrate that while there is a significant difference in π between the two genotypes at 20° there is no difference in π when plants are grown at 10 °C. Therefore, in terms of π, growth of the *rht3* wild-type at 10 °C produces a mimic of the *Rht3* mutant.

5.3.2.2 Osmotic pressure of plants treated with gibberellin and paclobutrazol

The application of plant growth regulators to the wild-type and mutant genotypes growing at 10° and 20 °C may help to elucidate whether the product(s) of the *Rht3* allele have an effect on the water relations of expanding cells.

Grown at 20 °C, treatment of L2 with 5 µM GA_3 or 2*S*,3*S* paclobutrazol did not produce any obvious differences in π, in either L2 segment considered or, between the two genotypes (Fig. 5.3a & b). However, the osmotic pressures tended to be higher in the most basal 1 cm segment of L2 taken 0-1 cm from the leaf base, in both genotypes.

The equivalent experiment was repeated with L2 material grown at 10 °C. The reduction in temperature was associated with an overall increase in π in both genotypes, in both L2 segments and in both treatments, applied GA_3 and 2*S*,3*S* paclobutrazol (Fig. 5.3c & d). At 10 °C π was significantly higher ($P < 0.001$) in L2 treated with 2*S*,3*S* paclobutrazol, in both segments and in both genotypes compared to the untreated control leaves. Overall, both genotypes responded similarly to the applied growth regulators. Therefore, the increase in π in both genotypes in response to applied 2*S*,3*S* paclobutrazol at 10 °C was considered to be a specific interaction induced by application of 2*S*,3*S* paclobutrazol and low temperature and not one particularly associated with the *Rht3* allele. In contrast to the lack of effect at 20 °C, this observation could support the hypothesis that GA may be a more important regulator of low temperature growth than high temperature growth, as has been suggested previously (Chapter 4).

5.3.3 Measurement of the cell wall yield threshold

The remaining factor described in the Lockhart equation (eqn 2 section 5.1.1), as exerting an effect on the rate of cell extension was the wall yield threshold, Y, or critical turgor pressure which must be exceeded to permit cell extension.

Y was measured in L2 segments incubated in PEG solutions of different molarity. This was the bathing medium which appeared to give the most reliable results and those which were most comparable with published data (Malone *et al.*, 1989).

The break point in the segment extension curves, was *ca* 0.35-0.45 MPa in both the *rht3* wild-type and the *Rht3* mutant lines (Fig. 5.4). Hence Y, the difference in the internal and external osmotic pressure, was calculated to be *ca* 0.4 MPa in both genotypes.

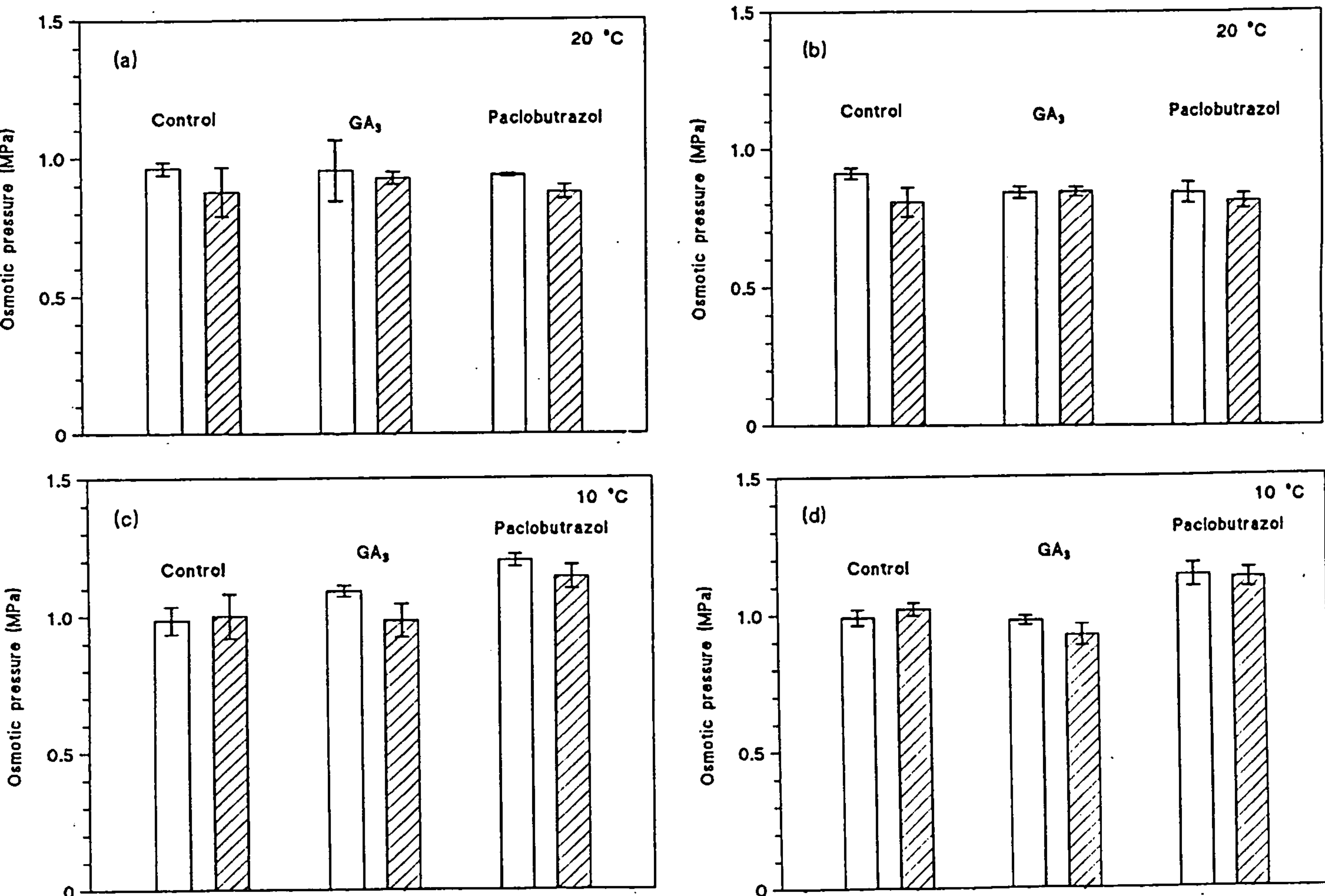


Fig. 5.3 Osmotic pressure (± s.e., n=3) in two successive segments of L2 0-1 cm (a & c) and 1-2 cm (b & d) in the *rht3* (wild-type), open bars, and *Rht3* (mutant), filled bars, growing 20 °C, sampled on d 9 and 10 °C, sampled on d 16. Plants treated with 5 µM GA_3 and 5 µM 2*S*,3*S* paclobutrazol.

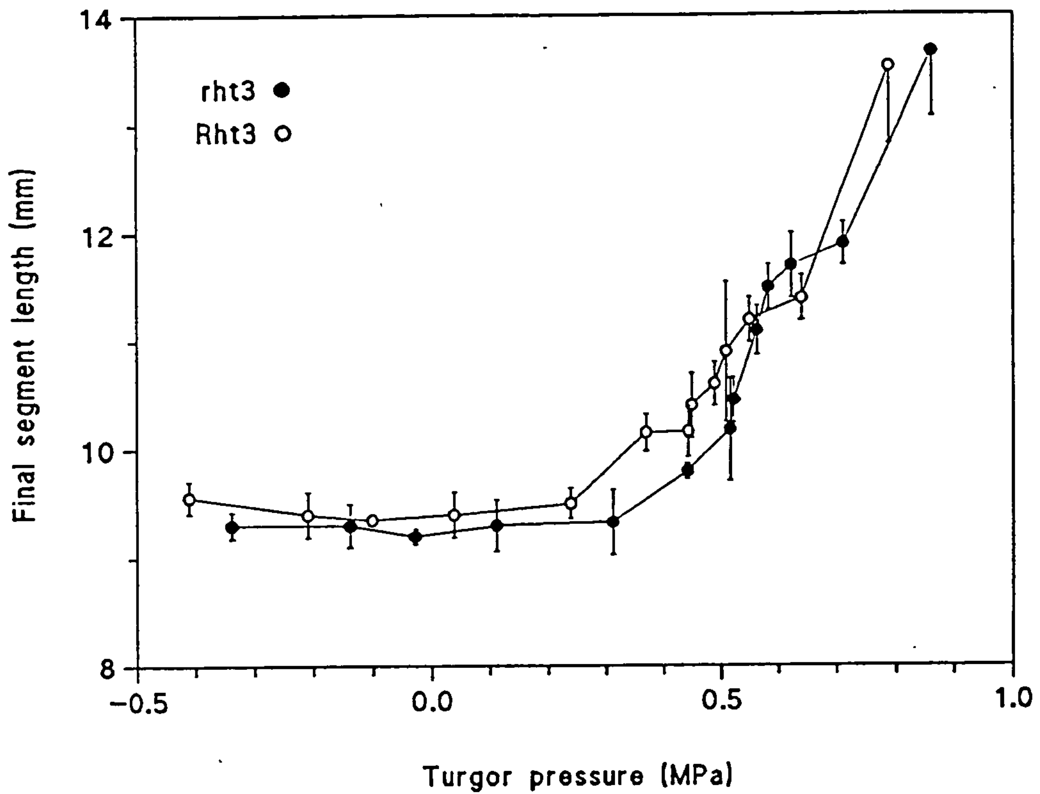


Fig. 5.4 Estimation of the wall yield threshold, Y (± s.e., n=7), in the *rht3* (wild-type) and *Rht3* (mutant) lines grown at 20 °C in a 1 cm segment of L2 taken 1-2 cm from the leaf base. Plants grown at 20 °C and sampled on d 9. Y measured as the balance point in different osmotic pressure solutions of PEG 4000 after an incubation period of 6 h. Original length of all segments was 1 cm.

At this osmotic pressure difference, it was assumed that the water potential of the interior of the cell and of the bathing medium was the same and P was thus zero. Therefore, the difference between the internal and external osmotic pressures of the cells was assumed to be equivalent to the wall yield threshold, or the turgor pressure which must be exceeded to permit cell expansion. Although the method used to determine Y was crude, the same approximate answer was derived in 3 repeat experiments; the position of the break point varied by *ca* 0.1 MPa. However, there was no consistent tendency for Y of the *Rht3* mutant to be higher than in the *rht3* wild-type.

The experiments were done on two successive 1 cm segments of L2. The results shown in Fig. 5.4 refer to the segments taken only from the region 1-2 cm from the leaf base. The first segments (0-1 cm from the leaf base) extended in solutions of very high osmotic strength up to 0.7 MPa PEG. It was concluded that a major cause of the observed extension was that cells in the segment may be capable of greater osmotic adjustment or variation in wall properties than cells in the 1 cm segment 1-2 cm from the leaf base, in both genotypes. The results also illustrate the apparent high osmotic pressures which may exist in the meristematic region of the L2 lamina.

The major conclusion was that there did not appear to be any obvious difference in the value of Y which must be exceeded to drive cell extension in the *rht3* wild-type and *Rht3* mutant lines. By implication the results suggest that the reduction in cell length associated with the *Rht3* allele is not a consequence of any effects the allele may have on the value of Y.

5.3.4 Conclusions

The biophysical parameters described in the Lockhart equation have been measured and compared between the *rht3* wild-type and the *Rht3* mutant lines. The main effects are the following.

1. The primary, and most significant, effect of the *Rht3* allele was to reduce P_{ex} of the cell walls within the L2 extension zone.

2. The *Rht3* allele was also associated with a significant reduction in π ($P < 0.05$) within the L2 extension zone. The *Rht3* allele did not appear to affect Y within the growing cells of the L2 extension zone.

3. Application of 2*S*,3*S* paclobutrazol significantly increased π in L2 of both genotypes at 10 °C.

The primary effect of the products of the *Rht3* allele appears to be an alteration in the rheological properties of the growing cell wall, such that compared to the *rht3* wild-type the cell walls in the *Rht3* mutant become less extensible earlier in development.

5.4 DISCUSSION

5.4.1 Effect of the *Rht3* allele on cell wall extensibility

How the *Rht3* allele may affect the rate of cell extension has been investigated by examining the biophysical parameters described in the Lockhart (1965) equation: 1/V.dv/dt = m (P - Y). When L2 was grown at 20 °C the *Rht3* allele significantly reduced P_{ex}, considered to relate to the m term of the Lockhart equation, (Cleland, 1971) while appearing to have only a slight effect in reducing P and no obvious effect on Y.

The results suggest that the primary effect of the *Rht3* allele is to stiffen the cell wall and therefore reduce its capacity to undergo continued plastic extension compared to the *rht3* wild-type. There are numerous reports which have linked changes in wall rheology to the biophysical control of growth in higher plants: in *Lolium* (Thomas *et al.*, 1989), in wheat roots (Jones *et al.*, 1987; Pritchard *et al.*, 1987), in mustard seedlings (Rich & Tomos, 1988), in pea internodes (Cosgrove & Cleland, 1983b), in maize leaves (Michelena & Boyer, 1982), in sunflower leaves (Matthews *et al.*, 1984), in birch leaves (Taylor & Davies, 1986) and in species of *Poa* (Woodward & Friend, 1988).

5.4.1.1 Correlation of Pex, the rate of growth and endogenous GA

The overgrowth *slender* mutant of barley, represents the opposite extreme to the GA-insensitive *Rht3* mutant of wheat. In *slender* there is a constitutive over-expression of GA-regulated genes (Chandler, 1988; Lanahan & Ho, 1988) and the plant behaves as though continually saturated with GA. P_{ex} was significantly increased in the *slender* mutant compared to the wild-type line (Pollock *et al.*, 1990; Pollock *et al.*, 1992). The suggestion that there may be a correlation between P_{ex}, the maximum rate of cell extension and sensitivity to endogenous GA is supported by the present results. Such a relationship has also been proposed following measurement of P_{ex} in the first leaves and sheaths of lines of wheat carrying the *Rht1* and *Rht2* semi-dwarfing alleles (Keyes *et al.*, 1990; Paolillo *et al.*, 1991). Increasing potency of the *Rht* gene dose was associated with a linear reduction in P_{ex} (Keyes, 1987; Keyes *et al.*, 1990) and in final sheath and epidermal cell length (Keyes *et al.*, 1989). In the same system applied GA_3 increased both leaf length and P_{ex}, and ancymidol reduced leaf growth and P_{ex}, compared to the untreated control leaves (Keyes, 1987; Keyes *et al.*, 1990).

5.4.1.1 The effect of low temperature growth on Pex in the mutant and the wild-type genotypes

Lowering the growth temperature to 10 °C was associated with a reduction in P_{ex} in both genotypes, although the effect was more severe in the *rht3* wild-type. However, both the method of expression of P_{ex} as a % increase in segment length per 10 g applied load, and the application of correction factors, tended to exaggerate the difference in P_{ex} values between

the two growing temperatures. Extensibility data at 10 °C was multiplied by a smaller correction factor than was used for L2 material grown at 20 °C (1.86 & 1.26 in *rht3* and *Rht3* lines respectively at 20 °C compared to 0.74 and 0.72 in the *rht3* and *Rht3* lines respectively at 10 °C). However, the results still clearly supported the conclusion of Chapter 4, that the *rht3* and *Rht3* lines are more similar to each other when grown at 10° than 20 °C. Growing the *rht3* wild-type at 10 °C produced a phenocopy of the *Rht3* mutant in terms of both L2 morphology and the P_{ex} of the cell wall. The results are compatible with the suggestion that both low temperature and the *Rht3* allele, may reduce the maximum rate of leaf elongation by a direct or indirect effect on the rheological properties of the cell wall. However, low temperature appears to act in addition to the effect of the *Rht3* allele on P_{ex}. The effect of temperature on the rate of leaf elongation was observed in *Lolium temulentum* where reducing the growing temperature from 20° to 5 °C resulted in a rapid decrease in the rate of leaf elongation (Thomas *et al.*, 1989). P_{ex} decreased as the growth temperature was reduced from 18 ° to 5 °C in different species of *Poa* (Woodward & Friend, 1988). It was concluded that a decrease in P_{ex}, rather than the slight decrease in turgor, was the major contributory factor associated with the observed reduction in the rate of leaf extension when the *Poa* species were grown at 5 °C compared to 18 °C. In wheat roots too, it was changes in the m term of the Lockhart equation, and not Y, which were associated with a reduced rate of growth as the temperature was reduced (Pritchard *et al.*, 1990).

A further consideration is that the extensiometer induces stress in only a uni-axial direction while in the intact plant stress is induced multi-axially by the internal cell turgor pressure (Tomos, 1985). If an effect of the *Rht3* allele were to be to reorientate the direction or polarity of cell growth, this would not easily be distinguished by estimates of P_{ex}. The problem may be more apparent when plants are grown at 10 °C. Various studies have associated growth at low temperature with significant morphological and anatomical changes (Terry *et al.*, 1983) and the production of broader, thicker leaves; in *Secale* and *Triticum* (White *et al.*, 1990), in *Zea mays* (Thiagarajah & Hunt, 1982) in *Avena* (Rooney *et al.*, 1989) and *Solanum* (Midmore *et al.*, 1992). However, the fundamental driving force for longitudinal growth described in the Lockhart equation is a force per unit area of wall acting in the direction of extension (Hsaio *et al.*, 1985).

5.4.1.2 Influence of the physical condition of tissue samples being stretched in the extensiometer

In the present study P_{ex} was observed to be higher in tissue segments which were stretched live compared to those which had been conditioned prior to stretching. The opposite effect was reported by Pollock *et al.*, (1990), P_{ex} being higher in previously conditioned than in live segments of barley. The increase in P_{ex} associated with previously conditioning leaf samples, in barley was higher in the wild-type than the *slender* mutant, which led the authors

to speculate that some constraint which was normally characteristic of living cells had been modified or lost in the mutant. However, for the present results in wheat, it may be unwise to infer too much from this observation as P_{ex} of conditioned L2 segments was measured only in the Edinburgh extensiometer, and not the Aberystwyth extensiometer, which would have allowed a more direct comparison. Furthermore, P_{ex} is a parameter which is very responsive to changes in environmental conditions such as light quality (Van Volkenburgh & Cleland, 1981; Van Volkenburgh *et al.*, 1985) and water stress (Green, 1968; Matthews *et al.*, 1984; Hsiao *et al.*, 1985; Tomos, 1985) and was thus likely to be slightly different between the two different sets of L2 growth conditions. However, the critical point is that despite these qualifications, at 20 °C P_{ex} was lower in the *Rht3* mutant than in the wild-type, whereas at 10 °C it was the same in both genotypes.

5.4.2 Difficulties in measurement of turgor pressure

The conclusions which may be drawn from the present results of π must be interpreted with reference to 3 different sources of error which may affect the magnitude of the result: (i) contents of the cell vacuole may have been diluted by up to 20 % by apoplastic fluid during the extraction process, which would tend to decrease the measured value of P (Tyree & Jarvis, 1982; Tomos *et al.*, 1984), (ii) low molecular weight compounds may have been formed during the extraction process (Turner, 1981) and (iii) the final value of osmotic pressure refers to the leaf segment as a whole, which represents a heterogeneous mixture of cells. In practice specific cells such as those in the upper and lower epidermal cell layers may have a controlling influence on the rate of cell extension within the leaf (Avery, 1933; Wenkert, 1980).

Precautions to eliminate such errors were taken, such as freezing the tissue segments immediately as they were dissected from the intact seedlings. However, the final results were still potentially subject to the sources of error as described above. Ideally it would have been better to measure the turgor pressure directly in the intact plant using the pressure probe method introduced by Green (1968) and modified by Steudle & Zimmerman (1974) which allows π to be measured in individual cells within the living plant.

5.4.2.1 The effect of the *Rht3* allele on the measured osmotic pressure of the cell sap

The *Rht3* allele significantly reduced π in the L2 segment taken 0-1 cm from the leaf base. Additional measurements of π made in sections of L2, distal to the extension zone showed an increasing tendency for π to be lower in the *Rht3* mutant than in the *rht3* wild-type by *ca* 0.15 MPa. A reduction in π was also found in lines of wheat carrying the *Rht1*, *Rht2* and *Rht3* alleles in both the Maris Huntsman and April Bearded cultivars (Hoogendoorn *et al.*, 1990). The same workers considered that the reduction in final cell and leaf extension associated with the *Rht* alleles may be, in part, be influenced by a lower osmotic pressure.

However, the interpretation of an effect of a reduced P on the rate of cell extension in the mutant genotype compared to the wild-type depends on Y being significantly lower than P (Pritchard *et al.*, 1987). The important factor is the magnitude of the difference between P and Y, because cell expansion is driven by turgor in excess of Y i.e. the term (P-Y), (Cosgrove, 1986). Thus, if P and Y are very close, a small change in P would elicit a large effect on the rate of cell extension, as found in *Nitella* where P exceeded Y by just 0.02 MPa (Green *et al.*, 1971). In the present work, in wheat, it is estimated that there may be a difference in the term (P-Y) of *ca* 0.4MPa. This may suggest that a relatively large change in P would be required to elicit an increase in the rate of extension, in both genotypes.

The osmotic pressures recorded in the present results within the L2 extension zone of both the *rht3* wild-type and the *Rht3* mutant lines are consistent with previous reports that the extension zone appears to be capable of large osmotic adjustments which occur independently of the more mature regions of the lamina (Maxwell & Redman, 1978; Cutler *et al.*, 1980a; Cutler *et al.*, 1980b; Matsuda & Raiza, 1981; Michelena & Boyer, 1982; Westgate & Boyer, 1985).

However, while increasing π in growing cells would tend to increase the rate of growth (Ray, 1987), the present results suggest that although π was reduced in the mutant compared to the wild-type line, the reduction in π was not as significant as it later became in more mature regions of the lamina, once cell extension had ceased. It is therefore suggested that the reduction in π in the extension zone of the *Rht3* mutant may play a secondary role in the mechanism through which cell length is reduced by the *Rht3* allele, the main effect being a reduction in the extensibility of the cell wall. The reduction in π in more mature regions of the lamina may be an indirect effect linked to physiological differences such as differences between the two genotypes in photosynthetic or transpiration rates.

5.4.3 Effect of the *Rht3* allele on the wall yield threshold

In the present study the *Rht3* allele was not particularly associated with an effect on the value of Y. However, many problems were encountered with the choice of a suitable osmoticum. Fig. 5.4 shows the results derived using PEG 4000 as an osmoticum. This was found to be more suitable than mannitol which was itself more suitable than sucrose. Segments bathed in sucrose were observed to show extremely large increases in length, especially the segment, which would include meristematic cells, taken 0-1 cm from the leaf base. It was concluded that the cells within this segment may have been actively metabolizing sucrose to drive cell extension during the period of incubation. Cleland (1959) showed that segments of *Avena* required an external source of sucrose for continued cell extension. The technique described in the present study does not however, measure turgor directly. Instead it relies on the fact that by changing the water potential of the experimental

solutions there will be an equivalent shift in the turgor pressure of the growing cells, as long as the change in cell volume remains negligible and the cell walls of the two genotypes have similar elastic properties, (Van Volkenburgh & Cleland, 1981). The method is only valid if the osmotic pressure within the cells remains constant during the experimental period (Cleland, 1959). However, there are reports of an increase of the osmotic pressure during the incubation period (Matsuda & Riazi, 1981; Westgate & Boyer, 1985). In addition, sucrose and mannitol have been shown to be taken up into the apoplast and symplast of the cell during the incubation period (Hohl & Schopfer, 1991). Basal 0-1 cm segments of the *Rht3* mutant line were observed to show a greater extension over the 6-h incubation period than those of the *rht3* wild-type when incubated in sucrose. The apparent differential effect of the sucrose may be explained as the result of a difference in the total number of cells present within a unit length of leaf in the two genotypes. Thus, a 1 cm length of L2 of the mutant genotype contains more cells than an equivalent 1 cm segment of the wild-type, because overall cell lengths are shorter in the mutant genotype. If each cell increased in volume by the same absolute amount during the incubation period, the final segment length of the *Rht3* mutant would be longer than that of the *rht3* wild-type line. Within the segment taken 0-1 cm from the leaf base the ability to extend in solutions of high water potential (0.7 MPa PEG, 1.8 MPa sucrose and 1.1 MPa mannitol) was assumed to be a demonstration of the ability of meristematic tissue to exhibit rapid osmotic adjustment even under conditions of extreme water stress which prevent cell expansion (Lawlor & Leech, 1985). Osmotic adjustment such as that which may have occurred in the present experiments has also been reported in growing leaves and floral apices (Munns & Weir, 1971; Barlow *et al.*, 1980)

The method used here to measure Y is also subject to the following constraints:

(1) It is assumed that the concentration of solutes within the cell wall is negligible, which it may not always be, this may lead to a buffering effect and thus an overestimate of Y.

(2) The walls of the cells are assumed to have a reflection coefficient of 0, therefore offering no resistance to the influx of water, in practice this may not be true and the slight resistance of the cell walls may tend to resist water movement and therefore overestimate Y.

(3) If the segment grows during the period of incubation, this may itself initiate an effect on reducing the hydraulic conductivity to cells within the centre of the tissue segment, such that they would cease to extend. This effect may also tend to result in underestimation of Y (pers. comm. J. Pritchard). Thus, the present results have to be interpreted with reference to the assumptions on which the method is based. However, in the present results, although the value of π varied between individual experiments, the same approximate answer was obtained several times. Ideally it would be most accurate to measure Y instantaneously in the living plant; unfortunately such techniques are not, as yet, available.

5.4.4 Effect of low temperature and plant growth regulators on the water relations within the expansion zone of L2

A reduction in the growing temperature from 20 ° to 10 °C resulted in a similar increase in π in both genotypes (Table 5.4). This effect of low temperature is not unusual and has often been associated with frost tolerance (Pollock, 1986; Pollock & Cairns, 1991). The osmotic pressure in leaves of *Secale* virtually doubled during cold acclimation. The increase was attributed to the accumulation of predominantly sucrose and raffinose (Koster & Lynch, 1992).

The effect of applied GA on the magnitude of π is inconsistent; in several higher plant systems such as that observed in this work π remains unchanged (Ordin *et al.*, 1956; Cohen & Atsmon, 1972; Stevenson & Cleland, 1981 & 1982). However in other systems π was increased (Katsumi & Kazama, 1978; Van den Ende & Koorneef, 1960).

In both genotypes, growth of L2 at low temperatures was associated with an apparent increased sensitivity of π to the application of the growth inhibitor, 2*S*,3*S* paclobutrazol. By implication the observation may be consistent with the hypothesis that GA may be a more important growth regulator at low temperatures than at higher temperatures (*cf.* Chapter 4; Stoddart & Lloyd, 1986; Pinthus *et al.*, 1989; Appleford & Lenton, 1991). Blocking the biosynthesis of endogenous GA by the application of 2*S*,3*S* paclobutrazol at 20 °C caused a marked increase in π (and the water potential, results not shown) of both the *Rht3* mutant and *rht3* wild-type lines. The similarity of the effect between the two genotypes further supports the suggestion that the growth inhibitor causes effects additional to those of the *Rht3* allele. 2*S*,3*S* paclobutrazol may affect both cell division and cell extension (Chapter 4) whereas the reduced sensitivity to endogenous GA associated with the *Rht* allele may only affect cell elongation as suggested by Paolillo *et al.* (1991).

5.4.5 Conclusions

Measurement of the biophysical parameters described in the Lockhart equation have illustrated the limitations to the experimental procedures presently available. Most of the results described in section 5.3 can be relied upon only to give a general estimation of a parameter and not an absolute value.

However, the primary effect of the *Rht3* allele was to significantly reduce the plastic extensibility of the cell wall, P_{ex}. It is concluded that the *Rht3* allele may primarily reduce final cell length via an effect on the rheological properties of the cell wall, while there may be a secondary effect to reduce P. The result suggest that the effect of the *Rht3* allele may be to modify the type and/or abundance of cross-links between structural polymers of the cell wall, thereby rigidifying the wall and prematurely reducing wall extensibility compared to the *rht3* wild-type.

It was therefore decided to investigate the two genotypes with respect to a potential type of covalent cross-link formed between polymers of the cell wall involving phenolic acid residues.

CHAPTER 6

POSSIBLE INVOLVEMENT OF PHENOLIC ACID CROSS LINKS IN MODIFYING CELL WALL EXTENSIBILITY IN THE *Rht3* MUTANT

6.1 INTRODUCTION

In the *Rht3* mutant, the length of the L2 extension zone is shorter than in the *rht3* wild-type because the rate of cell extension declines at a faster rate than in the *rht3* wild-type line (*cf.* Fig. 4.5, Chapter 4). This implies that the extensibility of the cell wall is lost more quickly in the *Rht3* mutant than in the *rht3* wild-type. Measurement of the plastic component (P_{ex}) of the wall extensibility in a basal segment of L2 including the whole extension zone, using an extensiometer, has indeed shown P_{ex} to be reduced by *ca* 60 % in the mutant genotype (*cf.* section 5.3, Chapter 5). Within the extension zone of L2 the *Rht3* allele has been demonstrated to reduce significantly the REGR subsequent to the position, *ca* 10 mm from the leaf base, when the maximum REGR was achieved. The main factor thought to cause wall stiffening and cessation of cell extension is the degree of cross-linking between polymers of the cell wall (Fry, 1986).

With the exception of cellulose, polymers present within the primary cell wall are intrinsically soluble. They are made insoluble by the formation of a variety of different intermolecular cross-links (Fry, 1986, 1988, 1989). The different types of cross-links formed can be classified into two major groups, non-covalent and covalent. Hydrogen bonding, ionic bonds and Ca^{2+} bridges represent non-covalent interactions within the cell wall (Preston, 1979; Fry, 1988, 1989). The second category of intermolecular cross-links includes oxidative phenolic coupling, glycosidic bonds, and ester cross-links (Fry, 1988; Brett & Waldron, 1990).

The present study has concentrated on one type of covalent cross-link, the oxidative coupling of phenolic acids linked to structural polymers of the cell wall (Fig. 6.1). Interest in the formation of such cross-links is based on their proposed ability to stiffen the cell wall and prevent continued wall extension (Cooper & Varner, 1984; Fry, 1983; Ilyama *et al.*, 1990; Kamisaka *et al.*, 1990; Tan *et al.*, 1991). The choice of this particular type of wall cross-link was influenced by the techniques available and the extent of knowledge concerning the potential influence on the mechanical properties of the cell wall that such cross-links may

have. Phenolic acids have been shown to represent *ca* 1-4 % of cell wall dry weight in cereal leaves (Hartley, 1987).

6.1.1 Occurrence of phenolic acid cross-links within the primary cell wall

The most common way in which phenolic acids (Fig. 6.1) are linked to cell wall polymers is through ester (Fig. 6.2) or ether linkages. Intermolecular cross-links involving phenolic acids could be of two types: phenol-polymer, such as the ester linkage between ferulic acid (FA) and a wall polysaccharide (Fry, 1982, 1983; Kato & Nevins, 1985); or phenol-phenol, forming dimerised phenolics such as diferulic acid (DFA) (Markwalder & Neukom, 1976).

Phenolic acids linked through ester bonds to wall polysaccharides have been identified in many different monocotyledons (Fausch *et al.*, 1963; Hartley, 1972, 1973, 1976; Hartley & Keene, 1984; Harris & Hartley, 1976; Hartley & Jones, 1976; Smith & Hartley, 1983; Shibuya, 1984; Kato & Nevins, 1985; Carpita, 1986; Mueller-Harvey *et al.*, 1986; Kamisaka *et al.*, 1990; Tan *et al.*, 1991) and also in different parts of the wheat plant: wheat germ (Markwalder & Neukom, 1976; Hartley & Keene, 1984), aleurone cells of the grain (Fulcher & O'Brien, 1972), stem internodes (Ilyama *et al.*, 1990; Lam *et al.*, 1990), and roots (Smith & O'Brien, 1979). Ferulic acid (FA), diferulic acid (DFA) and *p*-coumaric acid (PCA) have been identified as the phenolic acids most commonly bound to wall polymers in wheat internodes (Lam *et al.*, 1990). More particularly, the phenolic acids in monocotyledons have been shown to be linked to the arabinose residues of arabinoxylans in the hemicellulose fraction of the primary cell wall (Whitmore, 1974; Smith & Hartley, 1983; Kato & Nevins, 1985; Ishii *et al.*, 1990; Kamisaka *et al.*, 1990; Ishii, 1991; Tan *et al.*, 1991). If phenolic acid cross-links are important in decreasing the extensibility of the cell walls in the *Rht3* mutant line, then it may be expected that the number of phenolic cross-links would be greater in the *Rht3* mutant than in the *rht3* wild-type. A possible hypothesis concerning the involvement of phenolic acid cross-link formation with the reduced extensibility in the mutant genotype could be that the product(s) of the *Rht3* allele affected the rate at which phenolic acids were metabolised and/or the rate at which they were incorporated (predominantly feruloylated) into cell wall polymers. This was tested by feeding a precursor of FA, [^{14}C]cinnamic acid, to segments of L2 tissue in the *rht3* wild-type and *Rht3* mutant (*cf.* Fig. 6.3).

6.1.1.1 The formation of phenolic acid cross-links; the role of peroxidase

The formation of phenolic acid cross-links within the primary cell wall is assumed to be catalysed by peroxidase (Fry, 1979; Epstein & Lamport, 1984). The secretion of peroxidase into the cell wall is thought to be highly regulated. The concentration and sub-cellular distribution of peroxidase can change dramatically in response to stimuli such as temperature, drought, fungal infection, and applied plant hormones (Gaspar *et al.*, 1982; Fry,

1988) and Ca^{2+} (Penel & Greppin, 1979; Stitcher *et al.*, 1981). There are many reports of the occurrence of different isozymes of peroxidase present within the same plant (Leland & Shannon, 1966; Penel & Greppin 1979; Gaspar *et al.*, 1982; Goldberg *et al.*, 1986). It has been suggested that the secretion of different isozymes may represent an effective mechanism to control cell growth. It has also been suggested that the different peroxidase isozymes may be stored in different compartments within the cell (Ranjeva *et al.*, 1978) and/or they may have slightly different substrate molecules (Gaspar *et al.*, 1982). For the enzyme to be causally involved in the formation of cross-links between cell wall polymers it must be in the cell wall itself and therefore present in the apoplastic fluid (Fry, 1988; Sanchez *et al.*, 1989). Peroxidase activity has been shown to be present within the primary cell wall of plant tissue (Fry, 1986; Goldberg *et al.*, 1987).

6.1.1.2 The negative correlation of plant height and peroxidase concentration

If peroxidase is important in promoting wall cross-linking and limiting cell wall extension then a negative correlation between peroxidase activity and cell extension might be expected. This is indeed the case in many plants, in which there is a negative correlation between peroxidase activity and plant height (Kamerbeek, 1956; McCune & Galston, 1959; Evans & Alldrige, 1965; Schertz *et al.*, 1971; Cunningham *et al.*, 1975; Singhal *et al.*, 1979). This is also the case in many stature mutants (Jones, 1987; Jupe & Scott, 1989, 1992). A reduction in peroxidase activity has been suggested as being involved in the promotion of growth and cell extension by applied GA. Fry (1979, 1980) proposed that GA suppressed peroxidase secretion into the cell wall and so reduced the number of phenolic acid cross-links formed, therefore allowing extension growth to continue for longer. In support of this hypothesis, application of GA_3 to spinach cells was observed to suppress peroxidase secretion and permit the accumulation of extracellular phenolics in the unlinked form within the cell walls (Fry, 1979, 1980). Conversely, application of GA biosynthesis inhibitors increased peroxidase activity (Gaspar & Lacoppe, 1968; Biggs & Fry, 1987).

6.1.1.3 The role of phenolics and peroxidase in cell extension and the effects of the *Rht3* allele

If increased phenolic acid cross-linking is responsible for the reduced cell wall extensibility associated with the *Rht3* allele then cross-links involving phenolic acid residues may be expected to be more abundant in the cell walls of the *Rht3* mutant than in the *rht3* wild-type. In addition, the role of phenolic acid wall cross-links in the regulation of cell wall extensibility will be further investigated by the application of GA_3 and 2*S*,3*S* paclobutrazol, which may be expected to reduce and increase the abundance of such cross-links in the GA-responsive wild-type.

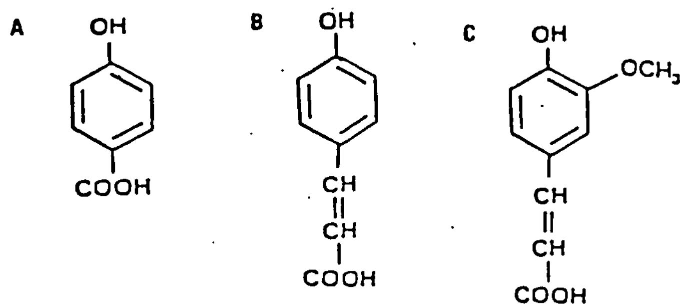


Fig. 6.1 Chemical structures of (A) *p*-hydroxybenzoic, (B) *p*-coumaric acid, and (C) ferulic acid.

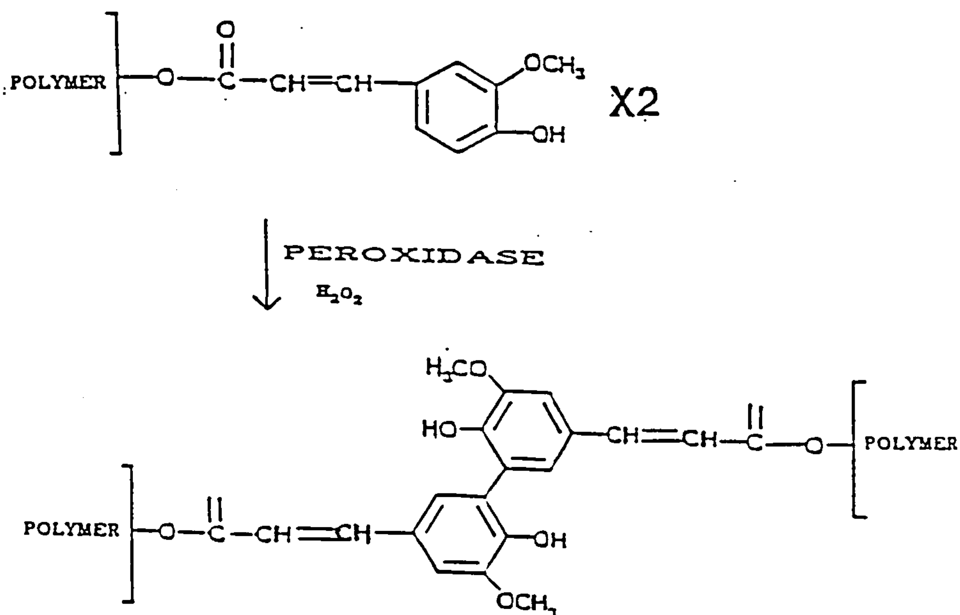


Fig. 6.2 Formation of diferulic acid wall cross-link between two cell wall polymers, catalysed by peroxidase

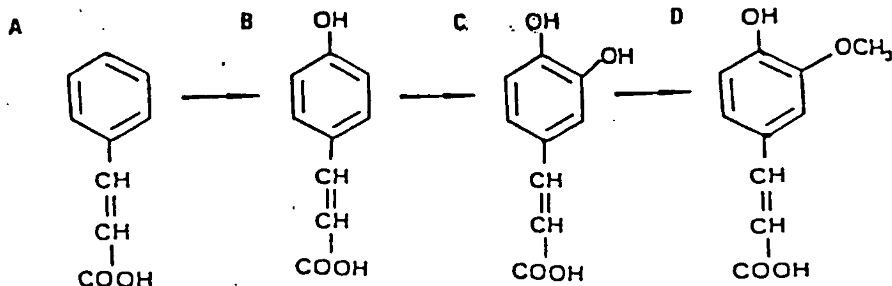


Fig. 6.3 The biosynthetic pathway through which cinnamic acid is converted to ferulic acid (A) cinnamic acid (B) p-coumaric acid (C) caffeic acid and (d) ferulic acid.

The objectives of the present work were therefore to compare L2 of the *rht3* wild-type and *Rht3* mutant in order to determine:

a) the number and concentration of different phenolic acid compounds bound to the cell wall in 1 cm long segments taken through the extension zone;

b) the rate of phenolic acid metabolism and incorporation of phenolic precursors into the cell wall using a presumed precursor of FA, [^{14}C]cinnamic acid;

c) peroxidase activity and the relationship between peroxidase activity and final plant height; and

d) the relationship of peroxidase activity to the rate of growth and phenolic content of the cell walls.

6.2 MATERIALS AND METHODS

6.2.1 Plant material and growth conditions

Grain was sown as described in Chapter 2 section 2.2 and grown under conditions N° 1 described in Table 2.1 except for plant material used for HPLC analysis which was grown under condition N° 2 described in Table 2.1.

6.2.2 Sampling of leaf tissue

L2 samples were collected nine days after sowing. Intact L2 was dissected out from the encircling L1 sheath as described in chapter 2 section 2.3. Leaves were placed on a moist paper towel to prevent excessive drying. Using a sharp razor blade, three successive 1 cm segments were cut from each leaf. The first 1 cm segment was measured from the base of the leaf. L2 segments for analysis of phenolic acids were wrapped in aluminium foil and kept on ice prior to homogenisation. L2 segments that were to be used to assay enzyme activities were used immediately.

6.2.3 Extraction and analysis of the phenolic acids bound to the cell wall

The alcohol insoluble residue (AIR) of cell wall material was prepared by the method of Fry (1988). Each sample consisted of 0.2-0.5 g fresh weight of the appropriate 1 cm segments of L2 tissue, which was homogenised in a pestle and mortar in 2 ml of 80 % ethanol. The homogenate was filtered through a sintered glass filter. The residue, containing the cell wall material, was retained and the supernatant discarded. Cell wall material was dried in a Speedvac in a centrifuge tube which was covered with a punctured layer of Parafilm. Dried cell walls (10 mg) were saponified with 1 ml of 0.1 M NaOH at 25 °C for 1 h. The saponification products were acidified with 0.1 M trifluoroacetic acid and the released products partitioned into 2 ml of butanol.

6.2.3.1 Thin layer chromatography (TLC)

Glass-backed fluorescent silica gel plates (Kieselgel 60 F_{254}. Merck) were used. The solvent system used was (9:2, v/v) benzene:acetic acid. Phenolic acid compounds were dissolved in 80 % ethanol and spotted onto the TLC plates using glass capillary tubes. TLC plates were run under continuous exposure to 360 nm UV light to promote the co-running of the *cis* and *trans* isomers of the phenolic compounds under investigation (Fry, 1980). Each plate was developed until the solvent front was approximately 2 cm from the top of the plate. Phenolic acids were identified by recording their characteristic fluorescence under long wave UV light in the presence, or absence, of fuming ammonia. TLC plates were sprayed with a fine mist of Folin & Ciocalteu's phenol reagent to visualise phenolic compounds. Diferulic acid used as a standard in TLC and HPLC analysis was a gratefully acknowledged gift from

6.2.3.2 High pressure liquid chromatography (HPLC)

Reverse phase HPLC was performed on a Hypersil 5 ODS C_{18} silica column (250x4.5 mm i.d.) Phenolics were eluted in a 10-80 % methanol gradient with 0.8 mM aqueous acetic acid over 30 min. A 20 µl sample injection volume was used and the flow rate was 1 ml min^{-1}. The absorbance was monitored continuously at 320 nm, the absorbance maximum for ferulic acid, and peaks were integrated using a VG Mulichrom programming package. Compounds were identified from their retention times (t_r), compared to authentic standards.

6.2.3.3 Chemical synthesis of ferulic acid oxidation products

Oxidation products of ferulic acid, were synthesized for use as standards on TLC plates (Fry, 1984b). Ferulic acid was oxidised with 0.75 M ferric cyanide. The products were diluted 1:10 with distilled water prior to application on a TLC plate.

6.2.4 Labelling of cell wall phenolics with [^{14}C]cinnamic acid

6.2.4.1 Feeding of labelled [^{14}C]cinnamic acid to basal L2 segments

37 kBq of [^{14}C]cinnamic acid, (SA 2.07 TBqmol^{-1}), (Amersham International UK) was dispensed in 20 µl into each of 6 separate 200 ml glass conical flasks. The flasks were left overnight to allow the toluene, in which the [^{14}C]cinnamic acid was dispensed, to evaporate.

Three 1g fresh weight samples, of the basal 1 cm (0-1cm from the leaf base) of L2 tissue were collected from both the *rht3* wild-type and *Rht3* mutant lines. One 1 g tissue sample was added to each of 6 conical flasks containing the [^{14}C]cinnamic acid (i.e 3 flasks per genotype) as well as 15 ml of buffer (5mM tartaric acid pH 4.5; with 1 M NaOH). Three incubation periods with the label were used; 10 min, 1 h and 6 h. The experiment was run at room temperature (20 ° ± 2°C). The contents of the conical flasks were shaken every 30 min. As the reaction proceeded 20 µl samples of the buffer were extracted to monitor the uptake of the label by scintillation counting. After each incubation period L2 segments were removed from one flask of each genotype. The alcohol insoluble residue of the tissue was extracted and the phenolic acids released by the method described in section 6.2.3.

6.2.4.2 Autoradiography of [^{14}C] TLC plate and subsequent scintillation counting of the resolved phenolic compounds

A sheet of autoradiographic film (Hyperfilm-HP Amersham International UK), pre-flashed twice, was placed, in the dark, over the TLC plate containing the radioactive compounds. The plate and film were wrapped in 3 layers of aluminium foil and left to

expose, at 4 °C, for 13 months and then developed. The silica gel of the spots on the TLC plate containing the major phenolic acids was carefully scraped off into plastic scintillation bottles. The radioactivity of the phenolic compounds on the TLC plate was determined by adding 2.5 ml of a non-Triton scintillant and counting for 2 minutes.

6.2.5 Extraction and analysis of enzyme activity within the cell wall

6.2.5.1 Extraction of peroxidase activity

Peroxidase activity was extracted from the L2 segments using the method of vacuum infiltration as described Fry (1988) and Hendricks (1989). Segments of L2 (0-1 cm; 0.2-0.5 g fresh weight) were placed into the barrel of a 2 ml disposable syringe with a glass wool plug at the bottom. A plug of aluminium foil was used to retain the segments in the syringe during the vacuum infiltration. To release the soluble apoplastic peroxidase activity, the syringe was submerged in distilled water in a large stoppered Buchner flask. A vacuum line was attached to the flask for 10 min; once the vacuum was released the segments were left submerged to infiltrate for a further 5 min. Segments were removed from the syringe and gently surface dried on tissue paper. The segments were reloaded into the syringe and centrifuged at 1000 rpm for 10 min. The expelled apoplastic fluid was collected in an open Eppendorf tube positioned under the end of the syringe. Ionically-bound apoplastic peroxidase activity was removed by repeating the procedure, except that 100 mM NaCl was vacuum infiltrated instead of distilled water.

Covalently-linked peroxidase activity was measured in the L2 segments, after removal of the soluble and ionically-bound activities. Segments were ground in a pestle and mortar with 2 ml of 1 M NaCl, 1 % Triton X-100 and 0.5 % BSA, washed three times with the same solution and a further three times with distilled water. Cell walls were finally resuspended in 10 ml of distilled water prior to the assay (Fry, 1988).

6.2.5.2 Peroxidase assay

Peroxidase (EC 1.11.1.7) was assayed using *o*-dianasidine (Sigma) as a substrate (Fry, 1988). Prior to the assay all extracts were diluted with distilled water. The reaction was followed at 320 nm in a Philips PU8700 spectrophotometer. The initial 25 s of reaction was used to calculate the activity of the enzyme in samples extracted by vacuum infiltration. Covalently-bound peroxidase activity was assayed *in situ* on the cell walls. Due to severe sedimentation problems of the walls during the period of the assay, a shorter reaction period of just 10 s was used to calculate the enzyme activity.

6.2.5.3 Fumarase assay

Fumarase activity was monitored in the experiments to provide an indication of the

degree of cytosolic contamination. Fumarase (EC 4.2.1.2) was assayed by the method of Hill & Bradshaw, (1969) using L-malate (Sigma) as the reaction substrate. The assay was monitored, using the spectrophotometer as described above 6.2.5.2 for peroxidase activity and followed at 250 nm for a 2 min reaction period.

6.3 RESULTS

6.3.1 Analysis of phenolic acids

6.3.1.1 Analysis of phenolic acids using TLC

Ferulic acid (FA), *p*-coumaric acid (PCA) and diferulic acid (DFA) were identified by their Rf values and t_r chracteristics, as bound to the primary cell wall of L2 in both the *rht3* wild-type and the *Rht3* mutant lines (Fig. 6.4 & Table 6.1). The spot on the TLC plate corresponding to FA was the largest and the most intense in both genotypes. There were several spots in the tracks of both genotypes for which no standards were available and so were not identified. With the exception of spot labelled number 7, the unidentified spots had similar Rf values and fluorescence characteristics to spots within the track of the chemically synthesized FA oxidation products. A certain proportion of the L2 phenolic acids loaded onto the TLC plate always remained at the origin. This was found in all solvent systems tried (Benzene:acetic-acid (9:2); benzene:acetic-acid:water (7:3:3); chloroform:acetic-acid (9:1) and ethyl acetate:benzene (5:6). It was assumed that these may represent extremely large polymers which contained phenolic acids but remained immobile while the solvent front moved up the TLC plate. However, the spot corresponding to FA and DFA in Fig. 6.4 always showed the same fluorescence characteristics in each solvent system tried.

There were no apparent differences between the *rht3* and *Rht3* lines in the fluorescence characteristics or the intensity of the spots present on the TLC plate.

6.3.1.2 Analysis of phenolic acids using HPLC

The most abundant phenolic acid in all samples was FA, with t_r=18.6mins (Fig. 6.5). In both genotypes, it represented 70-80 % of the total phenolic compounds detected in the L2 segment taken 0-1 cm from the leaf base (Fig. 6.5). In the same segment DFA (t_r=23.6mins) represented 13 % of the total phenolic acids detected. PCA (t_r=13.6mins) was detected as only a very minor peak on the HPLC profile, representing 0.5 % of the total phenolic acids detected. It was assumed to be more abundant from the TLC analysis because it may have chromatographed with an oxidation product of FA that had a similar Rf value (Fig. 6.4). In the segment taken 2-3 cm from the leaf base the relative percentage of FA decreased to 60-70 % in both genotypes. There was a simultaneous increase in the abundance of DFA and several different, unknown phenolic acids detected with t_r *ca* 21-27 min. This observation would be compatible with an increase in the presence of FA oxidation products and the formation of cross-links between wall polymers involving these compounds as the cell wall matures.

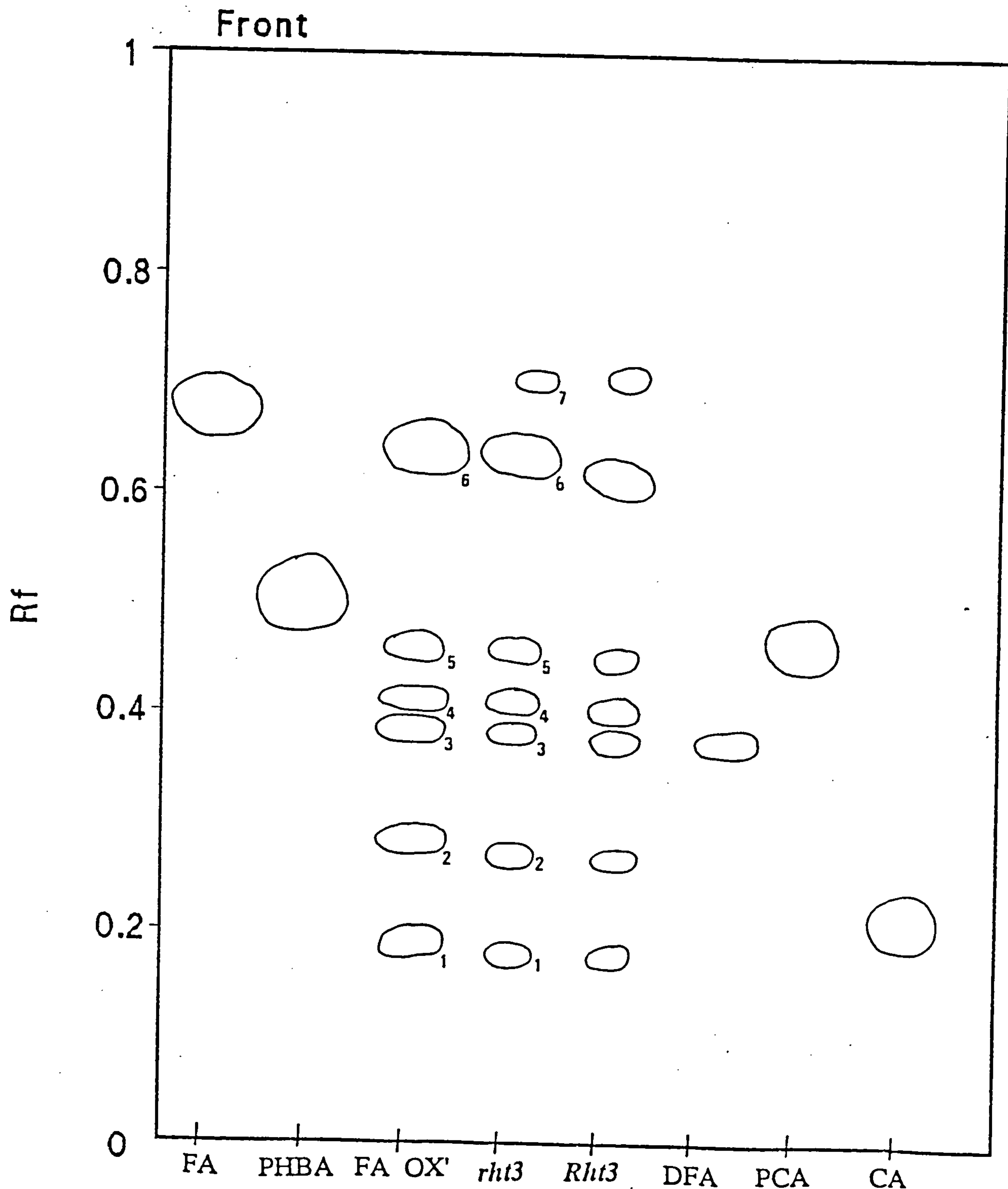


Fig. 6.4 TLC plate of phenolic acids released following saponification and acidification of the alcohol insoluble residue (AIR) of L2 cell wall material from the *rht3* (wild-type) and *Rht3* (mutant) lines grown at 20 °C and sampled on d 9. Phenolics were run in a (9:2) benzene:acetic-acid solvent system, under constant 360 nm UV illumination. For identification of numbered spots refer to Table 6.1. Key to standards used; FA, ferulic acid; PHBA, *p*-hydroxy benzoic acid; FA OX', ferulic acid oxidation products (chemically synthesised); DFA, diferulic acid; PCA, *p*-coumaric acid and CA, caffeic acid. (Front, indicates position of solvent front.)

Table 6.1 *Fluorescence characteristics of phenolic compounds detected by TLC, shown in Fig. 6.4. Spots or sample numbers correspond to the numbers of the spots identified on the TLC plate shown in Fig 6.4. Folin & Ciocalteu's phenol reagent was used to visualise the phenolic acid. Fluorescence notation: (-), no fluorescence; (b. prefix), bright; (d. prefix), dark. Standards identification; (FA), ferulic acid; (DFA), diferulic acid; (PCA), p-coumaric acid; (PHBA), p-hydroxybenzoic acid and (CA), caffeic acid. The rht3 (wild-type) and Rht3 (mutant) lines, samples were taken from saponified L2 cell wall material, grown at 20 °C and sampled 9 d after sowing.*

Phenolic acid fluorescence characteristics						
Sample / Spot N^o	Rf value value	Long λ UV	Long λ UV + NH_3	Phenol reagent	Reagent + NH_3	Possible phenolic
rht3 & Rht3						
1	0.08	-	-	-	grey	?
2	0.16	-	-	-	grey	?
3	0.37	-	-	-	grey	DFA
4	0.40	-	-	-	grey	?
5	0.44	-	violet	-	grey	PCA
6	0.59	grey	b.blue	grey	d.grey	FA
7	0.66	-	-	grey	?	
FA oxidation products						
1	0.08	-	-	-	grey	?
2	0.16	-	-	-	grey	?
3	0.35	-	-	-	grey	DFA
4	0.38	-	-	-	grey	?
5	0.59	grey	b.blue	grey	d.grey	FA
Standards						
FA	0.63	grey	b.blue	grey	d.grey	FA
DFA	0.35	-	-	-	grey	DFA
PCA	0.43	-	violet	-	PCA	
PHBA	0.44	-	b.blue	-	blue	PHBA
CA	0.20	white	b.yellow	grey	mustard	CA

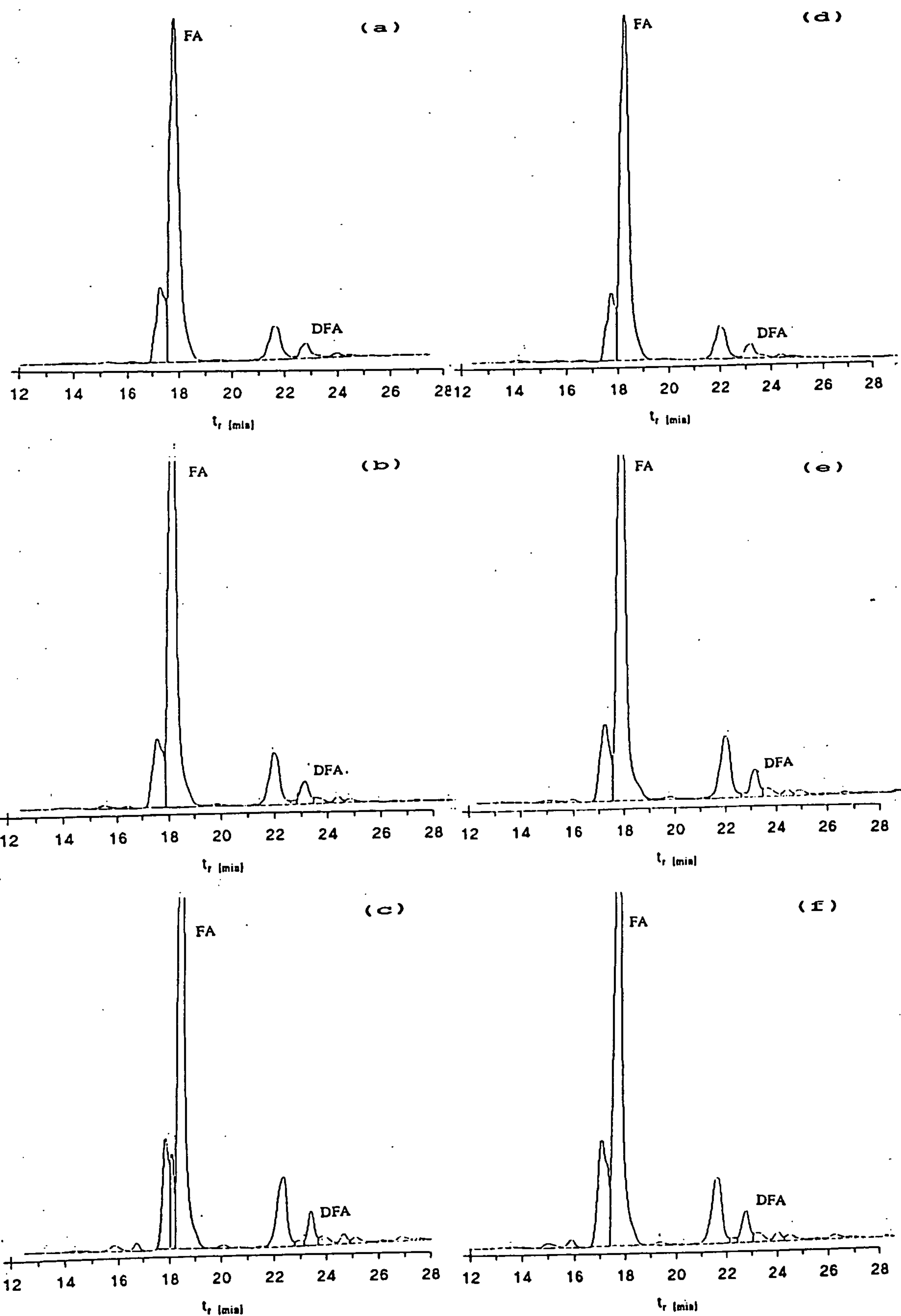


Fig. 6.5 HPLC profiles of phenolic acids released following saponification and acidification of L2 cell wall material taken from three successive 1 cm segments through the extension zone in the *rht3* (wild-type) and *Rht3* (mutant) lines grown at 20 °C and sampled 9 d after sowing. Distance from L2 base segments taken: 0-1 cm (a & d) 1-2 cm (b & e); 2-3 cm (c & f); *rht3* (a, b, c) and *Rht3* (d, e, f) respectively. Compounds were eluted from a C_{18} reverse phase column in an increasing MeOH gradient. The absorbance was monitored at a single wavelength of 320 nm. Peaks labelled FA & DFA correspond to ferulic and diferulic acid respectively.

The concentration of free FA bound to the cell wall tended to increase with distance from the leaf base reflecting an increase in cell maturity (Table 6.2). There was an indication of a greater FA concentration in the *Rht3* mutant genotype in each cm segment of L2 considered, although the difference between the two genotypes was not significant.

Table 6.2 *Concentration of ferulic acid (FA) bound to the cell wall in three successive 1 cm segments taken through the L2 extension zone. Plants of the rht3 (wild-type) Rht3 and (mutant) were grown at 20 °C and sampled 9 d after sowing. Results based on the mean (± s.e.) of two separate experiments, except those marked ([a]) which have been measured only once.*

Genotype	Total FA (mg g^{-1} dry wt)		
	Segment distance from L2 base (cm)		
	0-1	1-2	2-3
rht3	0.48 (0.19)	0.87 (0.10)	0.86[a]
Rht3	0.53 (0.03)	0.96 (0.09)	0.98[a]

DFA is the dimer of FA (Fig. 6.2) and is probably more significant than FA in regulating cell wall extensibility. DFA represents the possible residue of a cross-link which may have existed within the growing cell wall in the L2 segment between two FA acid molecules. If phenolic acid cross-linking is involved in reducing cell wall extensibility the ratio of FA:DFA may be expected to be lower in the slower growing *Rht3* mutant than the *rht3* wild-type line. That is, more FA cross-links would be present within the cell wall of the mutant than the wild-type line. However, the ratio of FA:DFA was not found to be consistently different between the two lines in the L2 segments analysed (Table 6.3). However, as implied by the FA:DFA ratio there was some indication of an increase in DFA in the 1-2 cm segment in the *Rht3* genotype that could have contributed to the reduced relative elemental growth rate (REGR) (Fig. 4.5) compared with the *rht3* wild-type. In both genotypes, the ratio of FA:DFA did not increase markedly in the mature leaf segments sampled 2-3 cm from the leaf base. These observations are consistent with an increased formation of FA oxidation products as the cell wall matures but not particularly with an increase in DFA or a clear difference between genotypes.

Table 6.3 *Comparison of the peak area (x10^5) arbitrary units, of ferulic acid (FA) and diferulic acid (DFA), and in brackets the FA:DFA ratio in 1 cm segments taken through the L2 extension zone of the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C and sampled 9 d after sowing.*

Genotype	Distance of segment from L2 base (cm)		
	0-1	1-2	2-3
	FA/DFA	FA/DFA	FA/DFA
rht3	93.2/5.1 (18.3:1)	256.2/15.2 (16.8:1)	234.0/17.6 (13.3:1)
Rht3	137.4/7.3 (18.8:1)	278.2/20.4 (13.6:1)	261.9/18.6 (14.0:1)

6.3.2 Incorporation of [^{14}C]cinnamic acid into cell wall phenolics of wild-type and mutant lines

An indication of the rate of phenolic acid metabolism and subsequent incorporation into polymers of the cell wall was tested by feeding a precursor of FA, [^{14}C]cinnamic acid 37kBq (SA 2.07 TBq mol^{-1}) to isolated 1 cm basal segments (0-1 cm only), of the *rht3* wild-type and *Rht3* mutant lines. Three different length incubation periods were used: 10 min, 1 h and 6 h.

[^{14}C] FA was detected in the bound form in the cell wall in both genotypes following an incubation period with the label of just 10 min (Fig. 6.6 & 6.7). This suggested that there was a rapid conversion of cinnamic acid to FA and subsequent feruloylation of cell wall polymers (Fig. 6.3) present in both genotypes (Fig. 6.7). Comparison of the equivalent tracks of labelled phenolics compounds shown on the autoradiographic film did not suggest any obvious differences in intensity of labelling of phenolic compounds between the two genotypes.

Measurement of the amount of [^{14}C] label incorporated into individual compounds showed that FA was the most heavily labelled compound in both genotypes, after each incubation period (Table 6.5). However, after 6 h there was significantly more activity incorporated into DFA (37 %), possible PCA (36 %), and FA (55 %), bound to cell wall material of the *Rht3* mutant compared to the *rht3* wild-type.

Table 6.5 *Comparison of the activity, cpm (± s.e.), of [^{14}C]cinnamic acid label incorporated into the presumed DFA, ^{a}PCA (+FA oxidation product) and FA released by saponification and acidification of basal L2 cell wall material. Basal 1 cm L2 segments of the rht3 (wild-type) and Rht3 (mutant) lines were incubated with [^{14}C]cinnamic acid precursor for 10 min, 1 h and 6 h. Plants were grown at 20 °C and sampled 9 days after sowing. (Sig. shows level of significance compared between the two genotypes).*

Phenolic compound	Incubation period	[^{14}C] cpm detected in TLC spot *rht3*	*Rht3*	Sig.
DFA	10 min	65 (4)	61 (11)	n.s.
^{a}PCA	"	48 (7)	54 (4)	n.s.
FA	"	110 (6)	170 (16)	**
DFA	1 h	75 (9)	79 (4)	n.s.
^{a}PCA	"	62 (9)	85 (2)	n.s.
FA	"	484 (9)	485 (11)	n.s.
DFA	6 h	204 (8)	321 (7)	***
^{a}PCA	"	173 (5)	270 (17)	***
FA	"	917 (27)	2070 (34)	***

All ^{14}[C] cpm were significantly above the background level. **/*** = difference significant at $p < 0.01/0.001$ compared to *rht3* wild-type

The [^{14}C] feeding results suggest that the *Rht3* allele may be associated with a faster rate of metabolism of phenolic acids and/or a more rapid feruloylation into polymers within the cell wall. The effect of the *Rht3* allele was clear only after 6 h incubation. However, the results must be interpreted with caution because of several potential sources of error: (1) experimental; the relative efficiency of the phenolic acid extraction itself between the genotypes and the efficiency of transferring all of silica gel from the TLC plate containing the spot into the container using for scintillation counting, (2) theoretical; the possibility that the labelled precursor and/or labelled products may be differentially sequestered into endogenous pools of phenolic acids stored within the endomembrane system of the cell in the wild-type and the mutant lines, (3) the pool size of unlabelled CA may be different between the two different genotypes; and (4) physiological; a 1 cm basal segment of L2 contains a different total number of cells in the two genotypes. There may be more cell wall material capable of incorporating label present within the mutant genotype. However, it was considered that any differences between the two genotypes related to (1), (2) or (3) would also have been evident in samples taken at 10 min and 1 h, in addition to the 6 h incubation period. In addition, the difference in cell numbers between the genotypes (4) in a 1 cm

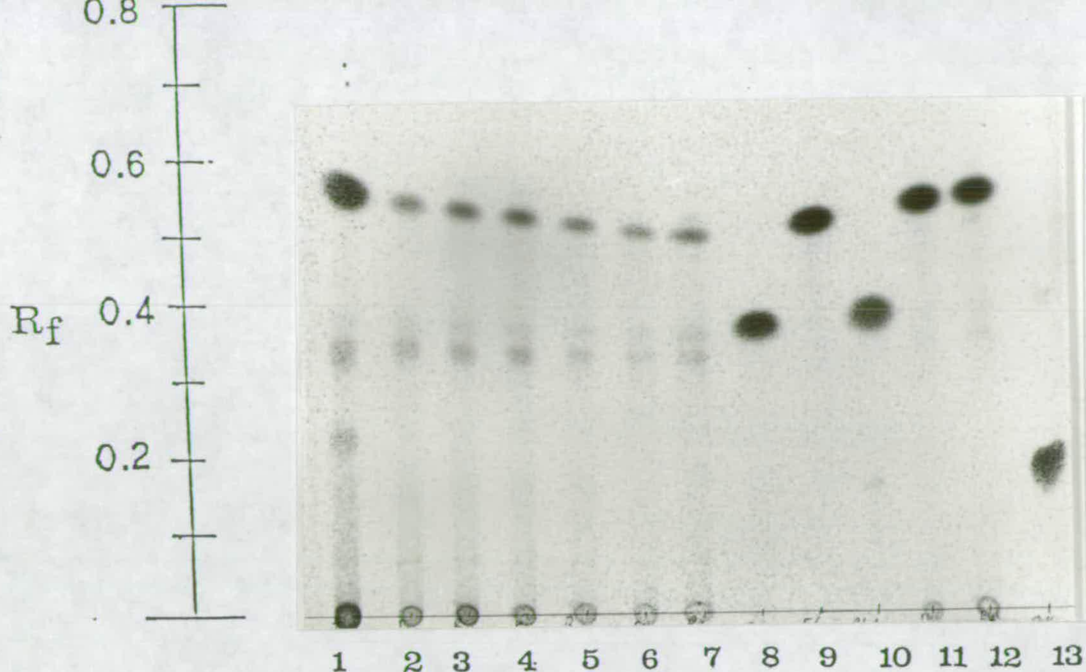


Fig. 6.6 Cell-wall bound phenolic compounds of L2 cell wall material taken 0-1 cm from the leaf base in *rht3* (wild-type) and *Rht3* (mutant) lines. The compounds were resolved by TLC using a solvent system of (9:2) benzene acetic-acid and were developed with Folin and Ciocalteu's reagent. Segments had been incubated for; 10 min, 1 h or 6 h with 37 kBq [^{14}C]cinnamic acid. Plant material was grown at 20 °C and sampled 9 d after sowing. Identification of samples in numbered tracks (incubation time with ^{14}[C] label, where appropriate); 1 = FA, 2 = *rht3* (10 min), 3 = *Rht3* (10 min), 4 = *rht3* (1 h), 5 = *Rht3* (1 h), 6 = *rht3* (6 h), 7 = *Rht3* (6 h), 8 = coumaric acid, 9 = FA, 10 = PHBA, 11 = *rht3* (1 h + FA), 12 = *Rht3* (1 h + FA), 13 = CA.

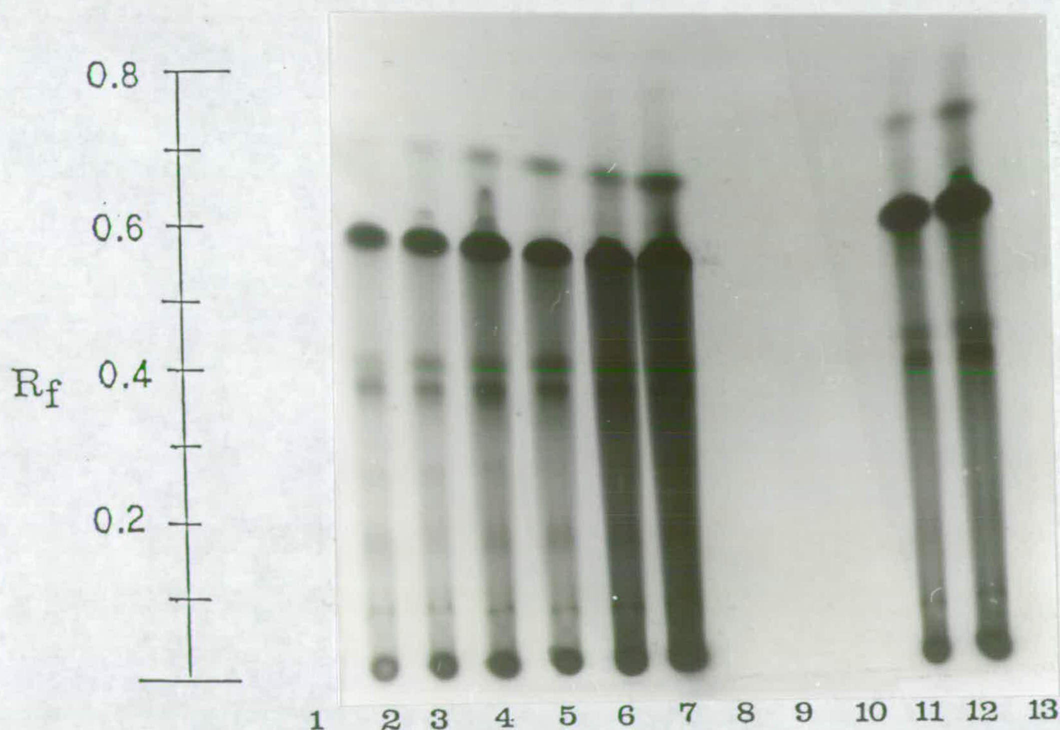


Fig. 6.7 Autoradiogram developed after 13 months exposure to the TLC plate shown in Fig. 6.6. Details of samples are given in figure legend 6.6. Track numbers and samples correspond to those described in Fig.6.6 legend.

segment of L2 is small compared to the differences in the rate of uptake of the ^{14}[C] label after 6 h.

Therefore, it was concluded that there was a significant increase in the rate of incorporation of the ^{14}[C] label in the *Rht3* mutant and therefore a more rapid synthesis of phenolic acids in the cell wall than in the *rht3* wild-type.

6.3.3 Effects of gibberellin and paclobutrazol on bound phenolic acids of the cell wall

Two of the pleiotropic effects of the *Rht3* allele are to confer an insensitivity to applied GA_3 and only a limited response to the growth retardant, 2*S*,3*S* paclobutrazol (Lenton *et al.*, 1987; Chapter 4). Applied growth regulators affect cell wall extensibility (Keyes, 1987 & 1990). They may therefore also be expected to affect the nature or abundance of phenolic cross-links within the primary cell wall of the *rht3* wild-type, where they have a marked effect on the length of the L2 extension zone (*cf.* Chapter 4, section 4.3.2).

Application of GA_3 to the *rht3* wild-type and *Rht3* mutant caused an overall increase in the concentration of phenolic acids bound to the cell wall, and also an increase in the number of different phenolics compounds bound to the cell wall (Fig. 6.8). In the *rht3* wild-type the concentration of saponified FA was increased by *ca* 5.5-fold compared to the untreated control. Surprisingly, GA_3 initiated a lesser, but similar effect in the *Rht3* mutant line. Compared to the untreated control the concentration of free FA bound to the cell wall was increased *ca* 2-fold. Since the *Rht3* mutant plants do not respond to GA_3 by extension growth (*cf.* section 4.3), the results suggest that only that part of the GA_3 response concerned with elongation may be blocked by the *Rht3* allele and that the initial recognition of GA_3 may remain unaffected. However, applied GA_3 only increases cell wall extensibility in wild-type lines (Keyes, 1987; 1990). It therefore seems unlikely that the increased amounts of wall bound phenolics observed following GA_3 treatment are important in regulating cell wall extension, because the changes are the same in both genotypes.

Application of 2*S*,3*S* paclobutrazol initiated a similar increase in phenolic acids covalently bound to the cell wall, in both genotypes (Fig. 6.8). The effect was not so marked as that observed in response to applied GA_3. The response by both genotypes to applied PGRs shows that control of phenolic acid linkage within the cell wall is affected by both GA_3 (a growth promoter) and 2*S*,3*S* paclobutrazol (a growth inhibitor) and that the *rht3* wild-type and *Rht3* mutant are affected in a similar way by their application. The phenolic content of the cell walls could apparently therefore, be altered in a way that was not correlated with the effect of the PGRs on growth and cell extension.

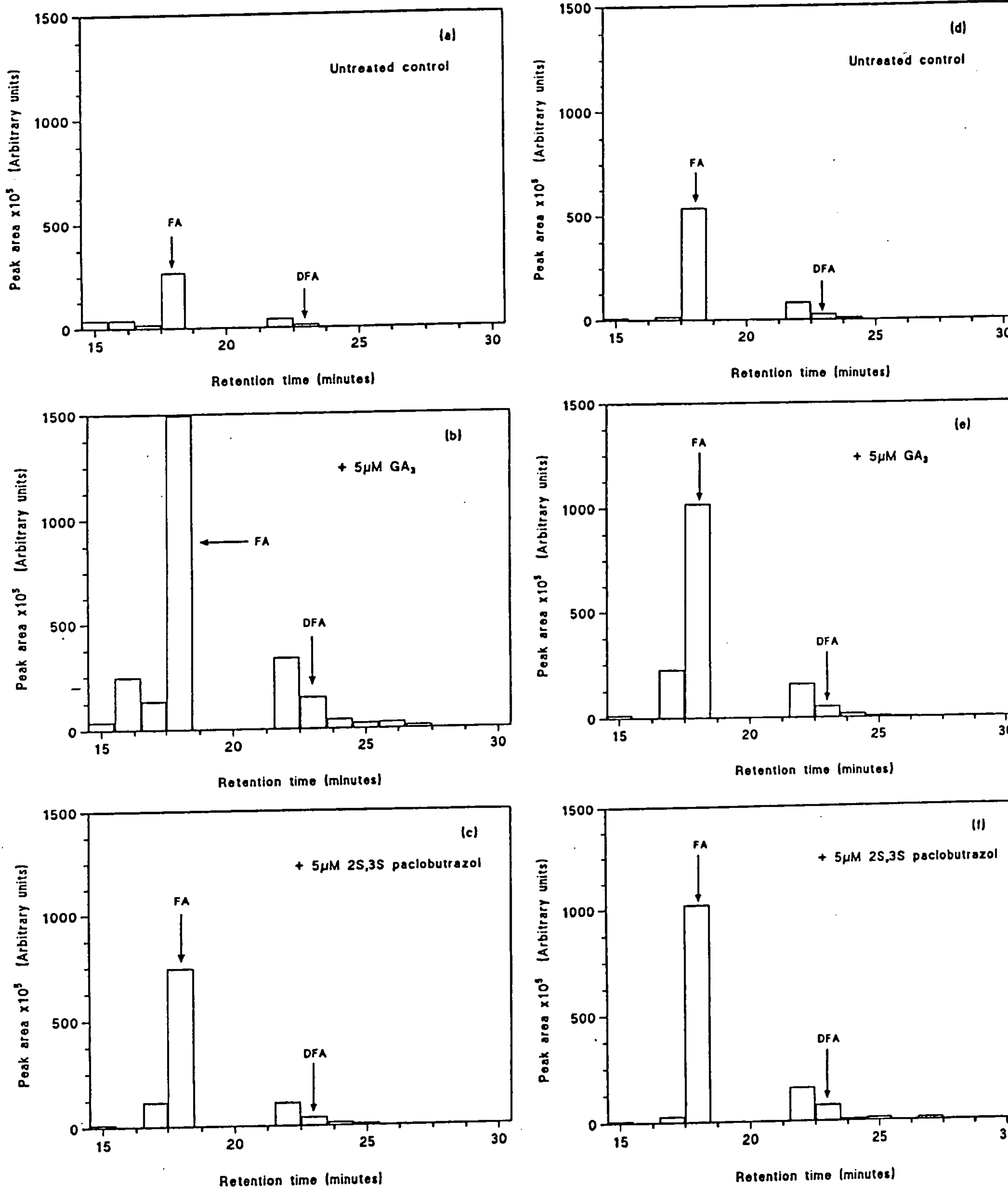


Fig. 6.8 Abundance of phenolic acids extracted into butanol following saponifiaction and acidification of L2 cell wall material taken from the segment 0-1 cm from the leaf base in the *rht3* (wild-type) (a, b and c) and and *Rht3* (mutant) (d, e and f) genotypes. L2 treated with 5 μM GA_3 (b & e) and 2*S*,3*S* paclobutrazol. The abundance of phenolic acids is expressed in arbitrary units of peak area calulated from the HPLC profile. Plants were grown at 20 °C and sampled 9 d after sowing.

6.3.4 Estimation of apoplastic peroxidase activity in the wild-type and mutant lines

Peroxidase is assumed to catalyse the formation of cell wall cross-links involving phenolic acids (Fry, 1979; Epstein & Lamport, 1984). Fumarase activity was assayed as a measure of cytosolic contamination and hence the efficiency of the vacuum infiltration procedure, used to extract peroxidase activity in extracting only apoplastic protein.

A higher proportion of peroxidase activity was found to be freely soluble or ionically linked to the cell walls in both genotypes than was covalently linked to the cell wall (Table 6.6). There were no consistent differences in peroxidase activity observed between the genotypes in either the form in which the enzyme was bound, or, in the different regions of the L2 extension zone from which the filtrate was extracted (Table 6.6).

Table 6.6 *Peroxidase activity (± s.e.) extracted from three successive 1 cm segments through the extension zone of L2 in the rht3 (wild-type) and Rht3 (mutant) lines. Plants were grown at 20 °C and sampled 9 d after sowing. Results show means from 3 different experiments, each including 3 replicate measurements.*

Distance from L2 base (cm)	Peroxidase activity (absorbance units min^{-1} g^{-1} dry wt)		
	Soluble	Ionic	Covalent
rht3			
0-1	363 (85)	407 (85)	109 (44)
1-2	310 (109)	432 (78)	88 (53)
2-3	458 (150)	423 (44)	-
Rht3			
0-1	322 (75)	361 (59)	135 (54)
1-2	300 (98)	342 (67)	101 (42)
2-3	514 (23)	472 (83)	-

- = impossible to obtain any reproducible results (see text).

The covalently linked peroxidase activity was difficult to assay. In the most mature tissue segment taken 2-3 cm, from the leaf base, the tissue was too highly lignified to be thoroughly homogenised and the walls began to sediment to the bottom of the reaction cuvette as the reaction was followed during the continuous monitoring of the assay in the spectrophotometer.

There were significant levels of intracellular contamination in the segments taken 0-1 cm from the leaf base. Fumarase activity, expressed as a % of the soluble peroxidase activity, represented a contamination level of approximately 20 % in both genotypes. However, this basal segment contained the meristematic zone of L2 and cells within this segment were probably structurally weak and particularly susceptible to disruption during the vacuum infiltration procedure. Additionally, there were unlikely to be many air spaces within

the L2 tissue in the basal segment capable of being vacuum infiltrated. Cytosolic contamination of the peroxidase extracts was lower in both genotypes in the two more mature L2 segments. It represented *ca* 10 % and 5 % of the total soluble peroxidase activity in the segment 1-2 cm and 2-3 cm from the leaf base, respectively.

Measurement of the peroxidase activity suggested that there were no significant differences between the *rht3* wild-type or the *Rht3* mutant lines, or between different regions of the growing leaf in either the activity of the enzyme, or the form in which it was bound to the cell wall.

6.3.5 Conclusions: possible involvement of phenolic acid cross-links in rigidifying the cell wall of the *Rht3* mutant

The major effects of the *Rht3* allele on the phenolic acid residues and peroxidase activity of the cell wall can be summarised as being:

1. no obvious qualitative or quantitative difference in the phenolic acids bound to the cell wall, FA being the most abundant phenolic acid present in both lines;

2. no marked effect on peroxidase activity, or the form in which it was bound to the cell wall;

3. an increased rate of incorporation label from ^{14}[C] cinnamic acid into cell wall-bound phenolic acids compared to the *rht3* wild-type;

4. a reduced, but otherwise similar, response to applied PGRs as the *rht3* wild-type.

6.4 DISCUSSION

6.4.1 The possible involvement of phenolic acid wall cross-links in rigidifying the cell wall of the mutant genotype

From the literature reviewed in the introduction a working hypothesis that covalently bound cell wall phenolics may restrict the extensibility of the cell wall was investigated, as well as the possible correlation with peroxidase activity thought to catalyse the formation of phenolic acid cross-links between structural polymers of the cell wall. Since the cell walls of the *Rht3* mutant were less extensible than the *rht3* wild-type line (*cf.* Chapter 5) it was predicted that they would have a greater number of diferulic cross-links per unit length of wall and a higher peroxidase activity than the *rht3* wild-type. However, no difference between the *rht3* wild-type and the *Rht3* mutant lines were found in either the concentration, or the variety of phenolic acids ester linked to L2 cell wall material. Similarly, no differences were found in the peroxidase activity extracted from apoplastic fluid of the two genotypes. Considered together these results do not support the idea that these phenolic acid cross-links perform an important role in rigidifying the cell wall of the *Rht3* mutant genotype. However, feeding labelled [^{14}C]cinnamic acid to equivalent L2 segments (of the basal 0-1 cm, only) appeared to indicate that there was a faster rate of phenolic acid metabolism and subsequent incorporation into cell wall material in the *Rht3* mutant line. Thus, the reduced wall extensibility associated with the *Rht3* allele and the involvement of phenolic acids in rigidifying the cell wall of the mutant may be more complex than predicted by the original hypothesis.

The results imply that the cell walls of the *rht3* wild-type and the *Rht3* mutant lines contain a similar amount of phenolic acids and peroxidase activity, but the phenolic acids which are present appear to be metabolised faster and incorporated into the cell wall at a faster rate in the *Rht3* mutant than in the *rht3* wild-type. Feruloylated matrix polysaccharides in spinach cell walls were shown to be resistant to the action of hydrolytic enzymes (Fry, 1979 & 1984b). Thus, the present results could suggest that a faster rate of feruloylation of cell wall polymers associated with the *Rht3* allele may be an important factor in the determination of cell wall extensibility by restricting the accessibility of other hydrolytic cell wall enzymes. A similar conclusion concerning the relationship between diferulic acid and wall extensibility has been suggested for both the oat (Kamisaka *et al.*, 1990) and rice coleoptiles (Tan *et al.*, 1991).

One problem associated with the results presented in this chapter is that segments of equal length were taken to compare the two genotypes. During the measurement of peroxidase activity it was apparent that the results could be interpreted in different ways depending on the basis used for the comparison. It was difficult to decide which method of expression had the most biological significance. The second leaves of the *rht3* wild-type and

Rht3 mutant lines do not have the same final length or fresh weight (*cf.* Fig. 4.3). Measurement of interstomatal cell length showed that there were approximately 344 ± 69 and 307 ± 35 cells within the most basal 1 cm of L2 lamina in the *rht3* wild-type and *Rht3* mutant respectively. Peroxidase activity could be expressed as organ^{-1}, cell^{-1}, cm^{-1} of organ, g^{-1} fresh wt or g^{-1} dry wt. The difficulty arose as to which method of expression represented the most biologically important factor in controlling wall cross-linking and its subsequent effect on wall extensibility. Table 6.7 illustrates the different ways in which extracted soluble peroxidase activity could have been expressed.

Table 6.7 *Different methods of expression of the soluble peroxidase activity present within the basal 1 cm segment of L2 in the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C. AU = absorbance units measured at 420 nm. (Based on results expressed in Table 6.6 and in chapter 4, sections 4.3.1).*

Basis of expressing peroxidase activity	Genotype *rht3*	*Rht3*
AU min^{-1} g^{-1}. dry weight	363 ±85	322 ±75
AU min^{-1} g^{-1} fresh weight	32.4 ±0.2	29.9 ±0.3
AU min^{-1} " " cell^{-1}	0.093 ±0.015	0.097 ±0.010
AU min^{-1} " " organ^{-1}	924.6 ±41.3	464.6 ±10.6
AU min^{-1} " " mm^2 L2	373.5 ±18.1	315.4 ±5.4

Values for organ peroxidase activity have been calculated by multiplying the activity g^{-1} fresh wt cm^{-1} by the final L2 length in the two lines

Thus, if the peroxidase activity is expressed on an organ (per leaf), basis there is apparently a very large difference in soluble peroxidase activity between the two genotypes. However, by all other bases of comparison shown in Table 6.7 there is no obvious difference in peroxidase activity between the two genotypes. It was considered that the most important bases to express peroxidase activity were either peroxidase activity per cell or activity per g dry weight. It has been shown that there is an approximately equivalent photosynthetic (Morgan *et al.*, 1990) and respiratory capacity (King *et al.*, 1983; Morgan *et al.*, 1990) between the two genotypes. This may suggest that there is the same net amount of wall material present in L2 of both genotypes, but that it is condensed into a shorter length in the *Rht3* mutant genotype compared to the *rht3* wild-type.

The comparison of the relative importance of phenolic cross-linking within the cell wall of the two genotypes is also complicated by the observation that there is a whole range of different FA oxidation compounds bound to L2 cell wall material of both genotypes (Fig. 6.4 & 6.5). Diferulic acid was the only oxidation product of FA for which an authentic standard was available. These other compounds may also be of relevance with respect to their potential ability to form cross-links between structural wall polymers and stiffen the cell wall. There may also be differences between the two genotypes in the amount of ether-linked phenolic acids, which have not been measured in the present experiments. In addition,

truxillic acids have also been identified in cell walls of *Lolium multiflorum* (Hartley *et al.*, 1988) and *Cynodon dactylon* (Hartley *et al.*, 1990). Truxillic acids may be formed by the photodimerization of *p*-coumaric or ferulic acid (Hartley *et al.*, 1988). Although not investigated in the present study, these compounds could form cross-links within the cell wall which may be important in rigidifying the cell wall of the *Rht3* mutant. Theoretically, all of these other types of phenolic cross-links should be investigated before the involvement of the *Rht3* allele with such compounds can be dismissed.

6.4.1.1 What quantitative difference in cross-linked phenolic acids would be sufficient to produce a significant effect on the extensibility of the cell wall ?

It is interesting to consider what increase in cross-linked phenolic acids would be required to reduce cell wall extensibility in the *rht3* wild-type line to that of the *Rht3* mutant (*cf.* Chapter 4). Fry (1979) calculated that the formation of one diferuloyl bridge per 3000 sugar residues would be sufficient to cause matrix gelation in the cell wall. He assumed that if the specific polysaccharide capable of gelation made up 10 % of the total cell wall polysaccharides, then a ferulate level of approximately 0.01 % dry weight of polysaccharides should allow gelation. If similar assumptions are made for the L2 system of wheat, there is *ca* 10 times sufficient feruloylation present within the cell walls of L2 tissue to cause matrix gelation in both the *rht3* wild-type and the *Rht3* mutant lines. Similar calculations of an apparent over-capacity for gelation have been made for spinach cells, (Fry, 1979), wheat flour (Fausch *et al.*, 1963) and *Lolium* (Hartley, 1973). Considered in isolation the apparent over-capacity for matrix gelation present in both genotypes of wheat implies that phenolic cross-links may not be primarily responsible for the reduction in wall extensibility associated with the *Rht3* allele.

6.4.2 The nature of the relationship between final plant height and the level of peroxidase activity

There were no clear differences in peroxidase activity between the two genotypes although there was an indication of an increase in soluble and ionic activity in the most mature segments (Table 6.6). In addition to the peroxidase assays in the segment of L2 taken through the extension zone (*cf.* section 6.3), the activity of the enzyme was also measured in other organs of the wheat plant; stem internodes, flag leaf, roots and the coleoptile. No consistent differences between the two genotypes were identified in the apoplastic enzyme activity in any of these other organs. No significant differences in the phenolic content between the two genotypes were found in response to the application of either GA_3 or 2*S*,3*S* paclobutrazol. This suggests that the suppression of peroxidase secretion in response to applied GA_3 as suggested by Fry (1979, 1980) may not provide an adequate explanation for the situation which exists in L2 cell wall material of wheat.

A tall phytochrome mutant, *aurea*, of tomato has been shown to contain the same level of covalently bound wall peroxidase activity as the wild-type (pers. comm. M. G. Jones) showing that there is no obligate relationship between peroxidase activity and rate of plant growth or final plant height. The observations based on *aurea* could imply that the response to light may be involved in the relationship of peroxidase and the rate of growth. The possible interaction of phytochrome, peroxidase and the rate of cell extension has also been suggested in other higher plant systems e.g. mustard internodes (Casal *et al.*, 1990) and cucumber hypocotyls (Cosgrove, 1988).

6.4.2.1 Localisation of phenolic acids and peroxidase

Valuable information about the localisation of phenolic acids and peroxidase is lost by tissue extraction. It has been suggested that the epidermal cells may be a primary target site for growth regulation, or may be the cells most directly involved with the control of growth (Brummel & Hall, 1980; Kutschera, 1987; Kutschera *et al.*, 1987; Kutschera & Briggs, 1987). FA has been shown to be ester-linked to cell wall polysaccharides particularly within the upper and lower epidermal layers (Fulcher & O'Brien, 1972; Whitmore, 1974; Harris & Hartley, 1976). In *Petunia* stems most of the peroxidase activity was located in the epidermis and a third of the activity was extracellular (Hendricks & Van Loon, 1990). In addition, in mung bean hypocotyl tissue, peroxidase was localized in junctions between neighbouring cells (Goldberg *et al.*, 1987). It was concluded that the products of peroxidase activity may be restricted to specific sub-cellular sites and that wall cross-links may be restricted to specific areas of the cell wall. Thus, in the present work it is possible that the *Rht3* mutant could have the same total amount of peroxidase activity present in L2 as the *rht3* wild-type, while the sub-cellular distribution of the activity may be different. More detailed measurements on tissue and subcellular localisation of phenolic acids should be conducted before dismissing these compounds as potential regulators of cell extensibility in wheat.

6.4.2.2 The relationship between gibberellins, peroxidase and the rate of growth

It has been proposed by Fry (1979, 1980) that GA_3 suppresses the secretion of peroxidase and so prevents the enzyme catalysing the formation of bonds rigidifying the cell walls. In the present work (section 6.3.3) application of GA_3 was found to cause a significant increase in the level of both free FA and DFA released from the cell walls of the *rht3* wild-type and, more surprisingly, initiate a similar but much reduced response in the *Rht3* mutant. The *Rht3* mutant line does not respond to applied GA_3 with an increase in the rate of growth or an increase in the length of the L2 extension zone as observed in the *rht3* wild-type (*cf.* Chapter 4). It is therefore suggested that a direct causal relationship between gibberellin, the abundance of bound phenolics, the activity of peroxidase and the rate of growth may be too simplistic. The observed increase in bound phenolic acids by the *Rht3* mutant in response

to applied GA_3 is interesting as it suggests that the *Rht3* allele may affect the transduction pathway of gibberellin action and not the initial process of GA recognition itself. There appear to be no published reports which show such an inverse relationship between gibberellin, peroxidase activity and final plant height in GA-insensitive mutants. The results could suggest that cells must display a responsiveness to an active form of gibberellin i.e GA_1 or GA_3, to induce the stimulus for the secretion of peroxidase into the cell wall. In GA-insensitive mutants the lesion in the endogenous GA biosynthetic pathway may prevent the putative stimulus from being induced. Jupe & Scott (1992) studying peroxidase release in the *procera* mutant of tomato recently proposed that internode extension may be regulated by a spatially controlled release of peroxidase, and/or that only those cells which have passed a critical stage of development may be able to respond to the enzyme. In the *Rht3* mutant the effect of the allele may occur before the position in the signal transduction pathway at which such a stimulus is induced, or they may never reach a state when they are responsive to peroxidase, as suggested by Jupe & Scott (1992).

6.4.3 Is there a causal relationship between phenolic acid cross-linking and the reduced wall extensibility present in the *Rht3* mutant ?

The potential existence of cross-links between wall polymers involving phenolic acids has been demonstrated for both the *rht3* wild-type and the *Rht3* mutant lines. The only evidence obtained in the present work to support the hypothesis that phenolic cross-links may be causally involved in the rigidification of the cell wall of the mutant genotype, was derived from the [^{14}C]cinnamic acid labelling experiment. The oxidation of wall-bound FA to DFA can be controlled at three different points: (i) feruloylation of matrix polysaccharides, (ii) peroxidase activity, and (iii) oxidant formation (Kamisaka *et al.*, 1990). The present results are compatible with the conclusions regarding phenolic acid cross-linking reached by Kamisaka *et al.*, (1990) and Tan *et al.*, (1991) based on the *Avena* coleoptile and rice coleoptile respectively, that wall extensibility may be controlled at the level of the feruloylation of the polysaccharide within the cell wall. Thus, wall extensibility in the *Rht3* mutant, genotype may be reduced as a consequence of the faster rate of phenolic acid incorporation (feruloylation) into cell wall polymers. This effect may make the polymers more resistant against other hydrolytic enzymes, which in the *rht3* wild-type line may continue to maintain the cell wall in a state capable of loosening and extending for longer and over a greater length of L2. The overall conclusion from both these sets of work was that while DFA may be an important factor in determining the mechanical properties of the cell wall, it is probably not the sole factor. It is also appreciated that there are other types of phenolic acid cross-link which have not been considered in the present work.

Considered overall, it is concluded that there is no convincing evidence to support the hypothesis that phenolic acid cross-links are primarily responsible for the reduced wall

extensibility of the *Rht3* mutant genotype. The [^{14}C]cinnamic acid feeding results however, do suggest that such linkages may mediate in some way in the effect of the *Rht3* allele.

CHAPTER 7

DISCUSSION

7.1 Summary of the present work

The *Rht3* allele had no significant effect on either cell number or cell length within developing stem internodes during the first 15 d of growth. However, subsequent to d 15 internode lengths were reduced in the *Rht3* mutant compared to the *rht3* wild-type (Chapter 3). The second leaf has been used as a model system to study the effects of the mutant allele at the cellular level. The predominant effect of the *Rht3* allele is to limit cell extension along the longitudinal axis of the leaf, whilst having no significant effect in reducing total cell number (Chapter 4). Applied GA_3 increased and 2*S*,3*S* paclobutrazol decreased, both the rate of leaf and cell elongation and the length of the L2 extension zone in the *rht3* wild-type. Neither treatment had any marked effect on the *Rht3* mutant. At 10 °C, growth of L2 in the *rht3* wild-type mimicked L2 growth in the *Rht3* mutant while little effect was seen in L2 growth in the *Rht3* mutant itself (Chapter 4). The effect of the *Rht3* allele on cell extension was correlated predominantly with a reduction in cell wall extensibility but with a lesser effect in reducing cell turgor pressure (Chapter 5). However, the reduction in wall extensibility associated with the mutant genotype did not appear to be related to any alteration in the frequency or type, of covalent cell wall cross links involving phenolic acid residues (Chapter 6).

The effect of the *Rht3* allele at the cellular level may be summarised as a premature slowing down of growth in the mutant cells compared to equivalent cells in the *rht3* wild-type. This suggests that the overriding effect of the *Rht3* allele, in the context of growth, is to control processes which regulate cell extension. Therefore, the measurement and interpretation of factors believed to control the rate of cell extension are central to the elucidation of the effect of the *Rht3* allele itself.

7.2 The control of cell extension: Difficulties in the interpretation of P_{ex}

The *Rht3* allele significantly reduced the P_{ex} component of the wall extensibility term m, in the Lockhart equation (*cf.* section 5.1.1), in segments of L2 grown at 20 °C. However, the biological significance of P_{ex} and how accurately it relates to the rate of cell extension in

the intact plant is not well understood (Cleland, 1971; Metraux *et al.*, 1980; Richmond *et al.*, 1980).

7.2.1 Is P_{ex} really an estimation of m, in the Lockhart equation ?

Cleland (1971) described P_{ex} as 'the potential of the wall at a given moment to undergo turgor driven expansion' and as 'being proportional to the average extensibility over the immediate past'. Thus, P_{ex} can be used to detect a change in m, even though it can not be used to obtain an absolute value of m (Cleland, 1984). The Instron method of measuring wall extensibility, and, the modified version used in this study applies a uni-axial force to the tissue sample. However, turgor pressure, the driving force of cell extension (Ray *et al.*, 1972; Tomos, 1985), exerts a multi-axial force. Application of a uni-axial stress to *Nitella* cell ghosts (cells filled with mercury) induced 3 to 4-fold more elastic extension than the same stress applied multi-axially. In addition, an equivalent interior cell pressure caused greater transverse than longitudinal extension of the cell wall (Kamiya *et al.*, 1963). Using the same system, a different study measured both the P_{ex} and E_{ex} components which may relate to the m term described in the Lockhart equation (Cleland, 1971). While P_{ex} was increased 10-fold, E_{ex} was only increased 2-fold when stress was applied in a uni-axial as opposed to multi-axial direction (Richmond *et al.*, 1980). The difference between the two extensibilities was believed to be the result of microfibril reorientation in response to a longitudinally applied force (Richmond *et al.*, 1980). Indeed, Cleland (1971) suggested that the difference in the plastic compliance (strain/stress) between *in vivo* and *in vitro* measurements may represent loosening events which occurred in the period of growth 60-90 min prior to the tissue sample being killed. In the present study, P_{ex} was also measured in live L2 segments. Hence, biochemical reactions which may loosen the cell wall could theoretically continue through the period of the test. In practice the process of segment excision itself probably reduced the measured wall extensibility (Cosgrove *et al.*, 1984; Pritchard *et al.*, 1988).

There is an obvious problem in the present work concerning how accurately P_{ex} reflects the ability of the cell wall to extend. The *Rht3* allele reduced P_{ex} by *ca* 70 % in L2 at 20 °C, while it reduced the maximum rate of L2 extension by *ca* 40 % compared to the *rht3* line. However, when L2 was grown at 10 °C, there was no reduction in P_{ex} while the maximum rate of L2 extension was still reduced by the *Rht3* allele by *ca* 17 %. It is possible that there may be a systematic error of *ca* 20-30 % in which both environmental and technical difficulties may affect the measured value of P_{ex}. However, in the present work, while it is appreciated that P_{ex} may not give an accurate representation of the ability of the cell wall to extend, P_{ex} can provide a good estimate of it.

7.2.2 The heterogeneous nature of the leaf segment

In the present work P_{ex} was determined from the heterogeneous L2 segment. To

produce a more complete comparison between the *rht3* and *Rht3* lines, ideally P_{ex} of the epidermal and inner cell layers should have been measured separately. P_{ex} of peeled maize coleoptiles and pea internodes was approximately twice that of non-peeled controls, while E_{ex} was only slightly changed (Kutschera & Briggs, 1987; Kutschera *et al.*, 1987). Using the creep test a similar result was found for cucumber hypocotyl sections (Cosgrove, 1988). The extensibility of outer epidermal and cortex layers was lower than that of the inner cortex and pith in segments of lupin hypocotyl (Penny *et al.*, 1972). However, the epidermal cell layers display morphological differences compared to internal cell layers of the leaf. Epidermal cell walls can be up to 10 times thicker (Fry, 1988) and can be coated with cutin and waxes (Northcote, 1972; Biggs & Fry, 1987). The existence of such morphological and biochemical differences between the external and internal cell layers of the leaf itself suggests that the mechanical properties of all cell types may not be the same. The importance of the epidermis in the control of growth has most often been considered in coleoptile or hypocotyl model systems in response to applied auxin. The outer epidermal layers have, in most cases, been considered to represent the controlling influence on the rate of growth (Thimann & Schneider, 1938; Tanimoto & Masuda, 1971; Firn & Digby, 1977; Darvill *et al.*, 1978; Brummell & Hall, 1980; Pope, 1982; Kutschera, 1987; Kutschera & Briggs, 1987; Kutschera *et al.*, 1987). By analogy, if the epidermal layers in the L2 system control the rate of organ growth, they could represent a likely target tissue for the effects of the *Rht3* allele.

If the epidermis had been peeled off L2 samples in the present work it would have allowed a direct comparison of whether the *Rht3* allele may specifically affect properties of the epidermal cell layers of L2. More preferable still, would be the ability to measure P_{ex} of individual cells, instantaneously, within the growing leaf.

7.2.3 Are tissue tensions important in the L2 system ?

It is believed that the yielding properties of the outer epidermal wall are critical as it restrains the stresses generated by inner cells and by the turgor of the epidermal cells themselves (Green, 1980). Steady growth may involve the co-ordinated action of wall loosening in the epidermis and regeneration of tension by the inner tissues (Kutschera *et al.*, 1987). Hence, the peripheral walls are under tension, imposed by the turgor pressure, while the extensible inner walls are under compression (Kutschera, 1989). The vacuolation of cells and the build-up of internal turgor pressure in a system in which cells are cemented together and not free to move, could be responsible for the generation of tissue tensions in the growing root system (Burstrom, 1977; Barlow, 1989).

The concept of differential tissue tensions may be important with respect to the effect of the *Rht3* allele as it was noticed that leaves of the mutant genotype were often crimped in the mature region (Plate 7.1).

Plate 7.1 'Crimping' observed in higher leaves of the *Rht3* mutant line grown at 20 °C.

This effect, while frequent in the *Rht3* line, was rare in the *rht3* wild-type. This observation suggests that the *Rht3* allele may influence growth of one cell type more than another so that different rates of growth occur in epidermal and inner cell layers. Alternatively there could be asynchronous cessation of cell extension in the different cell layers of the leaf. An example of a mutation causing such an effect is the knotted (*kn1*) mutant of maize. In mutant plants, finger-like projections grow in the region of the lateral veins of otherwise anatomically normal leaves. This dominant mutation has been shown to encode a novel protein which delays the normal differentiation of internal cell layers by a set amount of time, without altering the rate of cell division itself. As a consequence, developmentally younger cells are out of place in older tissue and continue to divide while cells in the surrounding tissue have stopped. The overall effect of the mutation is one in which the internal parenchyma cells do not recognise their chronological age (Freeling *et al.*, 1988). The crimping of leaves in the *Rht3* genotype may be consistent with the allele affecting the synchronization of cell division and extension in the different cell layers of the leaf and/or affecting the polarity of cell divisions.

Imposed stress has also been shown to affect the polarity of cell divisions (Yeoman & Brown, 1971; Lintilhac, 1974a & b). Externally applied compressive stress controlled the direction of cell wall formation in actively dividing *Coleus* internode tissue (Lintilhac & Vesecky, 1981). The results suggested that the stimulus controlling cell plate formation could be mechanically transmitted and sensed. The *Rht3* allele may therefore induce changes in the orientation of cell plate formation which may generate internal tissue tensions that are subsequently manifest as crimping of the leaves.

A detailed anatomical study of L2 development using sections through L2 in the *rht3* and *Rht3* lines could provide information about how the *Rht3* allele may, or may not, influence patterns of growth within internal cell layers of the leaf. Such a study would also give an estimate of how important tissue tensions may be in the mechanism through which the *Rht3* allele operates.

7.2.4 Wall synthesis and wall loosening

The reduction in P_{ex} induced by the *Rht3* allele is itself the end result of a relatively unknown sequence of events. Wall extensibility is thought to be controlled by both the rate at which wall loosening events occur, and by the physical extension which results from each loosening event (Cleland, 1981). Wall loosening may involve: (a) wall synthesis, involving the intussusception of wall polymers and/or (b) wall degradation, involving the cleavage of load-bearing bonds (Cleland, 1981). The relative importance of (a) and (b) has been a source of debate. The thickness of the cell wall in *Avena* internodes treated with GA_3 remained the same as the untreated controls, suggesting that wall synthesis must contribute to GA-induced cell elongation (Kaufman & Dayanadan, 1983). Application of GA_3 to dwarf pea internodes

increased the rate of protein synthesis but not sufficiently to keep pace with the rate of cell expansion. However, the amount of cell wall per unit length remained constant over the period of expansion (Broughton & McComb, 1967). In contrast, several studies of auxin-stimulated growth suggest that wall synthesis does not contribute to cell elongation (Rayle *et al.*, 1970; Edelmann *et al.*, 1989; Kutschera, 1990). A recent study suggested that two entirely separate auxin-stimulated mechanisms, synthesis and cleavage, may exist in the outer epidermal wall of the pea internode (Bret-Harte *et al.*, 1991). While auxin always appeared to initiate wall breakdown (bond cleavage), whether it simultaneously induced wall synthesis depended on the available substrate conditions, including the addition of glucose to the incubation medium.

A further level of potential control is via the wall loosening factor (WLF). While wall loosening is an extracellular event; the site of action of plant growth regulators appears to be intracellular (Cleland, 1981, 1986). Therefore, cellular control of wall loosening could be effected by (a) the rate of release of WLF and/or (b) the capacity of the walls to respond to the WLF (Cleland, 1981). Only two wall loosening factors have, as yet, been identified: protons (H^+) (Rayle & Cleland, 1977; Taiz, 1984) and calcium ions (Ca^{2+}) (Cleland & Rayle, 1977; Moll & Jones, 1981).

Despite the limitations to the interpretation of P_{ex} which have been discussed, different studies have correlated measurements of P_{ex} with the rate of growth (Cleland, 1967; Coutaney & Morre, 1980a). It has been shown that at 20 °C the *Rht3* allele significantly reduced both the rate of cell extension and P_{ex} compared to the *rht3* wild-type. Exactly how closely P_{ex} reflects the rate of cell extension is uncertain and the actual mechanism through which this is achieved is unknown.

Considered overall, the effect of the *Rht3* allele could be to induce a reduction in P_{ex} compared to the *rht3* line via a variety of mechanisms: wall synthesis and/or wall breakdown, the nature and release of the WLF and/or the amount of extension that occurs as a result of each wall loosening event. The present work has only identified the eventual outcome of a, (probably), complex and interactive sequence of events, any of which may be specifically affected by the *Rht3* allele and all of which could have led to the same final result of a reduction in P_{ex}. There is an obvious requirement for future research to resolve exactly which component(s) of such an integrated process may be specifically affected by the *Rht3* allele.

7.2.5 Involvement of specific cross-links or enzyme activities in stiffening the cell wall of the *Rht3* mutant line

The *Rht3* allele appears to stiffen the cell wall and effectively prevent continued wall extension. The results are compatible with the suggestion that one (or more) type(s) of biochemical cross-link within the primary wall of the *Rht3* mutant may be more abundant

and/or formed earlier in development than in the *rht3* wild-type. The present investigation appears to eliminate the direct involvement of cross-links involving phenolic acids in this role (Chapter 5). However, the increased incorporation of the ^{14}C label, from a cinnamic acid precursor, into cell walls of the mutant line suggested that the *Rht3* allele may increase the rate of feruloylation of wall polymers, and therefore perhaps protect such polymers from the subsequent activity of wall hydrolytic enzymes as suggested by Fry (1979). The continued activity of such hydrolytic enzymes in the *rht3* wild-type line may maintain cell wall extensibility for longer. A similar involvement of phenolic acid residues in the process of wall stiffening has been suggested to occur in both *Avena* and *Oryza* coleoptiles (Kamisaka *et al.*, 1990; Tan *et al.*, 1991).

An alteration in the structure of the primary cell wall has also been linked to the effect of the *slender* overgrowth mutant in barley (Pollock *et al.*, 1990). *Slender* increased P_{ex} by *ca* 65 % compared to the wild-type line (Pollock *et al.*, 1990; Pollock *et al.*, 1992). Three lines of evidence indicated that the *slender* mutation induced biochemical changes within the wall; *slender* showed higher levels of sugar autolysis, a lower uptake of ^{14}C into the acid-hydrolysable glucose preparations following a 5 min pulse of $^{14}CO_2$, and a decreased release of uronic acids following treatment with purified hydrolytic enzymes (Pollock *et al.*, 1990). All factors were consistent with the *slender* mutation altering the architecture of the cell wall (Pollock *et al.*, 1990).

The difference in P_{ex} between the two genotypes in the present work suggests that the structure of the primary wall and/or activity of a cell-wall-bound enzyme in the mutant line is modified in some way by the *Rht3* allele. While the identification of such a cross-link or enzyme is hampered by a poor understanding of plant primary cell walls (Fry, 1986), certain wall constituents do appear of interest because of their apparent capacity to influence the rate of cell wall expansion.

7.2.5.1 β-glucans

The metabolism of β-D-glucans has been implicated in the control of cell expansion in grasses (Taiz, 1984; Carpita & Kanabus, 1988; Masuda, 1990; Gibeaut & Carpita, 1991). It has been suggested that the degradation of β-glucans in the cell wall is responsible for wall loosening and cell elongation in maize and oat coleoptiles (Goldberg, 1980; Huber & Nevins, 1980; Hatfield & Nevins, 1987; Labrador & Nevins, 1989). The highest (1-4),(1-3)-β-D-glucan content of *Zea* coleoptiles occurred during rapid elongation. However, when growth ceased the content of (1-4),(1-3)-β-D-glucan decreased (Luttenegger & Nevins, 1985). The activity of β-glucosidase in peas was correlated with the rate of growth in sections of increasing developmental maturity taken from positions below the apical hook (Murray & Bandurski, 1975). The activity of β-glucosidase did not change, expressed on a per cell basis during the process of cell elongation. However, the specific activity declined as a result of the

deposition of new wall material. It is possible that the product of the *Rht3* allele may reduce the activity of enzymes responsible for cleavage of β-D-glucan molecules. The effect of this may be to prevent or reduce the frequency of wall loosening events within the cell wall of the *Rht3* mutant line.

7.2.5.2 Extensins

It is also possible that the stiffening of the cell wall in the *Rht3* mutant line could be related to a greater abundance of cell wall-bound extensin or hydroxyproline rich proteins. Extensins have been implicated in the control of wall architecture and the organisation of wall microfibrils (MF) (Fry, 1988). In particular, it has been suggested that the formation of isodityrosine cross-links cell wall polymers and thereby stiffens the cell wall (Epstein & Lamport, 1984; Fry, 1982, 1984b & 1986; Wilson & Fry, 1986; Biggs & Fry, 1987). The hydroxyproline content of the cell wall increased significantly from rapidly extending regions of pea epicotyl into regions of non-expanding mature tissue (Cleland & Karlsnes, 1967). A faster rate of coleoptile extension underwater, compared to above water, has also been associated with a reduction in the hydroxyproline content of the cell walls (Hoson & Wada, 1980). While more abundant in the cell walls of dicotyledons (Fry, 1989) extensins are present in the primary cell walls of monocotyledons (McNeil *et al.*, 1984; Fry, 1989). The premature decline in the rate of cell extension associated with the *Rht3* allele may be consistent with a greater hydroxyproline content of the cell wall, occurring earlier in development compared to the *rht3* wild-type but information on this is not yet available.

7.2.5.3 Xyloglucans

The activity of the enzyme xyloglucan endotransglycosylase (XET) could also be involved in the mechanism through which the *Rht3* allele may reduce cell wall extensibility. Xyloglucan molecules can hydrogen-bond to cellulose MF (Hayashi *et al.*, 1987), and may cross-link MF and thereby restrict cell extension (Fry, 1989; McCann *et al.*, 1990). It has been proposed that XET performs a role in the initial integration of new xyloglucan chains into the cell wall by linking them onto the ends of older chains (Fry *et al.*, 1992). Transglycosylase enzymes also allow cell extension to proceed via controlled, discrete steps. Xyloglucan molecules can be cleaved and their cut ends reformed with new partners. Despite no net change in the number of bonds being formed, the transglycosylase activity has allowed the cell wall to extend (Cleland & Rayle, 1972; Albersheim, 1976). Although xyloglucan represents only a very small proportion of the primary cell wall of monocotyledons compared to dicotyledons (Fry, 1988), the activity of XET was proportionally much higher in monocotyledons than dicotyledons (Fry *et al.*, 1992). In addition, the highest XET activity was recorded in the fastest growing tissues (Fry *et al.*, 1992). The results suggest that a comparison of XET activity at successive positions through the L2 extension zone of the *rht3* and *Rht3* lines of wheat would be a useful experiment in order to determine the relative

importance of transglycosylation in the premature stiffening of the cell wall initiated by the *Rht3* allele.

7.2.5.4 Deposition of the secondary cell wall

A different hypothesis to explain why cells may stop extending is that based on mechanical limitation. In the *Rht3* line the deposition of secondary cell wall may begin earlier and/or more material may be deposited compared to the *rht3* line. The occurrence of either could effectively restrict the capacity of the primary wall to undergo continued extension. Such a mechanism was proposed by Burstrom (1974) to describe why *Pisum* internode cells ceased to extend. A simultaneous increase in the deposition of cellulose, and later hemicellulose, was observed as cell extension proceeded; the cessation of deposition subsequently coincided with the cessation of cell extension.

This list is by no means exhaustive although it does present some potentially more likely candidates (or combinations of them) which could be responsible for the premature stiffening of the cell wall in the *Rht3* line, and the reduction of final cell length, compared to that in the *rht3* line.

7.3 Possible involvement of fructan biosynthesis in the control of cell extension by the *Rht3* allele

The *Rht3* allele also reduced the turgor pressure term of the Lockhart equation (*cf.* section 5.1.1). However, this effect was considered to be secondary to the more significant reduction in P_{ex} in contributing to the reduced cell extension associated with the *Rht3* allele.

It has been suggested that the metabolic control of fructan metabolism within the extension zone of the leaves of monocotyledons may be significant in the control of the patterns of leaf growth and cell wall deposition (Pollock & Cairns, 1991). Fructans accumulate in high concentrations within the extension zone in leaves of *Festuca* (Schnyder & Nelson, 1987 & 1989; Schnyder *et al*, 1988). A gradient of fructan accumulation has been shown to be correlated with both the rate of water uptake and the leaf elongation rate within the extension zone of these leaves. It is suggested that the *Rht3* allele may reduce the activity of the enzyme which catalyses the formation of fructan molecules or increase the activity of enzymes responsible for fructan hydrolysis. A developmentally regulated decline in the activity of such an enzyme could explain why the reduction in the turgor pressure appears to be greater in more mature regions of the lamina. Either effect may reduce the driving force for cell extension within cells of the *Rht3* mutant line and thus partially explain the reduced rate of cell extension associated with the *Rht3* allele.

It is also interesting to speculate whether the *Rht3* allele may be associated with an effect on the activity of enzymes related to sucrose/starch inter-conversions. The shrunken-2 (*sh2*) mutant of maize lacks any adenosine diphosphate glucose pyrophosphorylase activity.

Kernels of *sh2* accumulate high levels of sucrose and have low levels of starch (Tsai & Nelson, 1966). In addition kernels of *sh2* have a poorly developed endosperm, are shrunken in appearance and are lighter in weight than kernels of the wild-type line. In the present study grains of the *Rht3* mutant were characteristically more shrivelled and weighed less than grains of the *rht3* wild-type line (0.0394 ±0.0018 & 0.0506 ±0.0022 g per grain (n=50) respectively). The severity of the *Rht* gene dose has a proportional effect in reducing grain weight (Allan & Prichett, 1980; Pinthus & Levy, 1983; Law, 1989; Pinthus & Gale, 1990). However, although this feature has often been attributed to increasing competitive effects within the spike, a reduction in grain weight is also observed in lines which showed no increase in yield compared to wild-type lines (Fischer & Stockman, 1986) or decreased yield (Pinthus & Levy, 1983).

There is evidence to suggest that the *Rht3* allele affects growth within the embryo of the grain during embryogenesis (Flintham, 1981). Cell number was reduced in the coleoptile of the *Rht3* mutant compared to the *rht3* wild-type line prior to the phase of development when cell division began. It is possible that the *Rht3* allele may, in addition to its other pleiotropic effects (*cf.* Chapter 1), also alter starch metabolism. Such an effect may be manifest in the ear by the shrunken appearance of the grain and in the shoot by the reduced turgor pressure of expressed cell sap. However, the putative effect of the *Rht3* allele to reduce the cell turgor pressure, through whatever mechanism this may be achieved, is only considered to be a subsidiary mechanism through which the *Rht3* allele reduces the rate of cell elongation compared to the *rht3* wild-type line. The main effect of the *Rht3* allele appears to be to reduce the extensibility of the cell wall.

7.4 Could the *Rht3* allele induce a reorientation of growth?

The reduction in P_{ex} associated with the *Rht3* allele could perhaps be explained in part through an effect of the allele in increasing cell width and wall thickness, at the expense of cell length. If so, the effect of the *Rht3* allele may be to alter the polarity of cell growth. This may be consistent with the slight increase in epidermal cell width and L2 thickness in the *Rht3* line compared to the *rht3* wild-type at 20 °C. In the present work, stem width, measured in the third internode below the peduncle was shown to be greater in the *Rht3* mutant line compared to the *rht3* wild-type (0.36 & 0.27 cm respectively). Therefore, the *Rht3* allele may compensate for a reduction in internode length with an increase in internode diameter. Both the *rht3* and *Rht3* lines have similar specific leaf areas of L2 at both 10 & 20 °C (Table 4.8 & 4.18). However, as the *Rht3* allele reduces the final length of L2 this suggests that the total growth is different between the two lines. In the *rht3* wild-type the polarity of cell growth may be along the longitudinal axis of the lamina, while in the *Rht3* mutant, cell polarity may be shifted slightly towards the transverse axis of the leaf. This may

induce a greater degree of internal tissue tension. An increase in the thickness of the cell wall or, an increasing tendency for the orientation of longitudinal MF would also be consistent with the premature reduction of cell wall extensibility associated with the *Rht3* allele. Subsequently cell ageing would render the cell wall totally inextensible in both genotypes (*cf.* section 5.3) as has been demonstrated in *Lolium* leaves (Pollock *et al.*, 1990) and in graminaceous coleoptiles (Furuya *et al.*, 1972; Kamisaka *et al.*, 1990). The phenocopy of the mutant line produced in the wild-type by the application of 2*S*,3*S* paclobutrazol also showed reduced cell length but increased cell width (Table 4.12). Other studies have demonstrated that application of GA-biosynthesis inhibitors induce increased lateral growth at the expense of longitudinal extension (Mita & Shibaoka, 1984; Goa & Hofstra, 1987; Tanimoto, 1987). Such results suggest that endogenous GA may regulate the polarity of cell growth rather than have any effect in increasing growth *per se*, as also suggested by Keyes (1987).

Also compatible with such a hypothesis is the observation that the basic metabolism of the mutant genotype remains unaffected by the *Rht3* allele in terms of rates of respiration, amino acid uptake and protein synthesis (Ho *et al.*, 1981). In addition, the Norin 10 semi-dwarfing alleles, *Rht1* and *Rht2*, have been shown to contain effectively the same photosynthetic capacity as the wild-type lines but packed into a smaller cell (Morgan *et al.*, 1990). A brief study was made in the present work of the chlorophyll content of L2 in the *rht3* and *Rht3* lines, measured using a chlorophyll meter. On a unit area basis, the *Rht3* mutant genotype had 38 % more total chlorophyll per unit area and 40 % less surface area in L2. The effect of the *Rht3* allele would appear to be to condense the same total chlorophyll into a leaf of smaller surface area than that in the *rht3* line. Such a conclusion would be consistent with cells being smaller in the *Rht3* line (which they are except when both lines have been treated with 2*S*,3*S* paclobutrazol (Table 7.1)).

Table 7.1 *Comparison of calculated cell volumes in abaxial interstomatal cells of L2 in the rht3 (wild-type) and Rht3 (mutant) lines grown at 20 °C and sampled on d 9. Plants treated with 5 µM* GA_3 *and 5 µM 2S,3S paclobutrazol. Cell volume calculated using the equation,* $V = \pi r^2 l$*, where r = cell diameter/2 and l = cell length. Data of Table 4.12.*

Treatment	Cell volume (x10^4 µm^3) *rht3* (wild-type)	*Rht3* (mutant)
Untreated	10.04	6.82
GA_3	9.48	7.56
Paclobutrazol	7.45	6.10

Treatment with GA_3 does not appear to markedly affect total cell volume in either genotype (Table 7.1). The reduction in cell volume in leaves of both genotypes treated with

2*S*,3*S* paclobutrazol may be due to the fact that the growth inhibitor had an additional effect in reducing cell growth (*cf.* section 4.3.2). The important comparison, with cell volumes in the untreated controls does suggest that final cell volume is *ca* 30 % less in the *Rht3* line than the *rht3* line (Table 7.1). However, a true comparison between the two lines requires an estimation of the cell wall thickness. It is possible that the *Rht3* allele may have a dual effect, to reorientate the polarity of cell growth and to direct a greater proportion of assimilate to the side walls of the cell. Unfortunately such measurements were not made in the present study. It is suggested that interferometric techniques recently described by Baskin *et al.*, (1987) and Bret-Harte *et al.*, (1991) could provide an efficient way of determining whether the *Rht3* allele may be associated with the production of thicker cell walls. If such a study did suggest that the *Rht3* allele increased wall thickness compared to that in the *rht3* line, the evidence would imply that the *Rht3* allele does reorientate growth to some extent. By implication, such a result would suggest that one of the functions of endogenous GA, or sensitivity to it, is to specifically promote growth along the longitudinal axis of the leaf. Thus, an important distinction between the opposite effects of the *Rht3* allele and applied GA_3 is that the *Rht3* allele obviously does reduce the total growth potential of the mutant line compared to the *rht3* wild-type. This is shown by the reduced final L2 dry weight at both growth temperatures compared to the *rht3* line (Fig. 4.3 & 4.12). However, applied GA_3 may only affect the polarity of cell extension and not the growth potential *per se*.

The *gib-1* mutant of tomato which is insensitive to endogenous GA initiates a change in the polarity of growth. While cortical cell length was reduced in *gib-1* cells, there was a compensatory increase in cell width. Cortical cell volume was calculated to be the same in both the *gib-1* mutant and wild-type cells (Barlow *et al.*, 1991). Moreover, it was suggested that the function of endogenous GA was to affect the polarity of cell growth and the relative amounts of growth at the longitudinal and transverse walls of the cells thereby regulating cell shape.

Overall, it is proposed that there may be a certain amount of reorientation of growth associated with the *Rht3* allele. The absolute effect of minor alterations in the polarity of cell growth on both P_{ex} and the rate of cell extension is difficult to determine. Any reorientation of growth which may be associated with the *Rht3* allele is, however, considered to be a secondary effect to the major one in which the allele affects some aspect of cell wall biochemistry.

7.5 The importance of GA in controlling cell extension

It could be concluded from the present work that the physiological effects induced by the *Rht3* allele are as a consequence of the influence that the *Rht3* allele has on processes which control longitudinal cell extension. It is therefore logical to conclude that they may be

the same processes which are also influenced by endogenous GA_1 and can be stimulated further in the GA-sensitive *rht3* line by applied GA.

Indeed, application of GA to GA-sensitive plants or plant segments has, in most cases increased wall extensibility (Kamisaka *et al.*, 1972; Adams *et al.*, 1975; Kawamura *et al.*, 1976; Keyes, 1987; Taylor & Cosgrove, 1989; Paolillo *et al.*, 1991). Conversely, application of the GA biosynthesis inhibitor, ancymidol reduced wall extensibility in leaves and sheaths of wheat (Keyes, 1987; Paolillo *et al.*, 1991). It would therefore appear that the *Rht3* allele specifically blocks mechanism(s) through which endogenous GA or applied GA in sensitive plants may prevent wall stiffening (or initiate wall loosening), and irreversible cell extension.

However, the effect of *Rht3* in decreasing, and GA in increasing, wall extensibility may not be exactly opposite processes. Applied GA_3 increased cell length in *Avena* internodes and wall synthesis occurred simultaneously so that wall thickness remained the same in treated and control cells (Kaufman & Dayanadan, 1983). However, the increased wall extension initiated in response to applied GA_3 has been associated with a reduced deposition of silica (Soni *et al.*, 1980; Kaufman *et al.*, 1981) and lignin (Kaufman & Dayanadan, 1983). In the present work leaves of the *rht3* line treated with GA_3 were weaker and more prone to collapse than leaves of the untreated control which themselves were observed to be less rigid than leaves of the *Rht3* mutant. The observed effects may be because silica and lignin deposition cannot keep pace with the increased rate of cell extension stimulated by GA_3.

Growth of tomato cells in suspension culture in the presence of the cellulose inhibitor 2,6 dinitrobenzonitrile produced cells in which the primary cell wall lacked any cellulose/xyloglucan network and so lacked any major load-bearing bonds. The ability of the cells to survive in the absence of such bonds was cited as evidence of the flexibility plant cell walls possess for tolerating changes in cell wall composition (Shedletzky *et al.*, 1990).

By analogy it could be suggested that the *Rht3* allele in wheat may specifically promote the formation of load-bearing cell wall cross-links, perhaps of the cellulose and xyloglucan network within the primary cell wall. Gibberellin, however, may specifically prevent the formation of load-bearing bonds, or promote the formation of weaker-load-bearing cross-links within the cell wall. Thus, the presence of the *Rht3* allele or, in the opposite extreme, application of GA_3, to the *rht3* wild-type may be affecting the formation of different types of cross-links within the primary cell wall. Therefore, either the *Rht3* allele, or exogenous GA_3 (or 2*S*,3*S* paclobutrazol) in the *rht3* wild-type may disrupt the balance which normally exists between the formation of load-bearing and non-load-bearing bonds within the cell wall.

7.5.1 What processes of L2 growth may be blocked by the *Rht3* allele ?

It has been shown that at the organ level the growth processes affected by the *Rht3*

allele and endogenous GA are those which, directly or indirectly, regulate final leaf length, the maximum rate of leaf extension and the rate of cell extension (Chapters 4 & 5). All of these ultimately relate back to the extensibility of the cell wall. However, at the cellular level, the important determinants of final cell length are those mechanisms or processes of growth which affect cells as they pass through the extension zone. The *Rht3* allele - and conditions which mimic the allele, appear to affect cellular development on two superimposable scales, (1) linear, with increasing distance from the leaf base and (2) temporal, the length of time a cell takes to cross the extension zone. The length of the extension zone appears to be controlled directly by the *Rht3* allele (section 4.3.1) and by the semi-dwarfing *Rht1* and *Rht2* alleles (Paolillo *et al.*, 1991). The latter study showed that an increasing *Rht1* and *Rht2* gene dose (and therefore increasing insensitivity to GA) proportionally decreased both the extension zone length and the maximum rate of leaf extension.

On a temporal scale, in the present study, the time spent by a cell moving from the point of maximum REGR to the outer edge of the extension zone was always reduced in the mutant genotype or in conditions which mimicked its effect. This time period is critical in establishing final cell length in wheat (Fig 4.7), in *Lolium* (Schnyder *et al.*, 1990) and in *Festuca* (MacAdam *et al.*, 1989). This particular period of time may represent a developmental window in normal cellular development in which cells are most likely to be controlled by sensitivity to endogenous GA and therefore by the product(s) of the *Rht3* allele.

In order to understand how the *Rht3* allele may prematurely arrest cell extension, in this region of the extension zone it is useful first to consider how GA may promote cell extension in the L2 system of the GA-sensitive *rht3* wild-type, and, by implication what is being prevented from happening in the *Rht3* line.

7.5.2 How may GA affect cell extension in the *rht3* wild-type - what does the *Rht3* mutant line not do ?

The present work has suggested that GA may function in a more inhibitory than promotory manner, to delay the onset of cell maturation and cessation of cell extension (Table 4.11), or to delay processes involved in leaf (Fletcher & Osborne, 1965), or apical (Proebsting & Davies, 1977; Proebsting *et al.*, 1978) senescence.

This idea is compatible with the observations in the present work that application of GA_3 to the *rht3* wild-type increased both the distance from the leaf base at which maximum REGR was achieved and also the extent of L2 extension (Table 4.12). Conversely the *Rht3* allele and *2S,3S* paclobutrazol had the opposite effects and promoted events which stop a cell extending (section 4.3.1). It is therefore suggested that the *Rht3* mutant may represent the 'basic model' of GA-independent growth, without the topping up effect of endogenous GA. A similar conclusion was reached, for GA-independent growth for the *gib-1* tomato mutant (Butcher *et al.*, 1990). The difference in growth between the *gib-1* and the wild-type line was

considered to represent GA-dependent growth. In wheat, it may be that the response of a cell, or sensitivity (in terms of the number of functional receptors or affinity of those receptors (Leopold, 1988; Roberts & Hooley, 1988) for the GA molecule), may only be switched on around the time (or position) of maximum REGR (Fig. 7.1). In terms of the original hypothesis (that the *Rht3* allele and endogenous GA may be affecting the same process(s), which control cell elongation), the dominant product of the *Rht3* allele would appear to saturate the mechanism(s) through which endogenous, or applied, GA may promote cell extension within this region of the L2 extension zone. The suggestion of a developmentally controlled sensitivity to GA is also supported by the effect of *Rht* alleles on stem internode extension.

7.5.3 Involvement of GA in the extension of stem internodes

The first genotypic effect of the *Rht3* allele in reducing cell and stem internode length was not observed until *ca* 15 d after sowing (section 3.3). This appears to coincide with the time when the main activity of cells within internode cell layers of the sub-apical meristem changes from predominantly one of cell division with cell extension, to one of cell extension alone. It is also before the double-ridge stage of apical development, once the initiation of all leaf primordia (and, therefore also initiation of all stem internodes), has been completed. The time when the genotypic effect is first observed may be an external indicator of when cells first become responsive to, or first utilize GA_1. A recent study comparing stem internode extension in the *Le* (tall) and *le* (dwarf) lines of pea found a significant relationship between the endogenous amount of GA_1 and internode length (Ross *et al.*, 1992). Levels of both GA_1 and its immediate precursor, GA_{20} were relatively low around the time of internode emergence and later at impending apical arrest. The results suggest that GAs were not required to control growth at these periods and the cells had therefore not yet acquired, or had lost, sensitivity to endogenous GA. The involvement of the *Rht3* allele however appears to be centred around how GA_1 is utilized to induce increased cell extension, a subject about which little is known.

7.5.4 How important is the GA concentration in the intact plant ?

The relative importance of PGR concentration as opposed to tissue sensitivity to PGRs, in the control of plant development has been a source of discussion (Trewavas, 1981; 1982; 1983). Previous to such debate, it had been often assumed that the concentration of a specific PGR within a plant tissue controlled the magnitude of the growth response that it induced. Such an assumption would appear to be false, especially in the *Rht3* mutant, where high levels of endogenous GA_1 are associated with a lack of any commensurate growth response (Stoddart, 1984; Lenton *et al.*, 1987; Appleford & Lenton, 1991). Scott (1990) has drawn an analogy between the control of the *lac* operon in yeast and the mechanism through

which GA may control plant growth. In GA-insensitive mutants, such as *Rht3* of wheat and d_8 of maize, normally GA-inducible genes may not be transcribed. As a consequence normally GA-repressed biosynthetic genes may be constitutively expressed. Conversely, in the *slender* mutant of barley there may be constitutive expression of GA-inducible genes and the complete shut-down of genes controlling GA-biosynthesis. There is evidence that GA-inducible genes may exist. Application of GA_3 to GA-deficient dwarf mutants of maize and pea initiated both increased and decreased levels of specific translatable mRNAs (Chory *et al.*, 1987). The *lac* operon analogy is also compatible with the suggestion that the product(s) of the *Rht3* allele may be an inhibitor of processes of growth normally regulated by GA. The concept of a growth inhibitor being encoded by a dominant GA-insensitive mutant allele has been suggested both for the *Rht3* mutant of wheat (Gale *et al.*, 1975; Gale & Youssefian, 1985) and the d_8 mutant of maize (Harberd & Freeling, 1988). In addition, evidence of the possible involvement of transcriptional repressor proteins in the control of gene expression in plants has been suggested based on evidence that protein synthesis inhibitors can induce expression of auxin-inducible mRNAs in pea epicotyls (Theologis, 1986). GA_1 is essential for normal leaf growth in wheat (Fig. 7.2; Lenton *et al.*, 1987). In the GA-sensitive *rht3* wild-type line, blocking the level of endogenous GA_1 using increasing doses of the GA biosynthesis inhibitor 2*S*,3*S* paclobutrazol, was correlated with reductions in final L2 length (Lenton *et al.*, 1987).

The accumulation of GA_1 in the *Rht3* mutant genotype has been explained in terms of a lack of action of GA_1 (Appleford & Lenton, 1991). In the *rht3* wild-type, one of the actions of GA_1 is to down-regulate the activity of the enzyme converting GA_{19} to GA_{20} (GA_{19} oxidase), thus resulting in the accumulation of GA_1 in the mutant line (*cf.* Fig 1.1). However, if GA action is blocked in the mutant genotype, GA_{19} would then be metabolised and GA_1 would accumulate before the next rate limiting step in the pathway, i.e. its conversion to GA_8 (Appleford & Lenton, 1991).

While the cellular response to endogenous GA may be developmentally controlled it may also be tissue specific.

7.5.5 Extension of internodes within the rachis of the ear; the effect of the *Rht3* allele

The *Rht3* allele does not reduce either the rate of growth or the final length of internodes within the ear (Youssefian, 1986; Webb, 1987). Therefore, irrespective of the fact that both stem and ear internodes are growing simultaneously for a period in development of the mutant plant, the extent to which they are influenced by the *Rht3* allele is very different. Youssefian (1986) observed that final ear length in the *Rht3* mutant was longer than in the *rht3* wild-type

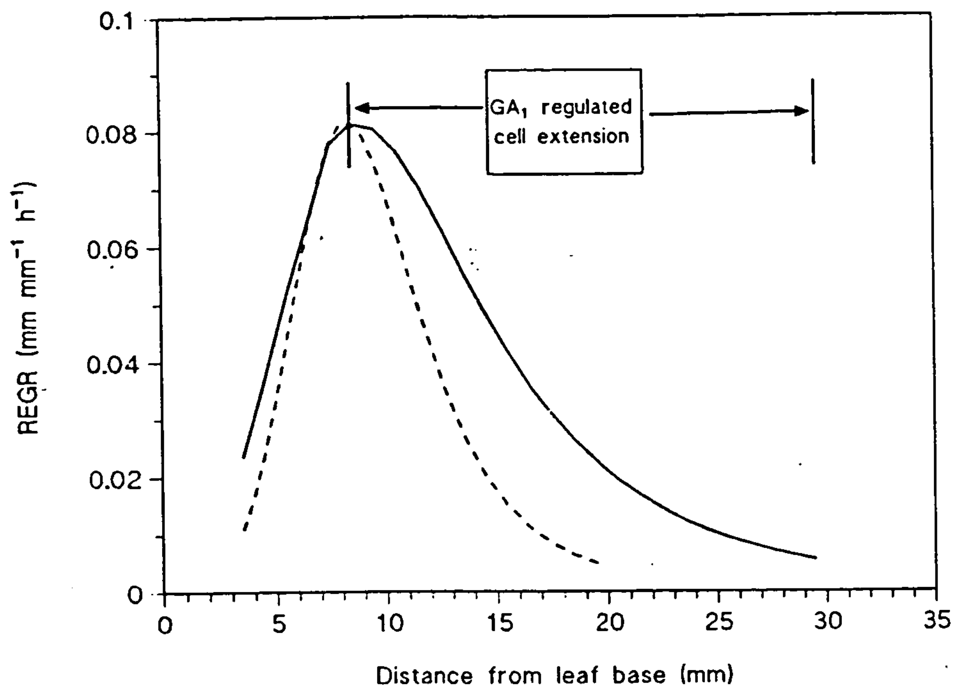


Fig. 7.1 Proposed GA-regulated region of cell expansion in the *rht3* (wild-type), solid line and *Rht3* (mutant), dashed line, of wheat grown at 20 °C.

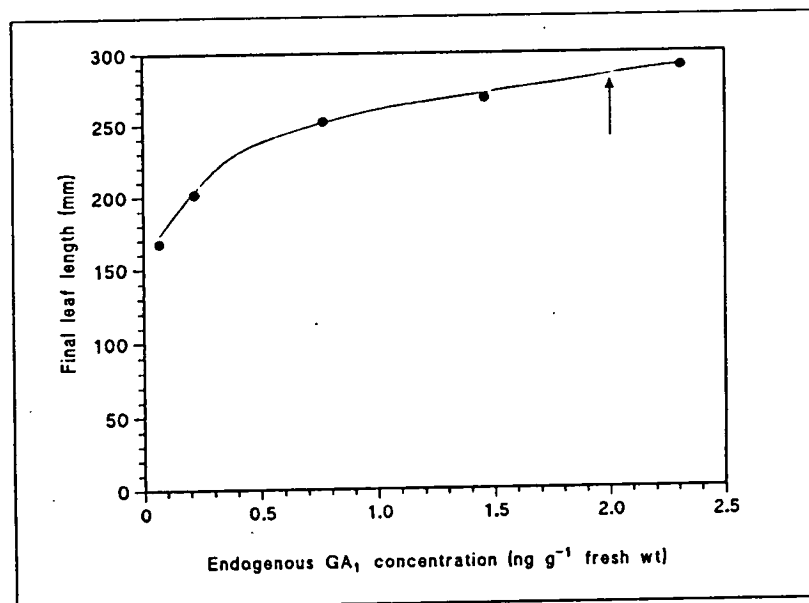


Fig. 7.2 Effect of reducing concentrations of endogenous GA_1 content within the L2 extension zone of the *rht3* wild-type line of wheat on final L2 length, grown at 20 °C and sampled 9 d after sowing, with increasing doses of 2*S*,3*S* paclobutrazol. Arrow indicated the measured level of endogenous GA_1. (Unpublished data of Richardson, Appleford & Lenton).

line. The apparent differential effect of the *Rht3* allele on stem and ear internode extension may be explained by the existence of two different sets of GAs which may regulate growth in vegetative and reproductive organs (Pharis & King, 1985; Kobayashi *et al.*, 1989). Thus, although the *Rht3* line may be insensitive to GA_1 (Radley, 1970; Stoddart, 1984; Lenton *et al.*, 1987) which regulates vegetative growth in wheat (Eckert *et al.*, 1978; Jenson & Juntilla, 1987) the *Rht3* mutant may be equally as sensitive as the *rht3* wild-type line to GA_{54} which is thought to control reproductive growth (Gaskin *et al.*, 1980). Moreover, while the *Rht3* line is associated with a 20-fold increase in the level of GA_1 in basal segments of developing stem internodes (Lenton *et al.*, 1987) and leaves (Chapter 3; Lenton *et al.*, 1987; Appleford & Lenton, 1991) compared to the *rht3* line there was no difference between the two genotypes in the level of GA_{54} in developing grains (Gale *et al.*, 1987).

It could be equally well argued that ear internode extension, and therefore cell extension within ear internodes, may be independent of endogenous GAs in which case it would be misleading to suggest that the *Rht3* allele has different effects in vegetative and reproductive organs. Rather, the *Rht3* allele may block the specific mechanism through which endogenous GA_1 initiates further cell extension, mechanisms which are not involved in the development of ear internodes, in either genotype.

7.5.6 Possible evidence that the *Rht3* genotype is not totally insensitive to GA

The present work has suggested that the *Rht3* allele is not entirely insensitive to GA because:

1) Application of GA_3 increased the concentration of phenolic acid residues bound to the cell wall in a similar way in both the *rht3* wild-type and *Rht3* mutant genotypes (Fig. 6.8, section 6.3).

2) Treatment of the *Rht3* mutant with 2*S*,3*S* paclobutrazol reduced both the final length of L2 and the length of the extension zone (Table 4.10, section 4.3.2).

Therefore, the effects of the *Rht3* allele may be within the transduction pathway of GA rather than on the initial detection of GA. A worthwhile experiment would have been to apply GA_3 + 2*S*,3*S* paclobutrazol to the *Rht3* mutant line. If the small reduction in the length of the L2 extension zone initiated by 2*S*,3*S* paclobutrazol alone could be reversed, this would provide positive evidence that the *Rht3* genotype is not completely insensitive to GA.

7.6 Signal transduction: possible involvement of second messengers and the *Rht3* allele

It is generally accepted that PGRs may work through second messenger systems (Brummel & Hall, 1987). There are several candidates as possible second messengers in

PGR-regulated processes: Ca^{2+}, calmodulin, cAMP, inositol phospholipids (Roberts & Hooley, 1988) or pectic fragments of xyloglucan (York *et al.*, 1984; Farkas & Machlachlan, 1988; McDougall & Fry 1989 & 1990). It is possible that the *Rht3* allele could affect the activity of any one or more of these messengers, preventing the transduction of the GA_1 stimulus and therefore preventing processes essential for continued wall extension. For example, plants do appear to closely regulate their cytoplasmic levels of Ca^{2+} and calmodulin (Gilroy & Trewavas, 1990) and changes in the level of membrane-bound Ca^{2+} can be linked with physiological events (Hepler & Wayne, 1985).

A new and potentially very rewarding technique, which may provide answers to the action of second messengers and the *Rht3* allele, is fluorescent imaging of intracellular Ca^{2+} in the intact plant. High levels of cytoplasmic Ca^{2+} were released virtually instantaneously when a tobacco leaf was placed at 4 °C (Knight *et al.*, 1991). This is interesting as auxanometric measurements of growth in *Lolium* (Pollock & Eagles, 1988; Thomas *et al.*, 1989), in *Avena* coleoptiles (Ray & Ruesnik, 1962) and in lupin hypocotyls have demonstrated an immediate reduction in the rate of growth as the temperature is reduced (Penny *et al.*, 1972). This rapid reduction in the rate of growth in response to low temperature has been cited as indirect evidence that low temperature must act at the level of cell extension rather than cell division (Pollock & Eagles, 1988; Thomas *et al.*, 1989).

It is proposed that the application of such a technique to the *rht3* and *Rht3* lines of wheat grown at 10 ° and 20 °C could yield extremely useful results toward the final elucidation of the mechanism of action of the *Rht3* allele, if Ca^{2+} is involved, not least because experiments could be performed on the live plant. Theoretically, if the *Rht3* allele specifically modified the release of Ca^{2+} ions into the cell vacuole the effect could be instantaneously visualized and the response quantified.

7.7 Is the *Rht3* mutant more of a high temperature insensitive mutant than a GA-insensitive mutant ?

It is proposed that the *Rht3* allele could be described as a mutation conferring insensitivity to high temperature as well as an insensitivity to GA. Application of GA_3 to the *rht3* wild-type at 10 °C restores the phenotype to that at 20 °C. In this way applied GA_3 can substitute for increased temperature. However, the *Rht3* allele appears to genetically restrict the phenotype of the mutant to that expressed in the *rht3* wild-type at low temperatures (or when treated with 2*S*,3*S* paclobutrazol). Grown at 10 °C, the *rht3* wild-type and *Rht3* mutant were more similar to each other than they were at 20 °C in several respects, in morphology, in length of the L2 extension zone, in cell residence time within the extension zone and in terms of cell wall extensibility.

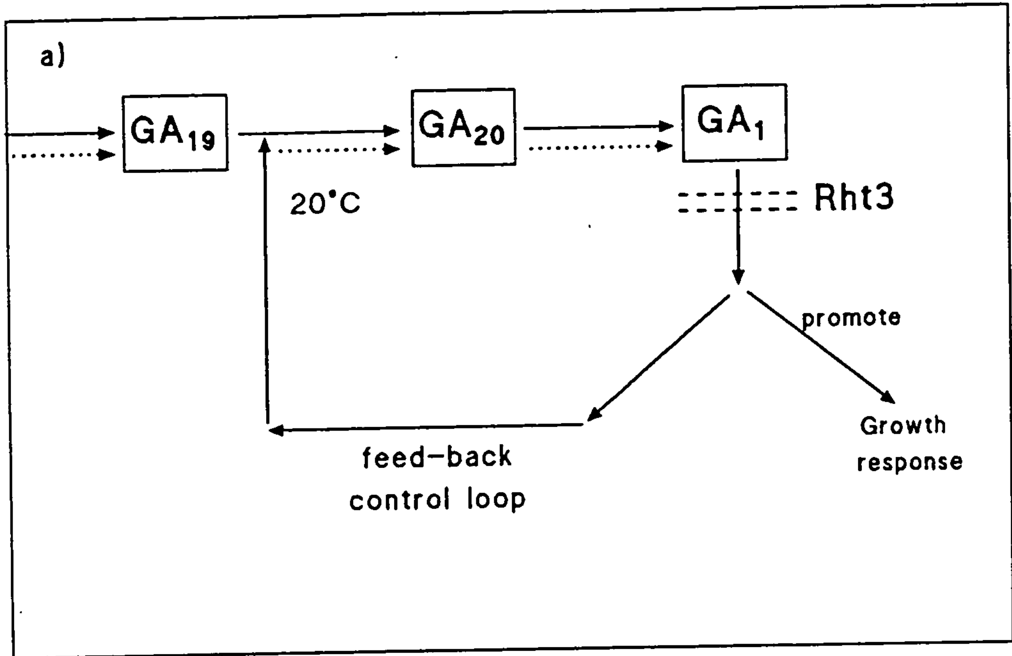
However, the effect of low temperature does act in addition to the *Rht3* allele. This is shown by the reduction in the maximum REGR, meristem length, P_{ex} and cell turgor in the

mutant at 10 °C compared to 20 °C.

It could be suggested that at 10 °C tissue sensitivity to endogenous GA is increased compared to that at 20 °C. Thus, at 20 °C the (dominant) product(s) of the *Rht3* allele may saturate or block the activity of the GA receptors or transduction pathway as suggested by Baroncelli *et al.*, (1980) and Keyes (1987). However, at 10 °C more receptors may be functional and/or their sensitivity to endogenous GA may be increased. The probability may be higher that the dominant product of the *Rht3* allele may not block the transduction stimulus from all of the putative GA receptors. Hence the likelihood of a partial functioning of the GA feedback control loop may be increased (Fig. 7.3). In the *rht3* wild-type grown at 10 °C, despite a *ca* 65 % reduction in the REGR compared to that at 20 °C there was no change in the level of GA_1. This could suggest that the control of GA level is very tightly regulated by the plant and that extensive regulatory adjustments may be made which are fully compensatory, in order that the GA level remains the same at the lower temperature.

Studies on sugar-cane (Bull, 1964; Mongelard & Mimura, 1972; Moore & Buren, 1968; Moore & Ginzoa, 1980), panolagrass (Karbassi *et al.*, 1971), sorghum (Yen & Carter, 1972) maize (Rood *et al.*, 1985) and wheat aleurone tissue (Singh & Paleg, 1984b) have also implied that responsiveness to GA_3 may be increased at low temperature. In addition, applied GA_3 hastens germination in maize (Gubbles, 1976) and potato (Lam, 1968) under cool conditions. Also, application of 10^{-5}M GA_3 to panolagrass completely reversed the effect of 10 °C night temperatures and restored the plant phenotype to that of plants grown at the higher 30 °C night temperatures (Karbassi *et al.*, 1971). Application of GA_3 also mobilized more than 50 % of the starch which had accumulated as a result of low night temperatures. It was suggested that the applied GA_3 increased the activity of a starch degrading enzyme.

Indeed enzymes activities of plants subject to low temperature do show changes in activity, freeze stability and isozymic variation compared to their properties at high temperature (Guy, 1990). Specific enzymes appear to be up-regulated in response to low temperature. Enzymes involved in starch-sucrose interconversions are presumed to be likely targets (Guy, 1990). Sucrose synthase activity was increased in wheat plants following a period of cold shock at 4 °C (Calderon & Pontis, 1985). If the *Rht3* allele is insensitive to high temperature, it is possible that certain enzymes, whose activities are specifically up-regulated by low temperature, may be expressed constantly in the *Rht3* mutant line. The same enzymes may be absent or less active in the *rht3* line at 20 °C. The idea would also be compatible with the fact that the two lines are much more similar at 10 °C when the putative enzyme activity would be similarly up-regulated in the *rht3* line. The possible involvement of enzymes responsible for starch-sucrose interconversions are of particular interest with



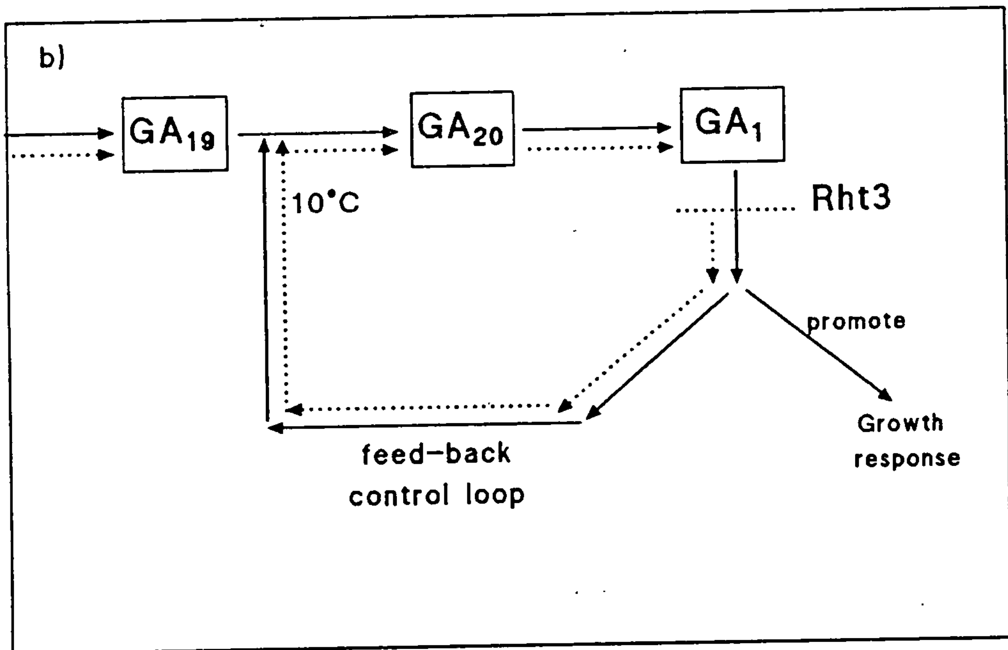


Fig. 7.3 Proposed feed-back control loop in the *rht3* (wild-type), solid line and *Rht3* (mutant) dotted line grown at two different temperatures (a) 20 °C and (b) 10 °C. Showing proposed partial functioning of the feed-back control loop in the *Rht3* (mutant).

respect to the mechanism of action of the *Rht3* allele. Such enzymes may be able, indirectly, to affect cell turgor pressure and therefore the driving force of cell extension.

While speculative, the available evidence is compatible with the *Rht3* allele having an additional effect on enzyme activities which may be promoted by low temperature and further research into this area could be worthwhile. However, it is not likely that the up-regulation of any such enzyme activity could explain all of the effects induced by the *Rht3* allele. Even at 10 °C L2 of the *Rht3* line has a lower, dry mass, final leaf length and surface area than the *rht3* wild-type (Fig. 4.13). In many respects the *Rht3* allele is insensitive to high temperature. As a consequence, many of the effects initiated by the *Rht3* allele may be better understood if they are considered in terms of being induced by low temperature as opposed to GA-insensitivity. The effects of GA-sensitivity and low temperature may, however, be closely interrelated. Growth responses to high temperature may be mediated through endogenous GAs. Such an inter-relationship may explain why the high temperature-insensitive trait of the *Rht3* allele has been relatively neglected compared to that of its GA-insensitivity.

7.8 Is the control of growth regulated by the *Rht3* allele adequately defined by premature wall stiffening, or is there an additional controlling factor ?

The reduction in P_{ex} of the cell wall in the *Rht3* mutant could be adequately explained by alterations in wall rheology with or without changes in the orientation of growth. However, such effects do not explain why REGR of cells in the *Rht3* mutant always tended to be higher than in the *rht3* wild-type in the initial 5-10 mm of L2 (Fig. 4.5 & 4.12). In addition, the *Rht3* line appeared to be associated with precocious early growth in developing stem internodes, as also observed in the developing coleoptile of the *Rht3* line (Flintham, 1981). It is suggested that in addition to any effects that the *Rht3* allele may have on the rheological properties of the cell wall, it may also initiate compensatory growth responses. Cells of the *Rht3* mutant genotype appear to display a form of phenotypic plasticity, perhaps as a permanent adaptation to low temperature growth, as discussed by Trewavas (1986). It could be suggested that every cell in both genotypes may receive a genetic dose of positional information, (analogous to each cell being in receipt of a map, a compass and a finishing time). A similar idea was suggested by Allan & Trewavas (1985), based on growth within the root of *Pisum* that cells acquired a programme of instructions within the root meristem and these were sequentially but independently expressed as the cells matured. In wheat, it is suggested that this basic, positional, information may not be influenced by the *Rht3* allele. However, cells of the mutant line may compensate for their insensitivity to endogenous GA by precocious early cell extension and by permitting a greater flexibility in the polarity of cell growth compared to equivalent cells in the *rht3* line. The situation may exist in which there is a feedback mechanism through which cells of the

Rht3 line sense that subsequent cell extension will be prematurely slowed down. They therefore may initiate more rapid early growth, before the processes which eventually stop the cell extending become fully functional.

Leaves of plants of *Festuca* grown with low nitrogen (N) levels and with a low leaf elongation rate (LER) showed similar effects in cell development to those induced by the *Rht3* allele in wheat in the present work. Compared to the controls with high LER and high N, the plants with low N showed: (1) an initial rate of cell elongation that was greater, although cell elongation stopped nearer the leaf base so that final cell length was shorter and (2) a reduction in the length of the leaf basal meristem (MacAdam *et al.*, 1989). It was suggested that low N levels may also initiate compensatory cellular responses which may be genetically only loosely controlled. Hence cell elongation proceeded faster in leaves with low N treatment than with high N. A significant feature of *Rht* lines of wheat is that they do require high levels of N to achieve their maximum yield potential (Gale & Youssefian, 1985; Austin & Arnold, 1989; Law, 1989). By analogy, it could be suggested that the *Rht3* allele may reduce the efficiency of N utilization and/or that both the *Rht3* allele and low N may inhibit cell elongation in a similar way.

Gamma irradiation of wheat leaves prevented the formation of new cell cross walls but did not affect the highly directional growth of the leaf (Haber, 1962). Haber concluded that genetic information encoded the specific leaf form independently of any influence on cell size or the extent or orientation of cell divisions.

Similarly, in wheat epiblasts it was the final, or critical mass that a cell attained rather than the rate and duration of growth that appeared to be biologically determined (Haber *et al.*, 1964). Thus, although the total time for the epiblast to reach its final mass varied 15-fold over the range of temperature studied (5 ° to 23 °C), the final size of the epiblast was the same at each temperature.

These examples do illustrate that different parameters of growth may be subject to differing levels of genetic control. While final cell mass and the duration of growth may be highly regulated, the polarity of cell division and cell length may only be loosely controlled. It is possible that the mutant line in wheat may demonstrate phenotypic plasticity. Those parameters which may be highly regulated, for example, the duration of L2 growth, cell number and the maximum REGR achieved within the extension zone, are conserved while other parameters such as cell length and width may be varied.

There are other possible explanations for the present results in wheat. The *Rht3* allele may also influence cellular responses to auxin (Gale & Youssefian, 1985). In coleoptile development IAA initially inhibits cell extension in young tissue while subsequently promoting cell extension in older tissue. The early inhibition response to IAA was absent in the *Rht3* line (Flintham, 1981). There is also evidence that the action of the *Rht* alleles may

also be modified by light (Baroncelli *et al.*, 1984; Gale & Youssefian, 1985; Baroncelli *et al.*, 1988). Although neither the effects of IAA nor irradiance have been considered directly in this thesis, the eventual understanding of the mechanism through which the *Rht3* allele operates may require an integrated approach explaining how the allele interacts with these other factors.

7.9 Final conclusions: the mechanism of action of the *Rht3* allele ?

The predominant effect of the *Rht3* allele is to reduce the rate of cell extension after the maximum rate has been reached. Thus, compared to the *rht3* wild-type cell length and not cell number is reduced in vegetative organs of the *Rht3* mutant. Unlike the *rht3* wild-type, the length of L2 extension zone is permanently reduced in the *Rht3* mutant and cannot be altered by applied PGRs and/or growth at low temperature. The *Rht3* allele promotes growth inhibitory processes which restrict continued cell extension. Thus, the *Rht3* has no effect on the initiation of leaf primordia and stem internodes by the shoot apex. Only when cells of the developing stem internodes begin to extend rapidly does the genotypic effect of the *Rht3* allele become obvious. It is also suggested that the *Rht3* allele may have an additional effect to reduce the length of the cell cycle, although this requires further investigation. The *Rht3* allele significantly reduces both the plastic extensibility of the cell wall and cell turgor pressure compared to the *rht3* wild-type. The effect of the *Rht3* allele appears to be one which blocks processes of growth through which GA_1 controls wall extensibility, and therefore cell extension.

The present study has outlined the limitations to our knowledge of the regulatory mechanisms which determine a fundamental process of growth - cell extension. The work has also emphasized the need for instantaneous measurements to be made on the intact plant. Often in the present work the technique used to measure a parameter interfered with the very process that it was designed to investigate. The deleterious effect of piercing the L2 extension zone to assess the spatial distribution of growth is an obvious example. In addition, the work has illustrated the possible hierarchy of differing levels of control processes which may govern cell growth. In addition while certain processes may be genetically highly regulated, such as cell number, others may be only loosely regulated such as final cell length. It is therefore difficult to try and ascribe a single mechanistic basis for effects observed within such a complex, interactive system.

The mechanism(s) through which the *Rht3* allele operates may be those through which endogenous GA_1 initiates a biophysical response to maintain wall loosening events within the cell wall. It is assumed that the processes of growth which are blocked by the *Rht3* allele are fundamental to normal cell extension in growing stem internodes, laminae and coleoptile, hence the similarity of the effect of the *Rht3* allele in these organs. It is also suggested that

the *Rht3* mutant may demonstrate GA-independent growth, which, by implication suggests that the function of endogenous GA, while essential for normal leaf growth, may assume more of a regulatory back-up function within the plant. This could be for environmental conditions which may act to prevent continued cell extension such as low temperature. While the present work has not identified the primary effect of the *Rht3* allele, it strongly implies that the processes affected by the *Rht3* allele are targeted at the primary cell wall, which reduce the rate of cell extension.

There is still a lot of work to be done for a full understanding of the mechanism through which the *Rht3* allele operates in relation to growth. More promising directions of future research could include comparative studies in the *rht3* and *Rht3* genotypes which investigate: the different components of the primary cell wall, developmental anatomy of the internal leaf structure, the release of second messengers such as Ca^{2+}, particularly in response to temperature, GA_3 and 2*S*,3*S* paclobutrazol and an investigation of enzyme activities involved in sugar-starch conversions.

CHAPTER 8

REFERENCES

ADAMS, M.W. (1967) Basis of yield component compensation in crop plants with specific reference to the field bean *Phaseolus vulgaris*. *Crop Sci.* **7**, 505-510

ADAMS, P.A., MONTAGUE, M.J., TEPFER, M., RAYLE, D.L., IKUMA, H., KAUFMAN, P.B. (1975) Effect of gibberellin in the plasticity and elasticity of *Avena* stem segments. *Plant Physiol.* **56**, 757-760

ALBERSHEIM, P. (1976) The primary cell wall. *In*. Plant biochemistry. (eds. J. Bonner & J.E. Varner) pp 225-274 3rd edition. Academic Press, New York

ALLAN, E.F., TREWAVAS, A. (1986) Tissue-dependent heterogeneity of cell growth in the root apex of *Pisum sativum*. *Bot. Gaz.* **147**, 258-269

ALLAN, R.E., (1980) Influence of semi-dwarfism and genetic background in stand establishment in wheat. *Crop Sci.* **20**, 634-638

ALLAN, R.E. (1986) Agronomic comparisons among wheat lines nearly isogenic for three reduced height genes. *Crop Sci.* **25**, 707-710

ALLAN, R.E. (1990) Agronomic comparisons between *Rht1* and *Rht2* semi-dwarf genes in winter wheat *Crop Sci.* **29**, 1103-1108

ALLAN, R.E., PRITCHETT, J.A (1980) Registration of 16 lines of club wheat germ plasm. *Crop Sci.* **20**, 832-833

ALLAN, R.E., VOGEL, O.A., CRADDOCK, J.C. (1959) Comparative responses of gibberellin of dwarf, semi-dwarf and standard short and tall winter wheat varieties. *Agron. J.* **51**, 737-740

ALLAN, R.E., VOGEL, O.A., BURLEIGH, J.R. (1962) Length and estimated number of coleoptile parenchyma cells of six wheat selections grown at two temperatures. *Crop Sci.* **2**, 522-524

ALLARD, G., NELSON, C.J. (1991) Photosynthate partitioning in basal zones of tall fescue leaf blades. *Plant Physiol.* **95**, 663-668

APPLEFORD, E.J., LENTON, J.R. (1990) Gibberellins and leaf expansion in near-isogenic wheat lines containing *Rht1* and *Rht2* dwarfing alleles. *Planta* **183**, 229-236

AUSTIN, R.B., ARNOLD, M.H. (1989) Variability in wheat yields in England: Analysis and future prospects. *In*. Variability in grain yields; Implication for agricultural research and policy in developing countries. (eds. J.R. Anderson & P.B.R. Hazell). pp 100-117. The John Hopkins University Press. Published for the Inst. Food Policy Res. Instit, Baltimore

AVERY, G.S. (1933) Structure and development in the tobacco leaf. *Am. J. Bot.* **20**, 565-592

BAINS, K.S., GILL, K.S. (1973) The genetics of coleoptile size in spring wheat. Proc. 4th Int. wheat Genet. Symp. (eds. E.R. Sears & L.M.S. Sears). pp 27-28 University of Missouri. Columbia

BARLOW, E.W.R. (1986) Water relations of expanding leaves. *Aust. J. Plant Physiol.* **13**, 45-58

BARLOW, E.W.R., MUNNS, R.E., BRADY, C.J. (1980) Drought response of apical meristems. *In*. Adaptation of plants to water and high temperature stress. (eds. N.C. Turner & P.J. Kramer). pp 191-205 Wiley Interscience, New York

BARLOW, P.W. (1989) Anatomical controls of root growth. *Aspects Appl. Biol.* **22**, 57-66

BARLOW, P.W., BRAIN, P., PARKER, J.S. (1991) Cellular growth in roots of a gibberellin - deficient mutant of tomato (*Lycopersicum esculentum.* Mill) and its wild-type. *J. Exp. Bot.* **42**, 339-351

BARONCELLI, S.M., BUIATTI, M., MAGNANI, G. (1980) Control of gibberellin action by semidwarf genes in durum wheat. *Zeitschrift fur Pflanzenzuchtung* **84**, 219-225

BARONCELLI, S., LECARI, B., CIONINI, P.G., CAVALLINI, A., D'AMOTO, F. (1984) Effect of light and gibberellic acid on coleoptile and first-forage leaf growth in durum wheat (*Triticum durum* Desf.) *Planta* **160**, 298-304

BARONCELLI, S., CAVALLINI, A., LECARI, B., CIONINI, P.G., D'AMOTO, F.D. (1988) Effect of light and gibberellic acid on cell division in the first foliage leaf of durum wheat (*Triticum durum* Desf.) *Planta* **173**, 257-262

BASKIN, T.I., BRET-HARTE, M.S., GREEN, P.B. (1987) Rates of cell wall accumulation in higher plants: Measurement with interference microscopy. *In*. Physiology of plant cell expansion. (eds. D.J. Cosgrove & D.P. Knievel). pp 258-261 The American Society of Plant Physiologists, Rockville

BEG, J.E., WRIGHT, M.J. (1962) Growth and development of leaves from intercalary meristems in *Phalaris arundinaceae* L. *Nature* **194**, 1097-1098

BERRY, J., BJORKMAN, O. (1980) Photosynthetic response and adaptation to low temperature in higher plants. *Ann. Rev. Plant Physiol.* **31**, 491-543

BIGGS, K.J., FRY, S.C. (1987) Phenolic cross-linking in the cell wall. *In*. Physiology of cell expansion during plant growth. (eds. D.J. Cosgrove & D.P. Knievel). pp 46-57 The American Society Plant Physiologists, Rockville

BOFFEY, S.A., ELLIS, J.R., SELLDEN, G., LEECH, R.M. (1979) Chloroplast division and DNA synthesis in light growth wheat leaves. *Plant Physiol.* **64**, 502-505

BOFFEY, S.A., SELLDEN. G., LEECH, R.M. (1980) Influence of cell age on chlorophyll formation in light grown and etiolated wheat seedlings. *Plant Physiol.* **65**, 680-684

BORRELL, A.K., INCOLL. L.D., DALLING, M.J. (1991) The influence of the *Rht1* and *Rht2* alleles on the growth of wheat stems and ears. *Ann. Bot.* **67**, 103-110

BOYER, J.S. (1985) Water transport. *Ann. Rev. Plant Physiol.* **36**, 473-516

BRANDLE, J.E., KNOTT, D.R. (1986) The effect of a gene for semidwarfism (*Rht1*) on various characteristics in a spring wheat cross. *Can. J. Plant Sci.* **66**, 529-533

BRETT, C., WALDRON, K. (1990) Cell wall structure and skeletal functions of the wall *In*. Physiology and biochemistry of plant cell walls. (eds. M. Black & J. Chapman) pp 4-57 Unwin Hyman, Boston. Sydney. Wellington

BRET-HARTE, M.S., BASKIN, T.I., GREEN, P.B. (1991) Auxin stimulates both deposition and breakdown of material in pea outer epidermal cell wall as measured interferometrically. *Planta* **185**, 462-471

BROCK, T.G., CLELAND, R.E. (1984) On the mechanism of rapid stimulation of expansion growth in bean leaves by cytokinins and gibberellin. *Plant Physiol.* **75**, Supplement p 160

BROOKING I.R., KIRBY, E.J.M. (1981) Interrelationship between stem and ear development in winter wheat: the effects of a Norin 10 dwarfing gene. *Gai/Rht*$_2$. *J. Agric. Sci.* **97**, 373-381

BROUGHTON, W.J., MCCOMB, A.J. (1967) The relation between cell wall and protein synthesis in dwarf pea plants treated with gibberellic acid. *Ann. Bot.* **31**, 359-366

BRUMMELL, D.A., HALL, J.L. (1980) The role of the epidermis in auxin-induced and fusicoccin-induced elongation in *Pisum sativum* stem segments. *Planta* **150**, 371-379

BRUMMEL, D.A., HALL, J.L. (1987) Rapid cellular responses to auxin and the regulation of growth. *Plant Cell Environ.* **10**, 523-543

BULL, T.A. (1964) The effects of temperature, variety and age on the response of *Saccharum* spp. to applied gibberellic acid. *Aust J, Agric. Sci.* **15**, 77-84

BUNCE, J.A. (1977) Leaf elongation in relation to leaf water potential in soybean. *J. Exp. Bot.* **28**, 156-161

BUREN, L.L., MOORE, P.H., YAMASAKI, Y. (1979) Gibberellin studies with sugarcane. II. Hand sampled field trials. *Crop Sci.* **19**, 425-428

BURSTROM, H.G. (1974) Patterns of synthesis during internodal growth of *Pisum* stems. *Z. Pflanzenphysiol.* **74**, 1-4

BURSTROM, H.G. (1977) Tissue tensions during cell elongation in wheat roots and comparison with contractile roots. *Physiol. Plant.* **25**, 509-513

BUSH, M.G., EVANS, L.T. (1988) Growth and development in tall and dwarf isogenic lines of spring wheat. *Field Crops Res.* **18**, 243-270

BUSH, R.H., CHAMBERLAIN, D.D. (1981) Effect of daylength responses and semidwarfism on agronomic performance of spring wheat. *Crop Sci.* **21**, 57-60

BUTCHER, D.N., CLARK, J.A., LENTON, J.R. (1990) Gibberellins and the growth of excised tomato roots: comparison of *gib-1* mutant and wild type and responses to applied GA_3 and 2*S*,3*S* paclobutrazol. *J. Exp. Bot.* **41**, 715-722

CALDERON, P., PONTIS, H.G. (1985) Increase in sugar synthetase activity in wheat plants after a chilling shock. *Plant Sci.* **42**, 173-176

CARPITA N.C. (1986) Incorporation of protein and aromatic amino acids into cell walls of maize coleoptiles. *Plant Physiol.* **80**, 660-666

CARPITA, N.C., KANABUS, J. (1988) Chemical structure of the cell walls of dwarf maize and changes mediated by gibberellin. *Plant Physiol.* **88**, 671-678

CASAL, J.J., WHITELAM, G.C., SMITH, H. (1990) Phytochrome control of extracellular peroxidase activity in mustard internodes; correlation with growth and comparison with the effect of wounding. *Phytochem. Phytobiol.* **52**, 165-172

CHANDLER, P.M. (1988) Hormonal regulation of gene expression on the *slender* mutant of barley (*Hordeum vulgare* L.) *Planta* **175**, 115-120

CHINOY, J.J. (1949) Correlation between growth and development. *Nature* **164**, 879-880

CHINOY, J.J., NANDA, K.K. (1951) Effect of vernalisation and photoperiodic treatments on growth and development of cereal crops. II. Varietal differences in stem elongation and tillering of wheat and their correlation with flowering under photo-inductive and post-photoinduction treatments. *Physiol. Plant.* **4**, 427-436

CHOLICK, F.A., WELSH, R.J., COLE, C.V. (1977) Rooting patterns of semi-dwarf and tall winter wheat cultivars under dryland field conditions. *Crop Sci.* **17**, 637-639

CHORY, J., VOYTAS, D.F., OLSZEWSKI, N.E., AUSUBEL, F.M. (1987) Gibberellin-induced changes in the populations of translatable mRNAs and accumulated polypeptides in dwarf maize and pea. *Plant Physiol.* **83**, 15-23

CIMMYT (1967) CIMMYT report. 1966-1967 p 78

CLARKE, G.M. (1980) Other tests of significance. In. Statistics and experimental design. 2nd edition. pp 58-76 Edward Arnold, London

CLELAND, R.E. (1958) A separation of auxin-induced cell wall loosening into its plastic and elastic components. *Physiol. Plant.* **11**, 599-609

CLELAND, R.E. (1959) Effect of the osmotic concentration on auxin action and on irreversible and reversible expansion of the *Avena* coleoptile. *Physiol. Plant.* **12**, 809-825

CLELAND, R.E (1967) Extensibility of isolated cell walls - measurement and changes during cell elongation. *Planta* **74**, 197-209

CLELAND, R.E. (1971) Cell wall extension. *Ann. Rev. Plant Physiol.* **22**, 197 - 222

CLELAND, R.E. (1981) Wall extensibility: hormones and wall extension. In. Plant carbohydrates II. Extracellular carbohydrates. Encyclopedia of plant physiology, New Series. Volume 13B (eds. W. Tanner & F.A. Loewus). pp 255-273 Spinger-Verlag, Berlin. Heidelberg. New York

CLELAND, R.E. (1984) The instron technique as a measure of immediate-past wall extensibility. *Planta* **160**, 514-520

CLELAND, R.E. (1986) The role of hormones in wall loosening and plant growth. *Aust. J. Plant Physiol.* **13**, 93-103

CLELAND, R., KARLSNES, A.M. (1967) A possible role of hydroxyproline containing proteins in the cessation of cell elongation. *Plant Physiol.* **42**, 689-671

CLELAND, R., RAYLE, D.L. (1972) Absence of auxin-induced stored growth in *Avena* coleoptiles and its implications concerning the mechanism of wall extension. *Plant Physiol.* **47**, 812-815

CLELAND, R.E., RAYLE, D.L. (1977) Reevaluation of the effect of calcium ions on auxin-induced elongation. *Plant Physiol.* **60**, 709-712

COARTNEY, J.S., MORRE, D.J. (1980a) Studies on the role of wall extensibility in the control of cell expansion. *Bot. Gaz.* **141**, 56-62

COARTNEY, J.S., MORRE, D.J. (1980b) Studies on the chemical basis of auxin induced cell wall loosening. *Bot. Gaz.* **141**, 63-68

COHEN, D., ATSMON, D. (1972) Measurement of water potential and hormone transport associated with the growth of cucumber hypocotyls. In. Plant Growth Substances 1970 (ed. D.J. Carr). pp 23-29 Spinger-Verlag, Berlin

COOPER, J.B., VARNER, J.E. (1984) Cross-linking of soluble extensins in isolated cell walls. *Plant Physiol.* **76**, 414-417

COSGROVE, D.J. (1985) Wall yield properties of growing tissues, evaluation by *in vivo* stress relaxation. *Plant Physiol.* **78**, 347-56

COSGROVE, D.J. (1986) Biophysical control of plant cell growth. *Ann. Rev. Plant Physiol.* **37**, 377-405

COSGROVE, D.J. (1987) Wall relaxation and the driving forces for cell expansive growth. *Plant Physiol.* **84**, 561-654

COSGROVE. D.J. (1988) The mechanism of rapid suppression of cell expansion in cucumber hypocotyls after blue light irradiation. *Planta* **176**, 109-116

COSGROVE, D.J., CLELAND, R.E. (1983a) Solutes in the free space of growing stem tissues. *Plant Physiol.* **72**, 326-331

COSGROVE, D.J., CLELAND, R.E. (1983b) Osmotic properties of pea internode in relation to growth and auxin action. *Plant Physiol.* **72**, 332-338

COSGROVE, D.J., VAN VOLKENBURGH, E., CLELAND, R.E. (1984) Stress relaxation of cell walls and the yield threshold for growth: Demonstration and measurement by micro-pressure probe and psychrometer techniques. *Planta* **162**, 46-54

CROKER, S.J., HEDDEN, P., LENTON, J.R. (1990) Comparison of gibberellins in normal and *slender* barley seedlings. *Plant Physiol.* **94**, 194-200

CUNNINGHAM, B.A., LIANG, G.H., MOORE, R.M., HEYNE, E.G. (1975) Peroxidase activity in near isogenic height lines in *triticale*. *J. Hered.* **66**, 151-154

CUTLER, J.M., SHAHAN, K.W., STEPONKUS, P.L. (1980a) The influence of water deficits and osmotic adjustments on leaf elongation in rice. *Crop Sci.* **20**, 314-318

CUTLER, J.M., SHAHAN, K.W., STEPONKUS, P.L. (1980b) Alteration of the internal water relations of rice in response to drought hardening. *Crop Sci.* **20**, 307-310

DALE, J.E. (1976) Cell division in leaves. *In*. Cell division in higher plants (ed. M.M. Yoeman). pp 315-345 Academic Press, London

DALE, J.E. (1982) Some effects of temperature and irradiance of growth of the first four leaves of wheat, *Triticum aestivum*. *Ann. Bot.* **50**, 851-858

DALE, J.E. (1988) The control of leaf expansion, *Ann. Rev. Plant Physiol. Mol. Biol.* **39**, 267-295

DALRYMPLE, D.G. (1978) Development and spread of high-yielding varieties of wheat and rice in the less developed nations. *Foreign Agricultural Economic Report. USDA, N° 455. USDA, Washington DC*

DARVILL, A.G., SMITH, C.J., HALL, M.A. (1978) Cell wall structure and elongation growth in *Zea mays* coleoptile tissue. *New Phytol.* **80**, 503-516

DAVIDSON, J.L., MILTHORPE, F.L. (1966) Leaf growth in *Dactylis glomerata* following defoliation. *Ann. Bot.* **30**, 173-78

DAVIES, T.G.E., OUGHAM, H.J., THOMAS, H., ROGERS, L.J. (1989) Leaf development in *Lolium temulentum*: plastid membrane polypeptides in relation to assembly of the photosynthetic apparatus and leaf growth. *Physiol. Plant.* **75**, 47-54

DAVIES, W.J., VAN VOLKENBURGH, E. (1983) Solutes in the free space of growing stem tissue. *J. Exp. Bot.* **72**, 987-999

DEAN, C., LEECH, R.M. (1982) Genome expression during normal leaf development. I. Cellular and chloroplast numbers and DNA, RNA, and protein levels in tissues of different ages within a seven day old wheat leaf. *Plant Physiol.* **69**, 904-910

DENGLER, N. (1980) Comparative histological basis of sun and shade leaf dimorphism in *Helianthus anuus*. *Can. J. Bot.* **58**, 717-730

EAGLES, C. (1967) The effect of temperature on vegetative growth in climatic races of *Dactylis glomerata* in controlled environments. *Ann. Bot.* **31**, 31-39

ECKERT, H., SCHILLING, G., PODLESAK, W., FRANKE, P. (1978) Extraction and indentification of gibberellins (GA_1 and GA_3) from *Triticum aestivum* L. and *Secale cereale* L. and changes in contents during ontogenesis. *Biochemie und Physiologie der Pflanzen.* **172**, 475-486

EDELMAN. H., BERGFIELD, R., SCHOPFER, P. (1989) Role of the cell wall biogenesis in the initiation of auxin-mediated growth in coleoptiles of *Zea mays* L. *Planta* **179**, 486-494

ELLIS, R.J., JELLINGS, A.J., LEECH, R.M. (1983) Nuclear DNA content and the control of chloroplast replication in wheat leaves. *Planta* **157**, 376-380

EPSTEIN, L., LAMPORT, D.T.A. (1984). An intramolecular linkage involving isodityrosine in extensin. *Phytochem.* **23**, 1241-1246

ERICKSON, R.O., SAX, K.B. (1956) Elemental growth rates of the primary root of *Zea mays*. *Proc. Amer. Phil. Soc.* **100**, 487-498

ERICKSON, R,O., SILK, W.K. (1980) The kinematics of plant growth. *Sci. Amer.* **205**, 102-114

ESAU, K. (1977) Anatomy of seed plants. John Wiley & Sons, New York

EVANS, J.J., ALLDRIDGE, N.A. (1965) The distribution of peroxidase in extreme dwarf and normal tomato (*Lycopersicum esculentum* Mill.) *Phytochem.* **4**, 499-503

EVANS, L.T., WARDLAW, I.F., FISCHER, R.A. (1973) Wheat. *In*. Crop Physiology (ed. L.T. Evans). pp 101-150 Cambridge University Press, Cambridge

FARKAS, V., MACHLACHLAN, G. (1988) Stimulation of pea 1-4-β-glucanase activity by oligosaccharides derived from xyloglucan. *Carbohydr. Res.* **93**, 1042-1048

FAUSCH, H., KUNDIG, W., NEUKOM, H. (1963) Ferulic acid as a component of glycoprotein from wheat flour. *Nature* **199**, 287

FICK, G.N., QUALSET, C.O. (1973) Genes for dwarfness in wheat. *Triticum aestivum* L. *Genetics* **75**, 531-539

FICK, G.N., QUALSET, C.O. (1975) Genetic control of endosperm amylase activity and gibberellic acid responses in standard height and short statured wheats. *Proc. Natl. Acad. Sci. USA* **72**, 892-895

FIRN, R., DIGBY, J. (1977) The role of the peripheral cell layers in the geotropic curvature of sunflower hypocotyls. A new model of shoot geotropism. *Aust. J. Plant Physiol.* **4**, 337-347

FISHER, J.E. (1973) Developmental morphology of the influorescence in hexaploid wheat cultivars with and without the cultivar Norin 10 in their ancestry. *Can. J. Plant Sci.* **53**, 7-15

FISCHER, R.A., STOCKMAN, Y.M. (1986) Increased kernel number in Norin 10 derived dwarf wheat; evaluation of the cause. *Aust. J. Plant Physiol.* **13**, 767-784

FLETCHER, R.A., OSBORNE, D.J. (1965) Regulation of protein and nucleic acid synthesis by gibberellin during leaf senescence. *Nature* **207**, 1176-1177

FLINTHAM, J.E. (1981) The physiological role and plant breeding potential of the Tom Thumb dwarfing allele in wheat. *PhD* thesis, University of Cambridge

FLINTHAM, J.E., GALE, M.D. (1982) The Tom Thumb dwarfing gene *Rht3* in wheat. 1. Reduced preharvest damage to breadmaking quality. *Theoret. App. Gen.* **62**, 121-126

FLINTHAM, J.E., GALE, M.D. (1983) The Tom Thumb dwarfing gene *Rht3*. II. Effects on height, yield, and grain quality. *Theoret. App. Genet.* **66**, 249-256

FOSTER, C.A. (1977) Slender, an accelerated extension growth mutant of barley. *Barley Genetics Newsletter* **7**, 24-27

FREELING, M., BONGARD-PIERCE, D.K., HARBERD, N., LANE, B., HAKE, S. (1988) Genes involved in the patterns of maize leaf cell division. *In.* Temporal and spatial regulation of plant growth. (eds. D.P.S. Verma & R. B. Goldberg). pp 41-62 Spinger-Verlag, Wien. New York

FRIEND, D.J.C., HELSON, V.A., FISCHER, J.A. (1962) Leaf growth in Marquis wheat as regulated by temperature, light intensity and daylength. *Can. J. Bot.* **40**, 1299-1311

FRIEND, D.J.C., POMERY, M.E. (1970) Changes in cell sizes and number associated with the effects of light intensity and temperature on the leaf morphology of wheat. *Can. J. Bot.* **48**, 85-90

FRY, S.C. (1979) Phenolic components of the primary cell wall and their possible role in the hormonal regulation of growth. *Planta* **146**, 343-351

FRY, S.C. (1980) Gibberellin controlled pectinic acid and protein secretion in growing cells. *Phytochem.* **19**, 735-740

FRY, S.C (1982) Phenolic components of the primary cell wall; feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. *Biochem. J.* **203**, 439-504

FRY, S.C. (1983) Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta* **157**, 111-123

FRY, S.C. (1984a) Incorporation of [^{14}C]cinnamate into hydrolase resistant components of the primary cell wall of spinach. *Phytochem.* **25**, 59-64

FRY, S.C. (1984b) Isodityrosine - its detection, estimation and chemical synthesis. *Meth. Enzymol.* **107**, 388-397

FRY, S.C. (1986) Cross-linking of matrix polymers in the growing cell wall of angiosperms. *Ann. Rev. Plant Physiol.* **37**, 165-186

FRY, S. (1988) The growing plant cell wall. Longman Scientific and Technical, London

FRY, S.C. (1989) Analysis of cross-links in growing cell walls of higher plants. *In.* Modern methods of plant analysis. New Series. Vol. 10. Plant fibers. (ed. H.F. Linskens & J.F. Jackson) pp 11-35 Spinger-Verlag, Berlin. Heidelberg

FRY, S.C., SMITH, R.C., RENWICK, K.F., MARTIN. D.J., HODGE, S.K., MATTHEWS, K.J. (1992) Xyloglucan endotransglycosylase, a new wall loosening enzyme activity from plants. *Biochem. J.* **282**, 821-828

FUJIOKA, S., YAMANE, H., SPRAY, C.R., GASKIN, P., MACMILLAN, J., PHINNEY, B.O., TAKAHASHI, N. (1988a) Quantitative and qualitative analyses of gibberellins in vegetative and shoots of normal *dwarf-1*, *dwarf-2*, *dwarf-3* and *dwarf-5* seedlings of *Zea mays* L. *Plant Physiol.* **88**, 1367-1372

FUJIOKA, S., YAMANE, H., SPRAY, C.R., KATSUMI, M., PHINNEY, B.O., GASKIN, P., MACMILLAN, J. (1988b) The dominant non-gibberellin responding dwarf mutant (*D8*) of maize accumulates native gibberellins. *Proc. Natl. Acad. Sci. USA* **85**, 9031-9035

FUJIOKA, S., YAMANE, H., SPRAY, C.R., PHINNEY, B.O., GASKIN, P., MACMILLAN, J. (1990) Gibberellin A_3 is synthesized from A_{20} via gibberellin A_5 in shoots of *Zea mays* L. *Plant Physiol.* **94**, 127-131

FULCHER, R.G., O'BRIEN, T.P. (1972) Studies of the aleurone layer. I. Conventional and fluorescence microscopy of the cell wall with emphasis on phenol-carbohydrate complexes in wheat. *Aust. J. Biol. Sci.* **25**, 23-34

FURUYA, M., MASUDA, Y., YAMAMOTA, R. (1972) Effects of environmental factors on mechanical properties of the cell wall in rice coleoptiles. *Dev. Growth Diff.* **14**, 95-105

GALE, M.D. (1979) The effects of Norin 10 dwarfing genes on yield. Proc. 5th Int. Wheat Genet. Symp. (ed. S. Ramanujam). pp 978-987 Indian Soc. Genet. and Plant Breeding. New Delhi, India

GALE, M.D., GREGORY. R.S. (1977) A rapid method for early generation selection of dwarf genotypes in wheat. *Euphytica* **26**, 733-738

GALE, M. D., HANSON, P. R. (1982) The plant breeding potential and genetic variation in cereal phytochrome systems. In. Chemical manipulation of crop growth and development. (ed. J.S. McLaren). pp 425-449 Butterworths, London

GALE, M.D., MARSHALL, G.A. (1973a) Dwarf wheat and gibberellins In. Proc. 4th Int. Wheat Genet. Symp. (eds. E.R. Sears & L.M.S. Sears) pp 513-519 Missouri. Coumbia

GALE, M.D., MARSHALL, G.A. (1973b) Insensitivity to gibberellin in dwarf wheats. *Ann. Bot.* **37**, 729-735

GALE, M.D., MARSHALL, G.A. (1975) The nature and genetic control of gibberellin insensitivity of dwarf wheat grain. *Heredity* **35**, 55-65

GALE, M.D., MARSHALL, G.A. (1976) The chromosomal location of *Gai1* and *Rht1*, genes for gibberellin insensitivity and semi-dwarfism in a derivative of Norin 10 wheat. *Heredity* **37**, 283-289

GALE, M.D., YOUSSEFIAN, S. (1985) Dwarfing genes in wheat. In. Progress in plant breeding - 1 (ed. G.E. Russel) pp 1-35 Butterworths, London

GALE, M.D., LAW, C.N., MARSHALL, G.A., WORLAND, A.J. (1975) The genetic control of gibberellic acid insensitivity and coleoptile length in dwarf wheat. *Heredity* **34**, 393-399

GALE, M.D., MARSHALL, G.A., GREGORY, R.S., QUICK, I. (1981) Norin 10 semi-dwarfism in tetraploid wheat and associated effects on yield. *Euphytica* **30**, 347-354

GALE, M.D., SALTER, M., LENTON, J.R. (1987) The induction of germination alpha amylase during wheat grain development in unfavourable weather conditions. In. 4th Int. Symp. on pre-harvest sprouting in cereals. (ed. D.J. Mares) pp 273-282 Westview Press, Boulder. Colorado

GALSTON, A.W., DAVIES, P.J. (1969) Hormonal regulation in higher plants. *Science* **163**, 1288-1296

GANDER, P.W. (1980) The analysis of growth and cell production in root apices. *Bot. Gaz.* **141**, 131-138

GANDER, P.W. (1983) Growth in root apices. I. The kinematic description of growth. *Bot. Gaz.* **137**, 187-202

GASKIN, P., KIRKWOOD, P.S., LENTON, J.R., MACMILLAN, J., RADLEY, M.E. (1980) Identification of gibberellins in developing wheat grains. *Agric. Biol. Chem.* **44**, 1589-1593.

GASPAR, T., LACOPPE, J. (1968) The effect of CCC and AMO-1618 on growth, catalase, peroxidase and IAA activity in young barley seedlings. *Physiol. Plant.* **21**, 1104-1109

GASPAR, T., PENEL, C.L., THORPE, T., GREPPIN, H. (1982) Peroxidases. 1970-1980; A survey of their biochemical and physiological roles in higher plants. University of Geneva

GASPAR, T., PENEL, C., CASTILLO, F.J., GREPPIN, H. (1985) A two-step control of basic and acidic peroxidases and its significance for growth and development. *Physiol. Plant.* **64**, 418-423

GIBEAUT, D., CARPITA, N.C. (1991) Tracing cell wall biogenesis in intact cells and plant - selective turnover and alteration of soluble and cell wall polysaccharides in grasses. *Plant Physiol.* **97**, 551-561

GILROY, S., TREWAVAS, A. (1990) Signal sensing and signal transduction across the plasma membrane. *In*. The plant plasma membrane. (eds. C. Larsson & I.M. Moller). pp 204-232 Springer-Verlag, Berlin. Heidelberg

GOA, J., HOFSTA, G., FLETCHER, R.A. (1987) Anatomical changes induced by triazoles in wheat seedlings. *Can. J. Bot.* **66**, 1178-1185

GOLDBERG, R. (1980) Cell wall polysaccharidase activities and growth processes. *Physiol. Plant.* **50**, 261-264

GOLDBERG, R., IMBERTY, A., CHU-BA, L. (1986) Development of isoperoxidases along the growth gradient in the mung bean hypocotyl. *Phytochem.* **25**, 1271-1274

GOLDBERG, R. LIEBERMAN, M., MATHIEU, M., PIERRON, M., CASSON, M. (1987) Development of epidermal cell wall peroxidases along the mung bean hypocotyl; possible involvement in the cell wall stiffening process. *J. Exp. Bot.* **38**, 1378-1390

GOTO, N., ESASHI, Y. (1973) Diffusable and extractable gibberellins in bean cotyledons in relation to dwarfism. *Physiol. Plant.* **28**, 480-489

GRAHAM, D., PATTERSON, B.D. (1982) Response of plants to low nonfreezing temperatures; proteins, metabolism and acclimation. *Ann. Rev. Plant Physiol.* **33**, 347-372

GRAEBE, J.E. (1987) Gibberellin biosynthesis and control. *Ann. Rev. Plant Physiol.* **38**, 419-465

GREACON, E.L., OH. J.S. (1972) Physics of root growth. *Nature* **235**, 24-25

GREEN, P.B. (1968) Growth physics in *Nitella*: a method for continuous *in-vivo* analysis of extensibiltiy based on micro-manometer techniques for turgor pressure. *Plant Physiol.* **43**, 1169-1184

GREEN, P.B. (1976) Growth and cell pattern formation on an axis: critique of concepts, terminology and modes of study. *Bot. Gaz.* **137**, 187-202

GREEN, P.B. (1980) Organogenesis. *Ann. Rev. Plant Physiol.* **31**, 51-52

GREEN, P.B. (1984) Analysis of axis extension. *In*. Positional controls in plant development. (eds. P.W. Barlow & D.J. Carr). pp 53-82 Cambridge University Press, Cambridge

GREEN, P.B., ERICKSON, R.O., BUGGY, J. (1971) Metabolic and physical control of cell elongation rate. *In-vivo* studies in *Nitella. Plant Physiol.* **47**, 423-432

GUBBLES, G.H. (1976) Emergence seedling vigour and seed yield of corn after pre-sowing treatments and the addition of phosphorous with the seed. *Can. J. Plant Sci.* **56**, 749-751

GUPTA, A.P., VIRAMI, S.M. (1973) Note on the rooting pattern of three-gene-dwarf wheats. *Indian. J. Agric. Sci.* **43**, 971-973

GUY, C.L. (1990) Cold acclimation and freezing stress tolerance: Role of protein metabolism. *Ann. Rev. Plant Physiol. Mol. Biol.* **41**, 187-223

HABER, A. H. (1962) Nonessentiality of concurrent cell divisions for degree of polarization of leaf growth. 1. Studies with radiation induced mitotic inhibition. *Am. J. Bot.* **49**, 583-589

HABER, A.H., LONG, T.J., FOARD, D.E. (1964) Is final cell size determined by the rate and duration of growth ? *Nature* **201**, 479-480

HAMILTON, H.H. (1948) A developmental study of the apical meristem in four varieties of *Avena sativa* grown at two temperatures. *Am. J. Bot.* **35**, 656-665

HARBERD, N.P., FREELING, M. (1988) Genetics of dominant gibberellin insensitive dwarfism in maize. *Genetics* **121**, 827-838

HARRIS, P.J., HARTLEY, R.D. (1976) Detection of bound ferulic acid in cell walls of *Gramineae* by UV fluorescence microscopy. *Nature* **259**, 508-510

HARTLEY, R.D. (1972) p-coumaric and ferulic acid components of cell walls of ryegrass and the relationship with lignin and digestability. *J. Sci. Food Agric.* **23**, 1347-1354

HARTLEY, R.D. (1973) Carbohydrate esters of ferulic acid as components of cell walls of *Lolium multiflorum. Phytochem.* **12**, 661-665

HARTLEY, R.D. (1976) Diferulic acid as a component of the the cell wall of *Lolium multiflorum. Phytochem.* **15**, 1157-1160

HARTLEY, R.D. (1987) HPLC for the separation and determination of phenolic compounds in plant cell walls. *In*. HPLC in plant science. (eds. H.F. Linskens & J.F. Jackson). pp 92-103 Spinger-Verlag, Berlin, Heidelberg, New York

HARTLEY, R.D., JONES, E.C. (1976) Carbohydrates and carbohydrate esters of ferulic acid released from the cell walls of *Lolium multiflorum* by treatment with cellulytic enzymes. *Phytochem.* **15**, 305-307

HARTLEY, R.D., KEENE, A.S. (1984) Aromatic aldehyde constituents of *Graminaceous* cell walls. *Phytochem.* **23**, 1305-1307

HARTLEY, R.D., WHATLEY, F.R., HARRIS, P.J. (1988) 4,4'- dihydrotruxillic acid as a component of cell walls of *Lolium multiflorum. Phytochem.* **27**, 349-351

HARTLEY, R.D., MORRISON, W.H., BALZA, F., TOWERS, G.H.N. (1990) Substituted truxillic acid and truxininc acids in cell walls of *Cynodon dactylon*. *Phytochem.* **29**, 3699-3703

HATFIELD, R.D., NEVINS, D.J., (1987) Hydrolytic activity and substrate specificity of an endoglucanase from *Zea mays* seedling cell walls. *Plant Physiol.* **83**, 203-207

HAUGHAN, P.A., LENTON, J.R., GOAD, L.J. (1988) Sterol requirements and paclobutrazol inhibition of a celery cell culture. *Phytochem.* **27**, 2491-2500

HAYASHI, T., MARSDEN, M.P.F., DELMER, D.P. (1987) Pea xyloglucan and cellulose. V. Xyloglucan-cellulose interaction *in vitro* and *in vivo*. *Plant Physiol.* **83**, 384-389

HEDDEN, P. (1988) The action of plant growth regulators at the biochemical level. In. Plant growth substances 1988. (eds. R.P Pharis & S.B. Rood) pp 322-332 Spinger-Verlag, Berlin

HEDDEN, P., GRAEBE, J.E. (1985) Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm and *Malus pumila* embryos. *J. Plant Growth Reg.* **4**, 111-122

HEDDEN, P., LENTON, J.R. (1988) Genetical and chemical approaches to the metabolic regulation and mode of action of gibberellins in plants. In. Biomechanisms regulating growth and development. (eds. G.L. Steffens & T.S. Rumsey). pp 175-204 Kluwer Academic Press, New York

HEJNOWICZ, Z. (1959) Growth and cell division in the apical meristem of wheat roots. *Physiol. Plant.* **12**, 124-138

HENDRICKS, T. (1989) Occurrence and properties of *petunia* peroxidase a. *PhD* thesis, University of Wageningen

HENDRICKS, T., VAN LOON, C.C. (1990) The nature and heterogeneity of *Petunia* peroxidase. *J. Plant Phys*iol. **136**, 526-531

HEPLER, P.K., WAYNE, R.O. (1985) Calcium and plant development. *Ann. Rev. Plant Physiol.* **36**, 397-439

HETHERINGTON, P.R., LAIDMAN, D.L. (1991) Influence of gibberellic acid and the *Rht3* gene in choline and phospholipid metabolism in wheat aleurone tissue. *J. Exp. Bot.* **42**, 1357-1362

HEYN, A.J.N. (1933) Further investigations on the mechanism of cell elongation and the properties of the cell wall in connection with elongation. *Protoplasma* **19**, 78-96

HILL, R.L., BRADSHAW, R.A. (1969) Fumarase. *Meth. Enzymol.* **13**, 91-99

HO, T.D.H., NOLAN, R.C., SHUTE, D.E. (1981) Characteristion of a gibberellin-insensitive dwarf wheat, D6899. Evidence for a regulatory step common to many diverse responses to gibberellins. *Plant Physiol.* **67**, 1026-1031

HOAGLAND, D.R., ARNON, D.I. (1938) The water culture method for growing plants without soil. *Circ. Calif. Agric. Expt. Stn. ext. serv.* **347**, 1-12

HOHL, M., SCHOPFER, P. (1991) Water relations in growing maize coleoptiles. *Plant Physiol.* **95**, 716-722

HOLMES, D.P. (1973) Influorescence development in short days and standard height wheat cultivars in different photoperiod and nitrogen treatments. *Can. J. Bot.* **51**, 941-956

HOOGENDOORN, J., RICKSON, J.M., GALE, M.D. (1990) Differences in leaf and stem anatomy related to plant height of tall and dwarf wheat (*Triticum aestivum* L.) *J. Plant Physiol.* **136**, 72-77

HOSON, T., WADA, S. (1980) Role of hydroxyproline rich cell wall protein in growth regulation of rice coleoptiles grown on or under water. *Plant Cell Physiol.* **21**, 511-524

HSIAO, T., SILK, W.K., JING, J. (1985) Leaf growth and water defecits: biophysical effects. In. Control of leaf growth. (eds. N.R. Davies, W.J. Davies, C.K. Ong). pp 239-266. S.E.B. seminar series 27. Cambridge University Press, Cambridge

HUBER, D.J., NEVINS, D.J. (1980) β-D-Glucan hydrolase activity in *Zea* coleoptile cell wall. *Plant Physiol.* **64**, 185-189

HUSSEY, G. (1971) In-vitro growth of vegetative tomato shoot apices. *J. Exp. Bot.* **72**, 688-701

ILYAMA, K., TUYET, T.B., STONE, B.A. (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochem.* **29**, 733-737

INGRAM, T.J., REID, J.B., MURFET, I.C., GASKIN, P., GIMOUR, S.J., MACMILLAN, J. (1984) Internode length in *Pisum*. The *Le* gene controls the 3β-hydroxylation of gibberllin A_{20} to gibberellin A_1. *Planta* **160**, 455-463

INGRAM, T.J., REID, J.B., MACMILLAN, J. (1986) The quantitative relationship between gibberellin A_1 and internode growth in *Pisum sativum* L. *Planta* **168**, 414-420

ISHII, T., HIROI, T., THOMAS, J.R. (1990) Feruloylated xyloglucan and *p*-coumaroyl arabinoxylan oligosaccharides from bamboo shoot cell walls. *Phytochem.* **29**, 1999-2003

ISHII, T. (1991) Isolation and characterisation of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell walls. *Carbohydr. Res.* **219**, 12-22

IZUMI, N., SAWADA, S., SASAKUMA, T. (1981) A dominant gene of dwarfism located on chromosome 4D in *Triticum aestivum* cultivar Ai-bian. Wheat information service **53**, 21-24

IZUMI, K., KAMIYA, Y., SAKURAI, A., OSHIO, H., TAKAHASHI, N. (1988) Studies of sites of action of a new plant growth regulator (E)-1-(4-chlorophenyl)-4, 4-dimethyl-2-(1, 2, 4-triazol-1-yl)-1-penten-3-ol. (S3307) and comparative effects of its stereoisomers in a cell free system from *Cucurbita maxima. Plant Cell Physiol.* **26**, 821-827

JAIN, H. K., SINHA, S. K., KULSHRESTHA, V.P., MATHUR, V.S. (1974) Breeding for yield in dwarf wheats. Proc. 4th Int. Wheat Genet. Symp. pp 527-531

JENSON, E., JUNTILLA, O. (1987) Endogenous gibberellins in young seedlings of wheat (*Triticum aestivum*) cultivars. *Physiol. Plant.* **71**, 277-280

JONES, M.G. (1987) Gibberellins and the *procera* mutant of tomato. *Planta* **172**, 280-284

JONES, R.L., MOLL, C. (1983) Gibberellin-induced growth in excised lettuce hypocotyls. In. The biochemistry and physiology of gibberellins. (ed. A. Crozier). pp 92-128 Volume 2 Preager, New York

JOPPA, L.R. (1973) Agronomic characteristics of near-isogenic tall and semi-dwarf lines of durum wheat. *Crop Sci.* **13**, 743-746

JOPPA, L.R., WALSH, D.E. (1974) Quality characteristics of tall and semi-dwarf near-isogenic lines of durum wheat. *Crop Sci.* **14**, 420-422

JUPE, S.C., SCOTT, I.M. (1989) The *slender* phenotype of pea: stem growth, peroxidase levels and ethylene responses. *Physiol. Plant.* **77**, 59-66

JUPE, S.C., SCOTT, I.M. (1992) Gibberellin and the *pro* gene suppresses peroxidase activity in elongating tomato (*Lycopersicum esculentum* Mill) stem tissue. *Ann. Bot.* **69**, 33-37

JUSAITIS, M., PALEG, L.G. (1982) The influence of gibberellic acid and temperature on the growth rate of *Avena sativa* segments. *Plant Physiol.* **70**, 532-539

KAMERBEEK, G.A. (1956) Peroxidase content of dwarf types and giant types of plants. *Acta. Bot. Neerl.* **5**, 257-263

KAMISAKA. S., SANO, H., KATSUMI, M., MATSUDA, Y. (1972) Effects of cyclic-AMP and gibberellic acid on lettuce hypocotyl elongation and mechanical properties of its cell wall. *Plant Cell Physiol.* **12**, 167-174

KAMISAKA, S., TAKEDA, S., TAKAHASHI, K., SHIBATA, K. (1990) Diferulic acid and ferulic acid in the cell wall of *Avena* coleoptiles - their relationship to mechanical properties of the cell wall. *Physiol. Plant.* **78**, 1-7

KAMIYA, N., TAZAWA, M., TAKETA, T. (1963) The effects of the turgor pressure to cell volume in *Nitella* with special reference to the mechanical properties of the cell wall. *Protoplasma* **57**, 501-521

KARBASSI, P., GARRARD, L.A., WEST, S.H. (1971) Reversal of low temperature effects on a tropical plant by gibberellic acid. *Crop Sci.* **11**, 755-757

KARSSEN, C. M., GROOT, S. P. C., KOORNEEF, M. (1987) Hormone mutants in Arabidopsis and tomato. In. Developmental mutants in higher plants. (eds. H. Thomas., D. Grierson). Cambridge University Press, Cambridge

KATO, Y., NEVINS, D.J. (1985) Isolation and identification of o-(5-o-feruloly-L-arabinofuranosyl) -(1-3)-o-β-D-xylopyranosyl-(1-4)-D-xylopyranose as a compound of *Zea* shoot cell wall. *Carbohydr. Res.* **137**, 139-150

KATZUMI, M., KAZAMA, H. (1978) Gibberellin control of cell elongation in cucumber hypocotyl sections. *Bot. Mag. Tokyo Special Issue* **1**, 141-158

KAUFMAN, P.B. (1965) The effects of growth substances on intercalary growth and cellular differentiation in developing internodes of *Avena sativa*. II. The effects of gibberellic acid. *Physiol. Plant.* **18**, 703-724

KAUFMAN, P.B., MOORE, P.H., GHOSHEH, N.S., LEE, M., CARLSON, T.J., BIGELOW, W.C., KRAUS, S., RIGOT, W. (1981) Regulation of silica deposition in sugarcane by gibberellic acid. *Plant Physiol.* **68**, 314-317

KAUFMAN, P.B., DAYANANDAN, P (1983) Gibberellin-induced growth in *Avena* internodes. *In.* The biochemistry and physiology of gibberellins. (ed. A. Crozier). pp 129-157 Volume 2 Praeger, New York

KAWAMURA, H., KAMISAKA, S., MASUDA, Y. (1976) Regulation of lettuce hypocotyl elongation by gibberellic acid. Correlation between cell elongation, stress-relaxation properties of the cell wall polysaccharide content. *Plant Cell Physiol.* **17**, 23-34

KEMP, D.R. (1980) The location of the extension zone of emerging wheat leaves. *New. Phytol.* **54**, 729-737

KEMP, D.R., BLACKLOW, W.M. (1980) Diurnal extension rates of wheat leaves in relation to temperature and carbohydrate concentration of the extension zone. *J. Exp. Bot.* **31**, 821-828

KEYES, G.J. (1987) Genetic control of sensitivity to gibberellic acid in wheat: some developmental consequences. *PhD* thesis, Cornell University

KEYES, G.J., PAOLLILO, D.J., SORRELLS, M.E. (1989) The effects of dwarfing genes on *Rht1* and *Rht2* on cellular dimensions and rate of leaf elongation in wheat. *Ann. Bot.* **64**, 683-690

KEYES, G.J., SORRELLS, M.E., SETTER, T.M. (1990) Gibberellic acid regulates cell wall extensibility in wheat (*Triticum aestivum* L.) *Plant Physiol.* **92**, 242-245

KHANNA, K.R. (1991) Crop improvement in the perspective of agricultural advancement and the necessity for investigations at the molecular level. In Biochemical aspects of crop improvement. (ed. K.R. Khanna). pp 3-36 C.R.C. Press, Boca Rato. Ann Arbor. Boston

KING, R.W., GALE, M.D., QUARRIE, S.A. (1983) Effects of Norin 10 and Tom Thumb dwarfing genes on morphology, physiology and abscisic acid production in wheat. *Ann. Bot.* **51**, 201-208

KIRBY, E.J.M., APPLEYARD, M. (1981) Cereal development guide. (ed. A. Barron). Plant Breeding Inst.

KOBAYASHI, M., SAKURAI, A., SAKA, H., TAKAHASHI, N. (1989) Quantitative analysis of endogenous gibberellins in normal and dwarf cultivars of rice. *Plant cell Physiol.* **30**, 963-969

KOORNEEF, M., VAN DER VEEN, J.H. (1980) Induction and analysis of gibberellin sensitive mutants of *Arabidopsis thaliana* L. Heynh. *Theoret. Appl. Genet.* **58**, 257-263

KOORNEEF, M, ELGERSMA, A., HANHART, C.J., VAN LOENEN-MARTINET, E.P., VAN RIJN, L., ZEEVAART J.A.D. (1985) A gibberellin insensitive mutant of *Arabidopsis thaliana* L. *Physiol. Plant.* **65**, 33-39

KONZAK, C.F. (1976) A review of semi-dwarfing gene sources and a description of some new short stature wheats. In. Induced mutations in Cross Breeding, Proceedings of an advisory group held in Vienna. October 1975 pp 79-93 IAEA Vienna Austria

KORNER, CH., LARCHER, W. (1988) Plant life in cold climates. In. Plants and temperature. Symposia for the Society of Experimental Biologists. (eds. F.I. Woodward & S.P. Long). pp 25-58 Cambridge University Press, Cambridge

KOSTER, K.L., LYNCH, D.V. (1992) Solute accumulation and compartmentation during cold acclimation of Puma rye. *Plant Physiol.* **98**, 108-113

KNIGHT, M.R., CAMPBELL, A.K., SMITH, S.M., TREWAVAS, A.J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**, 524-526

KUTSCHERA, U. (1987) Cooperation between outer and inner tissues in auxin-mediated plant organ growth. *In*. Physiology of cell expansion during plant cell growth. (eds. D.J. Cosgrove & D.P. Knievel). pp 215-226 The American Society Plant Physiologists, Rockville

KUTSCHERA, U. (1989) Tissue stresses in growing plant organs. *Physiol. Plant.* **77**, 157-163

KUTSCHERA, U. (1990) Cell-wall synthesis and elongation growth in hypocotyls of *Helianthus annus* L. *Planta* **181**, 316-323

KUTSCHERA, U., SCHOPFER, P. (1986) Effect of auxin and abscisic acid on cell wall extensibility in maize coleoptiles. *Planta* **67**, 527-535

KUTSCHERA, U., BRIGGS, W.R. (1987) Differential effect of auxin on *in vitro* extensibility of cortical cylinder and epidermis in pea internode. *Plant Physiol.* **84**, 1361-1366

KUTSCHERA, U., BERGFIELD, R., SCHOPFER, P. (1987) Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coleoptiles. *Planta* **170**, 168-180

LABRADOR, E., NEVINS, D.J. (1989) An exo-β-D-glucanase derived from *Zea* coleoptile walls with a capacity to elicit cell elongation. *Physiol. Plant.* **77**, 479-486

LAM, S.L. (1968) Interaction of temperature and gibberellin on potato seed germination. *Am. J. Bot.* **55**, 193-198

LAM, T.B.T., ILYAMA, K., STONE, B.A. (1990) Distribution of free and combined phenolic acids in wheat internodes. *Phytochem.* **29**, 429-433

LANAHAN, M.B., HO, T.D. (1988) Slender barley: a constitutive gibberellin response mutant. *Planta* **175**, 107-114

LANGER, R.H.M. (1972) Stem and root growth *In*. How grasses grow. Studies in biology N° **34**. Edward Arnold, London

LAW, C. N. (1989) Plant genetics and breeding research. *In*. Plant Breeding Institute Annual Report 1989. pp 1-2

LAW, C.N., PAYNE, P.I. (1983) Genetical aspects of breeding for improved grain protein content and type in wheat. *J. Cereal Sci.* **1**, 79-93

LAW, C.N., SNAPE, J,W., WORLAND, A.J. (1979) The genetical relationship between height and yield in wheat. *Heredity* **40**, 133-151

LAWLOR, D.W., LEECH. J.E. (1985) Leaf growth and water deficits: biochemistry in relation to biophysics. *In*. Control of leaf growth. (eds. N.R. Baker. & W.J. Davies). pp 267-294 S.E.B. seminar series 27. Cambridge University Press, Cambridge

LEECH, R.M. (1985) The synthesis of cellular components in leaves. _In_. Control of leaf growth. (eds. N.R. Baker, W.J. Davies., C.K. Ong). pp 93-113 S.E.B. seminar series 27. Cambridge University Press, Cambridge

LEECH, R.M., RUMSBY, M.G., THOMSON, W.W. (1973) Plastid differentiation, acyl lipid and fatty acid changes in developing green maize leaves. *Plant Physiol.* **52**, 240-245

LELAND, M., SHANNON, M., KAY, E., LEW, J.Y. (1966) Peroxidase isoenzymes from horseradish root. *J. Biol. Chem.* **241**, 2166-2172

LENTON, J.R. (1987) Mode of action of triazole growth retardents and fungicides - a progress report. *No 1. Vol 9. British Plant Regulator Group.* pp 1-12 Wantage

LENTON, J.R., HEDDEN, P., GALE, M.D. (1987) Gibberellin insensitivity and depletion in wheat - consequences for development. _In_. Hormone action in plant development - a critical appraisal. (eds. G.V. Hoad, J.R. Lenton, M.B. Jackson, R.K. Atkin). pp 145-160. Butterworths, London

LEOPOLD, A.C. (1988) Calcium and second messengers in hormonal regulation. _In_. Plant Growth Regulation 1988. (eds. R.P. Pharis. & S.B. Rood). pp 203-208 Spinger-Verlag, Berlin

LIANG, D.R., FISCHER, R.A. (1977) Adaptations of semi-dwarf wheat cultivars to rainfed conditions. *Euphytica* **26**, 129-139

LINTILHAC, P.M. (1974a) Differentiation, organogenesis, and the tectonics of cell wall orientation II. Separation of stresses in a two-dimensional model. *Am. J. Bot.* **61**, 135-140

LINTILHAC, P.M. (1974b) Differential organogenesis and the tectonincs of cell wall orientation. III. Theoretical considerations of cell wall mechanics. *Am. J. Bot.* **61**, 230-237

LINTILHAC, P.M., VESECKY, T.B. (1981) Mechanical stress and cell wall orientaion in plants. II. The application of controlled directional stress to growing plants with a discussion on the nature of the wound response. *Am. J. Bot.* **68**, 1222-1230

LOCKHART, J.A. (1965) An analysis of irreversible plant cell elongation. *J. Theoret. Biol.* **8**, 264-275

LOVETT, J.V., KIRBY, E.J.M. (1971) The effect of plant population and CCC on spring wheat varieties with and without a dwarfing gene. *J. Agric. Sci.* **77**, 499-510

LUPTON, F.G.A., OLIVER, R.H., ELLIS, F.B. BURNES, B.T., HOWSE, K.R., WELBANK, P.J., TAYLOR, P.J. (1974) Root and shoot growth of semi-dwarf and taller winter lines. *Ann. App. Biol.* **77**, 129-144

LUTTENEGGER, D.G., NEVINS, D.J. (1985) Transient nature of (1-3),(1-4)-β-d-glucan in *Zea mays* coleoptile cell walls. *Plant Physiol.* **77**, 175-178

LYNDON, R.F. (1987) Initiation and growth of internodes and stem and flower frusta in *Silene coeli-rosa*. _In_. Manipulation of flowering. (ed. J.G. Atherton). pp 301-314 Butterworths, London

MALONE, M., LEIGH, R.A., TOMOS, A.D. (1989) Extraction and analysis of sap from individual wheat leaf cells; the effect of sampling speed on the osmotic pressure of extracted sap. *Plant Cell Environ.* **12**, 917-926

MACADAM, J.W., VOLNEC, J.J., NELSON, C.J. (1989) Effects of nitrogen on mesophyll cell division and epidermal cell elongation in tall fescue leaf blades. *Plant Physiol.* **89**, 549-556

MACMILLAN, J. (1987) Gibberellin-deficient mutants of maize and pea and the molecular action of gibberellins. In. Hormone action in plant development - a critical appraisal. (eds. G.V. Hoad., J.R. Lenton., M.B. Jackson., R.K. Atkin). pp 73-87. Butterworths, London

MACMILLAN, J., PHINNEY, B. O. (1987) Biochemical genetics and the regulation of stem elongation by gibberellins. In. Physiology of cell expansion during plant growth. (eds. D.J. Cosgrove & D.P. Knievel). pp 156-171 The American Society of Plant physiologists, Rockville

MARKWALDER, H.M., NEUKOM, H. (1976) Diferulic acid as a possible cross-link in hemicellulose from wheat germ. *Phytochem.* **15**, 836-837

MARX, G.A. (1983) Developmental mutants in some annual seed plants. *Ann. Rev. Plant Physiol.* **34**, 389-417

MATTHEWS, M.A., VAN VOLKENBURGH, E., BOYER, J.S. (1984) Acclimation of leaf growth at low water potentials in sunflower. *Plant Cell Environ.* **7**, 199-206

MASUDA, Y. (1990) Auxin-induced cell elongation and cell wall changes. *Bot. Mag. Tokyo* **103**, 345-370

MATSUDA, K., RIAZI, A. (1981) Stress induced osmotic adjustment in growing regions of barley leaves. *Plant Physiol.* **68**, 571-576

MAXWELL, J.O. REDMAN, R.E. (1978) Water potential and component potentials in expanded and unexpanded xeric grasses. *Physiol. Plant.* **44**, 383-387

MCCANN, M.C., WELLS, M.C., ROBERTS, K. (1990) Direct visulalization of cross-links in the primary plant cell wall. *J. Cell Sci.* **96**, 323-334

MCCLUNG, A.M., CANTRELL, R.G., QUICK, J.S., GREGORY, R.S. (1986) Influence of the *Rht1* semi-dwarf gene on yield, yield components and grain protein in durum wheat. *Crop Sci.* **26**, 1095-1099

MCCUNE, D.G., GALSTON, A.W. (1959) Inverse effects of gibberellin on peroxidase activity and growth in dwarf strains of peas and corn. *Plant Physiol.* **34**, 416-418

MCDOUGALL, G., FRY, S.F. (1989) Structure activity relationships for xyloglucan oligosaccharides with anti-auxin activity. *Plant Physiol.* **89**, 885-887

MCDOUGALL, G., FRY, S.F. (1990) Xyloglucan oligosaccharides promote growth and activate cellulase; evidence for a role of cellulase in cell expansion. *Plant Physiol.* **93**, 1042-1048

MCINTOSH, R.A. (1979) Catalogue of gene symbols for wheat In. Proc. 5th Int. Wheat Genet. Symp. New Delhi. (ed. S. Ramanujam). p 1299-1309 Indian Soc. of Genet. and Plant Breeding. New Delhi. India

MCNEIL, M., DARVILL, A.G., FRY, S.C., ALBERSHEIM, P. (1984) Structure and function of the the primary cell walls in plants. *Ann. Rev. Biochem.* **53**, 625-663

MCVITTIE, J.A., GALE, M.D., MARSHALL, G.A., WESTCOTT, B. (1978) The intrachromasomal mapping of the Norin 10 and Tom Thumb dwarfing genes. *Heredity* **40**, 67-70

METRAUX, J.P. (1988) Gibberellins and plant cell elongation. In. Plant hormones and their role in plant growth and development. (ed. P.J. Davies). pp 296-317 Kluwer Academic Press, London

METRAUX, J.P., TAIZ, L. (1978) Transverse viscoelastic extension in *Nitella*. I. Relation to growth rate. *Plant Physiol.* **61**, 135-138

METRAUX, J.P., RICHMOND, P.A., TAIZ, L. (1980) Control of cell elongation in *Nitella* by endogenous cell wall pH gradients. *Plant Physiol.* **65**, 204-210

MEYER, R.F., BOYER, J.S. (1972) Sensitivity of cell division and elongation to low water potentials in soybean hypocotyls. *Planta* **108**, 77-88

MICHELENA, V., BOYER, J.S. (1982) Complete turgor maintenance at low water potentials in the elongation region of maize leaves. *Plant Physiol.* **69**, 1145-1149

MIDMORE, D.J., PRANGE, R.K. (1992) Growth responses of two *solanum* species to contrasting temperatures and irradiance levels: relations to photosynthesis, dark respiration and chlorophyll fluorescence. *Ann. Bot.* **69**, 13-20

MILLIGAN, S.P. (1986) Effects of root excision and root cooling on primary leaf expansion in *Phaseolus vulgaris* L. *PhD* thesis, University of Edinburgh

MITA, T., SHIBAOKA, H. (1984) Effects of S-3307, an inhibitor of gibberellin biosynthesis on swelling of leaf sheath cells and on the arrangement of cortical microtubules in onion seedlings. *Plant Cell Physiol.* **25**, 1531-1539

MOLL, C., JONES, R.L. (1981) Calcium and gibberellin induced elongation of lettuce hypocotyl sections. *Planta* **152**, 442-449

MOLZ, F.J., BOYER, J.S (1978) Growth-induced water potentials in plant cells and tissues. *Plant Physiol.* **62**, 423-429

MOLZ, F.J., FERRIER, J.M. (1982) Mathematical treatment of water movement in plant cells and tissues; a review. *Plant Cell Environ.* **5**, 191-206

MONGELARD, J.C., MIMURA, L. (1978) Gibberellin studies on the sugarcane plant. III. Some effects of root temperature and gibberellic acid and the interaction on growth. *Crop Sci.* **12**, 52-58

MOORE, P.H., BUREN, L.L. (1978) Gibberellin studies with sugarcane. I. Cultivar differences in growth responses to gibberellic acid. *Crop Sci.* **17**, 443-446

MOORE, P.H., GINZOA, H. (1980) Gibberellin studies with sugarcane. III. Effects of rate and frequency of gibberellic acid application on stalk length and fresh weight. *Crop Sci.* **20**, 78-82

MORGAN, J.A., LECAIN, D.R., WELLS, R. (1990) Semidwarfing genes concentrate photosynthetic machinery and affect leaf gas exchange of wheat. *Crop Sci.* **30**, 602-608

MORRIS, R., SCHMIDT, J.W., JOHNSON, V.A. (1972) Chromosomal location of a dwarfing gene in Tom Thumb derivative by monosomic analysis. *Crop Sci.* **12**, 247-249

MUELLER-HARVEY, I.M., HARTLEY, R.D., HARRIS, P.J., CURZON, E.H. (1986) Linkage of *p*-coumaroyl and feruloyl groups of cell wall polysaccharides of barley straw. *Carbohydr. Res.* **148**, 71-85

MUNNS, R., WEIR, R. (1981) Contribution of sugars to osmotic adjustment in elongating and expanding zones of wheat leaves during moderate water deficits at two light levels. *Aust. J. Plant Physiol.* **8**, 93-105

MURAKAMI, Y. (1972) Dwarfing genes in rice and their relation to gibberellin biosynthesis. In. Plant growth substances 1970. (ed. C.J. Carr). pp 166-174 Academic Press, London

MURRAY, A.K., BANDURSKI, R.S. (1975) Correlative studies of cell wall enzymes and growth. *Plant Physiol.* **56**, 143-157

NIKLAS, K.J., PAOLILLO, D.J. (1990) Biomechanical and morphometric differences in *Triticum aestivum* seedlings differing in *Rht* gene dosage. *Ann. Bot.* 65, 365-377

NILSON, E.B., JOHNSON, V.A., GARDNER, C.O. (1957) Parenchyma and epidermal cell length in relation to plant height and culm internode length in winter wheat. *Bot. Gaz.* **119**, 38-43

NORTHCOTE, D.H. (1972) Chemistry of the plant cell wall. *Ann. Rev. Plant Physiol.* **23**, 113-132

OKAMOTO, H., MIWA, C., MASUDA, T., NAKAHORI, K., KATOU, K. (1990) Effects of auxin and anoxia on the cell wall yield threshold determined by the negative pressure jumps in segments of cowpea hypocotyl. *Plant Cell Physiol.* **31**, 783-788

ORDIN, L., APPLEWHITE, T.H., BONNER, J. (1956) Auxin induced water uptake by *Avena* coleoptile sections. *Plant Physiol.* **31**, 44-53

OUGHAM, H.J., THOMAS, T.W.A., EVANS, M.L. (1987) Leaf development in *Lolium temulentum* L.: Progressive changes in soluble polypeptide complement and isoenzymes. *J. Exp. Bot.* **38**, 1689-1696

PAOLILLO, D.J., SORRELLS, M.E., KEYES, G.J. (1991) Gibberellic acid sensitivity determines the length of the extension zone in wheat leaves. *Ann. Bot.* **67**, 479-485

PAULS, K.P., CHAMBERS, J.A., DUMBROFF, E.B., THOMPSON, J.E. (1982) Perturbation of phospholipid membranes by gibberellins. *New Phytol.* **91**, 1-17

PEACOCK, E.M.M. (1975) Temperature and leaf growth in *Lolium perenne*. II. The site of temperature perception. *J. App. Ecol.* **12**, 115-123

PEARSE A.G.E (1968) The Periodic Acid-Sciff reaction. In. Histochemistry. Theoretical and applied pp 659-660 Appendix 10. 3rd edition Vol. 1. Churchill Press, London

PENEL, C., GREPPIN, H. (1979) Effect of calcium on subcellular distribution of peroxidases. *Phytochem.* **18**, 29-33

PENNY, D., MILLER, K.F., PENNY, P. (1972) Studies on the mechanism of cell elongation of lupin hypocotyl segments. *N. Z. J. Bot.* **10**, *97-111*

PEPE, J.F., HEINER, R.E. (1975) Plant height, protein percentage and yield relationships in spring wheat. *Crop Sci.* **15**, 793-797

PHARIS, R.P., KING, R.W. (1985) Gibberellins and reproductive development in seed plants. *Ann. Rev. Plant Physiol.* **36**, 517-568

PHINNEY, B. O. (1956) Growth responses of single gene mutants in maize to gibberellic acid. *Proc. Natl. Acad. Sci. USA* **43**, 398-404

PHINNEY, B.O. (1984) Gibberellin A_1 dwarfism and the control of shoot elongation in higher plants. In. The biosynthesis and metabolism of plant hormones. (eds. A. Crozier & J. R. Crozier). pp 17-41. S.E.B. seminar series 23. Cambridge University Press, Cambridge

PHINNEY, B.O., SPRAY, C. (1982) Chemical genetics and the gibberellin pathway in *Zea mays* L. In. Plant Growth Substances 1982 (ed. P.F. Wareing) pp 101-110 Academic Press, London

PHINNEY, B.O., WEST, C.A. (1957) The growth response of a single gene dwarf mutants of *Zea mays* to gibberellins and gibberellin like substances. *Proc. Int. Genet. Symp.* pp 384-385

PINTHUS, M.J., GALE, M.D. (1990) The effects of gibberellin insensitive dwarfing allelles on wheat in grain weight and protein content. *Theoret. App. Genet.* **79**, 108-112

PINTHUS, M.J., LEVY, A.A. (1983) The relationship between *Rht1* and *Rht2* dwarfing genes in *Triticum aestivum* L. spring wheat. *Theoret. App. Genet.* **66**, 153-157

PINTHUS, M.J., GALE, M.D., APPLEFORD, N.E.J., LENTON, J.R. (1989) Effect of temperature on gibberellin (GA) responsiveness and on endogenous GA_1 content of tall and dwarf wheat genotypes. *Plant Physiol.* **90**, 854-859

POLLOCK, C.J., CAIRNS, A.J. (1991) Fructan metabolism in grasses and cereals. *Ann. Rev. Plant Physiol. Mol. Biol.* **42**, 77-101

POLLOCK, C.J., EAGLES, C. F. (1988) Low temperature and the growth of plants. In. Plants and temperature. (eds. F.I. Woodward & S.P. Long). pp 157-180 Cambridge University Press, Cambridge

POLLOCK, C.J., LLOYD, E.J., STODDART, J.L., THOMAS, H., (1983) Growth, photosynthesis and assimilate partition in *Lolium temulentum* exposed to chilling temperatures. *Physiol. Plant.* **59**, 257-262

POLLOCK, C.J., LLOYD, E.J., THOMAS, H., STODDART, J.L (1984) Changes in the photosynthetic capacity during prolonged growth of *Lolium temulentum* at low temperatures. *Photosynthetica* **18**, 478-481

POLLOCK, C.J., TOMOS, A.D., THOMAS, H., SMITH.C.J., LLOYD, E.J., STODDART, J.L. (1990) Extension growth in a barley mutant with reduced sensitivity to low temperature. *New Phytol.* **115**, 617-623

POLLOCK, C.J., OUGHAM, H.J., STODDART, J.L. (1992) The slender mutation of barley. In. Barley; genetics, biochemistry, molecular biology and biotechnology. (ed. P.R. Shewry). C.A.B. International. (In press)

POPE. D.G. (1982) Effects of peeling on auxin-induced growth in *Avena* coleoptile. *Ann. Bot.* **49**, 493-501

POTTS, W.C., REID, J.B., MURFET, I.C. (1982) Internode length in *Pisum*. I. The effect of the *Le/le* gene differences on endogenous gibberellin like substances. *Physiol. Plant.* **55**, 323-328

POTTS, W. C., REID, J. B. (1983) Internode length in *Pisum* III. The effect and interaction of the *Na/na* and the *Le/le* gene differences on endogenous gibberellin-like substances. *Physiol. Plant.* **57**, 448-454

POTTS, W.C., REID, J.B., MURFET, I.C. (1985) Internode length in *Pisum*. Gibberellins and the slender phenotype. *Physiol. Plant.* **63**, 357-364

PRESTON, R.D. (1974) Wall extension and cell growth. In. The physical biology of plant cell walls. pp 383-421 Chapman and Hall, London

PRESTON, R. D. (1979) Polysaccharide conformation and cell wall function. *Ann. Rev. Plant Physiol.* **30**, 55-78

PRITCHARD, J., TOMOS, A.D., WYN JONES, R.G. (1987) Control of wheat root elongation growth. 1. Effects of ions on growth rate, wall rheology and cell water relations *J. Exp. Bot.* **38**, 948-95

PRITCHARD, J., WYN JONES, R.G., TOMOS, A.D. (1988) Control of wheat root growth. The effects of excision on growth, wall rheology and root anatomy. *Planta* **176**, 399-405

PRITCHARD, J., ADAMS, J.S., BARLOW, P.W., TOMOS, A.D. (1990) Biophysics of inhibition of root growth by low temperature. *Plant Physiol.* **93**, 222-230

PRITCHARD, J., WYN JONES, R.G., TOMOS, A.D. (1991) Turgor, growth and rheological gradients of wheat roots following osmotic stress. *J. Exp. Bot.* **42**, 1043-1049

PROEBSTING, W.M., DAVIES, P.J. (1977) Evidence for a graft transmissable substance which delays apical senescence in (*Pisum sativum* L.) *Planta* **135**, 93-94

PROEBSTING, W.M., DAVIES, P.J., MARX, G.A. (1978) Photo period induced changes in gibberellin metabolism in relation to apical growth and senesence in genetic lines of peas (*Pisum sativum* L.) *Planta* **141**, 231-238

RADLEY, M. (1970) Comparison of endogenous gibberellins and response to applied gibberellins of some tall and dwarf cultivars. *Planta* **92**, 292-300

RANJEVA, R., BOUDET, A.M., ALIBERT, G. (1978) Isoenzymes and compartmentation in the regulation of phenolic acid metabolism. In. Regulation of secondary product and hormone metabolism. (eds. M. Luckner & K. Schreiber). FEBS symposium S8 Vol. 55 pp 91-100 Pergamon Press, Oxford

RASHID, A.H., HALLORAN, G.M. (1984) Influence of photoperiod on culm elongation and apical development and semi-dwarf and standard height wheat. *Ann. Bot.* 54, 375-382

RAWSON, H.M., EVANS, L.T. (1971) The contribution of stem reserves to grain development in a range of wheat cultivars of different height. *Aust. J. Agric. Sci.* 22, 851-863

RAY, P.M. (1987) Principles of plant cell expansion. *In*. physiology of cell expansion during plant growth. (eds. D.J. Cosgrove, D.P. Knievel). pp 1-19 The American Society of Plant Physiologists, Rockville

RAY, P.M., RUESINK, A. W. (1962) Kinetic experiments on the nature of the growth mechanism in oat coleoptile cells. *Develop. Biol.* **4**, 377-397

RAY, P.M., GREEN, P.B., CLELAND, R. (1972) Role of turgor in plant cell growth. *Nature* **239**, 163-164

RAYLE, D.L., CLELAND, R. (1977) Control of plant cell enlargement by hydrogen ions. *Curr. Topics Devel. Biol.* **11**, 187-214

RAYLE, D.L., CLELAND, R. (1980) Evidence that auxin-induced growth of soybean hypocotyls involved proton excretion. *Plant Physiol.* **66**, 433-437

RAYLE, D.L., HAUGHTON, P.M., CLELAND, R.E. (1970) An *in vitro* system that simulates plant cell extension growth. *Proc. Natl. Acad. Sci. USA* **67**, 1814-1817

REDDI, M.W., HEYNE, E.G., LIANG, G.H.L (1969) Heritabilities and interrelationships of shortness and the agronomic characters in F3 and F4 generations of two wheat crosses (*Triticum eastivum* L. em Thell. *Crop Sci.* **9**, 222-225

REID, J.B. (1986) Gibberellin mutants. *In*. A genetic approach to plant biochemistry. (eds. A.D. Bondstein & P.J. King). pp 1-34 Springer-Verlag, Wien. New York

REITZ, L.P., SALMON, S.C. (1968) Origin, history and use of Norin 10 wheat. *Crop Sci.* **8**, 686-689

RHOADES, R.E. (1991) The worlds food supply at risk. *National Geographic Magazine* April 1991 pp 74-105

RICH, T.G.C., TOMOS, A.D. (1988) Turgor pressure and phototropism in *Sinapis alba* L. seedlings. *J. Exp. Bot.* 39, 291-299

RICHMOND, P.A., METRAUX J-P, TAIZ, L. (1980) Cell expansion patterns and directionality of wall mechanical properties in *Nitella*. *Plant Physiol.* **65**, 211-217

RIGGS, T.J., HAYTER, A.M. (1975) A study of the inheritance and interrelationships of some agronomically important characters in spring barley. *Theoret. Appl. Gen.* **46**, 257-264

ROBERTS, J.A., HOOLEY, R. (1988) Hormones and the concept of sensitivity, a rational approach. *In*. Plant growth regulators pp 49-67 Blackie. USA

ROBERTSON, D., LAETSCH, W.M. (1974) Structure and function of developing green maize leaves. *Plant Physiol.* **54**, 148-159

ROMANOVA, L.V., PRILYUK, L.V. (1975) Hormonal composition and and reaction of dwarf wheats of different origin to gibberellin. *Sel'skohozyaristvennaya Biologiya* **10**, 750-755

ROOD, S.B., MAJOR, D.J., JONES, M.D., PHARIS, R.P. (1985) Low temperature eliminated heterosis for growth of gibberellin content in maize. *Crop Sci.* **25**, 1063-1068

ROONEY, J.M., BRAIN, P., LOY, S.Y (1989) The influence of temperature on leaf production and vegetative growth of *Avena fatua* L. *Ann. Bot.* **64**, 469-479

ROSE, R.J., ADAMSON, D. (1969) A sequential response to growth substances in coleoptiles from γ irradiated wheat. *Planta* **88**, 274-281

ROSIELLE, A.A., FREY, K.J. (1975) Estimates of selection parameters associated with harvest index in oat lines derived from a bulk population. *Euphytica* **24**, 121-131

ROSS, J.J., REID, J.B., DUNGEY, H.S. (1992) Ontogenetic variation in levels of gibberellin A_1 in *Pisum*. Implications for the control of stem elongation. *Planta* **186**, 166-171

SACHS, R.M., BRETZ, C.F., LANG, A. (1959) Shoot histogenesis. The early effects of gibberellin upon stem elongation in two rosette plants. *Am. J. Bot.* **46**, 376-384

SAMPSON, D.R. (1971) Additive and nonadditive genetic variances and genotypic correlations for yield and other traits in oats. *Can. J. Gen. Cyt.* **13**, 864-872

SANCHEZ, O.J., PAN, A., NICOLAS, G., LABRADOR, E. (1989) Relation of cell wall peroxidase activity with growth in epicotyls of *Cicer arietinum*. Effect of calmodulin inhibitors. *Physiol. Plant.* **75**, 275-279

SCARISBRICK, D, CHATHAM, C., HILL, P. (1987) Technological change in the cereal sector. Dept. Agric. Hort. and Environ. Wye College. University of London

SCHERTZ, K.F., SUMPTER, N.A., SARKISSIAN, I.V., HART, G.E (1971) Peroxidase regulation by the 3-dwarf height locus in sorghum. *J. Hered.* **62**, 235-238

SCHNYDER, H., NELSON, C.J. (1987) Growth rates and carbohydrate fluxes within the elongation zone of tall fescue leaf blades. *Plant Physiol.* **85**, 548-553

SCHNYDER, H., NELSON, C.J. (1988) Diurnal growth in tall fescue leaf blades. I. Spatial distribution of growth, deposition of water and assimilate import in the elongation zone. *Plant Physiol.* **86**, 1070-1076

SCHNYDER, H., NELSON, C.J. (1989) Growth rates and assimilate partitioning in the elongation zone of tall fescue leaf blades at high and low irradiance. *Plant Physiol.* **90**, 1201-1206

SCHNYDER, H., NELSON, C.J., COUTTS, J.H. (1987) Assessment of the spatial distribution of growth in the elongation zone of grass leaf blades. *Plant Physiol.* **85**, 290-293

SCHNYDER, H., NELSON, C.J., SPOLLEN, W.G. (1988) Diurnal growth of tall fescue leaf blades. II. Dry matter partitioning and carbohydrate metabolism in the elongation zone and adjacent expanded tissue. *Plant Physiol.* **86**, 1077-1083

SCHNYDER, H., SEO, S., RADEMACHER, I.F., KUHBAUCH, W. (1990) Spatial distribution of growth rates and of epidermal cell lengths in the elongation zone during leaf development in *Lolium perenne* L. *Planta* **181**, 423-431

SCOTT, I.M. (1990) Plant hormone response mutants. *Physiol. Plant.* **78**, 147-152

SHARMAN, B.C. (1942) Developmental anatomy of the shoot of *Zea mays* L. *Ann. Bot.* **6**, 245-282

SHEDLETZKY, E., SHMUEL, M., DELMER, D.F., LAMPORT, D.T.A. (1990) Adaptation and growth of tomato cells on the herbicide 2,6 dichlorobenzonitrile leads to the production of unique cell wall lacking cellulose xyloglucan network. *Plant Physiol.* **94**, 980-987

SHIBUYA, N. (1984) Phenolic acids and their carbohydrate esters in rice endosperm cell walls. *Phytochem.* **23**, 2233-2237

SILK, W.K. (1984) Quantitative descriptions of development. *Ann. Rev. Plant Physiol.* **35**, 479-518

SILK, W.K., ERICKSON, R.O. (1979) Kinematics of plant growth. *J. Theoret. Biol.* **76**, 481-501

SILK, W.K., LORD, E.M., ECKARD, K.J. (1989) Growth patterns inferred from anatomical records. *Plant Physiol.* **90**, 708-713

SIMMONDS, N.W. (1979) Disease resistance. *In*. Principles of crop improvement. pp 242-278 Longman, London

SIMPSON, G.M. (1958) A colourimetric test for gibberellic acid and evidence from a dwarf-pea assay for the occurrence of a gibberellin-like substance in wheat seedlings. *Nature* **182**, 528-529

SINGH, S.P., PALEG, L.P (1984a) The low temperature induction of hormonal sensitivity in genotypically gibberellic acid-insensitive aleurone tissue. *Plant Physiol.* **74**, 437-438

SINGH, S.P., PALEG, L.P. (1984b) Low temperature induced GA_3 sensitivity of wheat. 1. Characterization of the low temperature effect on isolated aleurone of Kite. *Plant Physiol.* **76**, 139-142

SINGH, S.P., PALEG, L.P. (1984c) Low temperature-induced GA_3 sensitivity of wheat. 2. Changes in lipids associated with low temperature-induced GA_3 sensitivity. *Plant Physiol.* **76**, 143-147

SINGH, S.P., PALEG, L.G. (1986a) A possible role for indolacetic acid, low temperature, and phospholipid metabolism in the induction of GA_3 responsiveness in GA_3 insensitive (*Rht3*-containing) dwarf wheat aleurone. *Plant Physiol.* **82**, 688-694

SINGH, S.P., PALEG, L.G. (1986b) Low-temperature induced GA_3 sensitivity of wheat. VI. Effect of inhibitors of lipid biosynthesis on α-amylase production by dwarf (*Rht3*) and tall (*rht*) wheat, and on lipid metabolism of tall wheat aleurone tissue. *Aust. J. Plant Physiol.* **13**, 409-416

SINGH, S.P., PALEG, L.G. (1986c) Comparison of IAA-induced and low temperature induced GA_3 responsiveness and α-amylase production by GA_3 insensitive dwarf wheat aleurone. *Plant Physiol.* **82**, 685-687

SINGHAL, N.C., MEHTA, S.L., SINGH, M.P. (1979) Peroxidase activities in relation to plant height and grain weight in bread wheat. *Theoret. App. Genet.* **55**, 89-92

SMITH, M.M., O'BRIEN, T.P. (1979) Distribution of autofluorescence and esterase and peroxidase activities in the epidermis of wheat roots. *Aust. J. Plant Physiol.* **6**, 201-219

SMITH, M.M., HARTLEY, R.D. (1983) Occurrence of ferulic acid substitution in cell wall polymers in graminaceous plants. *Cabohydr. Res.* **118**, 65-80

SNAPE, J.W. (1987) Conventional methods of genetic analysis in wheat. In. Wheat breeding - its scientific basis. (ed. F.G.H. Lupton). pp 107-128 Chapman & Hall, London

SONI, R.L., KAUFMAN, P.B., BIGELOW, W.C. (1972) Regulation of silica deposition in *Avena* internodal epidermis by gibberellic acid and sucrose. *J. Exp. Bot.* **23**, 787-791

SPRAY, C., PHINNEY, B.O., GASKIN, P., GIMOUR, S.J., MACMILLAN, J. (1984) The *dwarf-1* mutation controls the 3β-hydroxylation to gibberellin A_{20} to gibberellin A_1. *Planta* **160**, 464-468

STEUDLE, E., ZIMMERMAN, U. (1974) Determination of the hydraulic conductivity and the reflection coefficients in *Nitella flexillis* by means of direct cell-turgor pressure measurements. *Biochem. Biophys. Acta.* **332**, *399-412*

STEVENSON, T.T., CLELAND, R.E. (1981) Osmoregulation in the *Avena* coleoptile in relation to auxin and growth. *Plant Physiol.* **67**, 749-753

STEVENSON, T.T., CLELAND, R.E. (1982) Osmoregulation in the *Avena* coleoptile. Control of solute uptake in peeled sections. *Plant Physiol.* **69**, 292-295

STITCHER, L., PENEL, C., GREPPIN, H. (1981) Calcium requirement for the secretion of peroxidases by plant cell suspensions. *J. Cell Sci.* **48**, 345-353

STODDART, J.L. (1984) Growth and gibberellin-A_1 metabolism in normal and gibberellin-insensitive (*Rht3*) wheat (*Triticum aestivum* L.) seedlings. *Planta* **161**, 432-438

STODDART, J.L. (1986) Gibberellin receptors. In. Hormones, receptors and cellular interactions in plants. (eds. C.M. Chadwick & D.R. Garrod). pp 91-114 Cambridge University Press, Cambridge

STODDART, J.L. (1987) Genetic and hormonal regulation of stature. In. Developmental mutants in higher plants. (eds. H. Thomas & D. Grierson). S.E.B. seminar series 32. pp 155-180 Cambridge University Press, Cambridge

STODDART, J.L. (1988) Gibberellin-insensitive and overgrowth mutants in temperate cereals. In. Plant Growth Substances. (eds. R.P. Pharis & S. B. Rood). pp 84-92 Spinger-Verlag, Berlin

STODDART, J.L, LLOYD, E.J. (1986) Modification by gibberellin of the temperature-growth relationship in mutant and normal genotypes of several cereals. *Planta* **167**, 364-368

STODDART, J.L., THOMAS, H., LLOYD, E.J., POLLOCK, C.J. (1986) The use of temperature profiled position transducer for the study of temperature growth in *Gramineae*. *Planta* **167**, 359-363

STUART, D.A., JONES, R.L. (1977) Role of the extensibility and turgor in dark stimulated growth. *Plant Physiol.* **59**, 61-68

SUGAVANAM, B. (1984) Diastereoisomers and enantiomers of paclobutrazol; their preparation and biological activity. *Pestic Sci.* **15**, 296-302

TAN, K.S., HOSON. T., MASUDA, Y., KAMISAKA, S. (1991) Correlation between cell wall extensibility and the content of diferulic and ferulic acid in cell walls of *Oryza sativa* coleoptiles grown under water and in air. *Physiol. Plant.* **83**, 397-403

TANIMOTO, E. (1987) Gibberellin-dependent root elongation in *Lactuca sativa*: Recovery from growth suppressed elongation with thickening by low concentration of GA_3. *Plant Cell Physiol.* **28**, 963-973

TANIMOTO, E., MASUDA, Y. (1971) Role of the epidermis in auxin-induced elongation of light-grown pea stem segments. *Plant Cell Physiol.* **12**, 663-673

TAYLOR, A., COSGROVE, D.J. (1989) Gibberellin stimulation of cucumber hypocotyl growth. *Plant Physiol.* **90**, 1335-1340

TAYLOR, G., DAVIES, W.J. (1986) Yield turgor of growing leaves of *Betula* and *Acer*. *New Phytol.* **104**, 347-353

TERRY, N., WALDRON, L.J., TAYLOR, S.E. (1983) Environmental influences on leaf expansion. In. The growth and functioning of leaves. (eds. Dale. J.E. & Milthorpe, F.L). pp 179-206 Cambridge University Press, Cambridge

TIAZ. L. (1984) Plant cell expansion; regulation of cell wall mechanical properties. *Ann. Rev. Plant Physiol.* 35, 585-567

THEOLOGIS, A. (1986) Rapid gene regulation by auxin. *Ann. Rev. Plant Physiol.* **37**, 407-438

THIAGARAJAH, M.R., HUNT, L.A. (1982) Effect of temperature on leaf growth in corn (*Zea mays*) *Can. J. Bot.* **60**, 1647-1652

THIMANN, K.V., SCHNEIDER, C.L. (1938) Differential growth in plant tissues. *Am. J. Bot.* **25**, 627-641

THOMAS, H. (1983a) Analysis of the nitrogen response of leaf extension in *Lolium temulentum* seedlings. *Ann. Bot.* **51**, 363-371

THOMAS, H. (1983b) Analysis of the response of leaf extension to chilling temperatures in *Lolium temulentum* seedlings. *Physiol. Plant.* **57**, 509-513

THOMAS, H., STODDART, J.L. (1984) Kinetics of leaf growth in *Lolium temulentum* at optimal and chilling temperatures. *Ann. Bot.* **53**, 341-347

THOMAS, J., ROSS, C.W., CHASTAIN, C.J., KOOMANOFF, N., HENDRIX, J.E., VAN VOLKENBURGH, E. (1981) Cytokinin-induced wall extensibility in excised cotyledons of radish and cucumber. *Plant Physiol.* **68**, 107-110

THOMAS, A., TOMOS, A.D., STODDART, J.L., THOMAS, H., POLLOCK, C.J. (1989) Cell expansion rate, temperature and turgor pressure in growing leaves of *Lolium temulentum* L. *New Phytol.* **112**, 1-15

THORNE, G.N., WELBANK, P.J., BLACKWOOD, G.C. (1969) Growth and yield of six short varieties of spring wheat derived from Norin 10 and two European varieties. *Ann. App. Biol.* **63**, 241-251

TOMOS, A.D. (1985) The physical limitations of leaf cell expansion. In. The control of leaf growth. (eds. N.R. Baker, W.J. Davies, C.K. Ong). pp 1-33 S.E.B. seminar series 27. Cambridge University Press, Cambridge

TOMOS, A.D., WYN JONES, R.G. (1988) Some transport properties of cells within tissues. *In*. Solute transport in plant cells and tissues. (eds. D.A.Baker & J.L. Hall). pp 220-250 Longman, London

TOMOS, A.D., LEIGH, R.A., SHAW, C.A., WYN JONES, R.G. (1984) A comparison of methods for measuring turgor pressures of cells of red beet storage tissue. *J. Exp. Bot.* **35**, 1675-1683

TOMOS, A.D., MALONE, M., PRITCHARD, J. (1989) The biophysics of differential growth. *Environ. Exp. Bot.* **29**, 7-23

TOTTMAN, R.D. (1977) The identification of growth stages in winter wheat with reference to the application of growth-regulator herbicides. *Ann. App. Biol.* **87**, 213-244

TOTTMAN, R.D. (1987) The decimal code for the growth stages of cereals with illustrations. *Ann. Appl. Biol.* **110**, 441-454

TREWAVAS, A.J. (1981) How do plant growth substances work ? *Plant Cell Environ.* **4**, 204-228

TREWAVAS, A.J. (1982) Growth substance sensitivity: the limiting factor in plant development. *Physiol. Plant.* **55**, 60-72

TREWAVAS, A.J. (1983) Is plant development regulated by changes in the concentration of plant growth substances or by changes in the sensitivity to growth substances ? *Trends Biochem. Sci.* **8**, 354-357

TREWAVAS, A.J. (1986) Resource allocation under poor growth conditions, a major role for plant growth substances in developmental plasticity. *In*. Plasticity in plants. (eds. D.H. Jennings & A.J. Trewavas). pp 31-76 Symposia of the Society for Experimental Biology XXXX. The Company of Biologists, Cambridge

TSAIO, C.Y., NELSON, O.E. (1966) Starch deficient maize mutants lacking adenosine diphosphate glucose pyrophosphorylase activity. *Science* **151**, 341-343

TURNER, N.C. (1981) Techniques and experimental approaches for the measurement of plant water relations. *Plant Soil* **58**, 339-366

TYREE, M.T., JARVIS, P.G. (1982) Water in tissues and cells. *In*. Physiological plant ecology - water relations and carbon assimilation. (eds. O.L. Lange, P.S. Nobel, C.B. Osmond, H. Ziegler). pp 33-77 Encyclopedia of plant physiology. New series. Volume 12B. Springer-Verlag, Berlin. Heidelberg. New York

VAN DEN ENDE, J., KORNEEF, P. (1960) Gibberellic acid and osmotic pressure. *Nature* **186**, 327

VAN VOLKENBURGH, E., CLELAND, R.E. (1980) Proton excretion and cell expansion in bean leaves. *Planta* **148**, 273-278

VAN VOLKENBURGH, E., CLELAND, R.E. (1981) Control of light-induced bean leaf expansion: role of osmotic potential, wall yield stress and hydraulic conductivity. *Planta* **153**, 572-577

VAN VOLKENBURGH, E., DAVIES, W.J. (1983) Inhibition of light stimulated leaf expansion by ABA. *J. Exp. Bot.* **39**, 411-420

VAN VOLKENBURGH, E., CLELAND, R.E., SCMIDT, M.G. (1985) The mechanism of light-stimulated leaf cell expansion. In. Control of leaf growth. (eds. N.R. Baker., W.J. Davies., C.K. Ong). pp 233-237 S.E.B. seminar series 27. Cambridge University Press, Cambridge

VAN VOLKENBURGH, E., HUNT, S., DAVIES, W.J. (1983) A simple instrument for measuring cell wall extensibility. *Ann. Bot.* **51**, 669-672

VERBELLEN, J.P., DE GREEF, J. A. (1979) Leaf development in *Phaseolus vulgaris* L. in light and darkness. *Am. J. Bot.* **66**, 970-976

VOLNEC, J.J., NELSON, C.J. (1981) Cell dynamics in leaf meristems of contrasting tall fescue genotypes. *Crop Sci.* **21**, 381-385

WANG, Y., XUE, X., GUOSHUN, T., QIUYING, W. (1982) Monsomic analysis of plant height in 'Ai-bian No 1'. *Acta agronomica sinica* **8**, 193-198

WATTS, W.R. (1974) Leaf extension in *Zea mays* III. Field measurements of leaf extension in response to temperature and leaf water potential. *J. Exp. Bot.* **25**, 1085-1096

WAYCOTT, W., TAIZ, L. (1991) Phenotypic characterisation of lettuce dwarf mutants and their response to applied gibberellin. *Plant Physiol.* **95**, 1162-1168

WEBB, S. (1987) Growth and gibberellins in Maris Huntsman wheat containing different dwarfing genes. Industrial placement report. Long Ashton Research Station

WEBSTER, P.L. (1980) Analysis of heterogeneity of relative division rates in root apical meristems. *Bot. Gaz.* **141**, 353-359

WENKERT, W. (1980) Measurement of tissue osmotic pressure. *Plant Physiol.* **65**, 614-617

WESTGATE, M.E., BOYER, J.S. (1985) Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. *Planta* **164**, 540-549

WHEELER, A.W. (1973) Longitudinal distribution of growth substances in leaves of wheat (*Triticum aestivum* L.) *Planta* **112**, 129-135

WHITE, P.J., COOPER, H.D., EARNSHAW, M.J., CLARKSON, D.T. (1990) Effect of low temperature on the development and morphology of rye (*Secale cereale*) and wheat (*Triticum aestivum*). *Ann. Bot.* **66**, 559-566

WHITMORE, F.W. (1974) Phenolic acids in wheat coleoptile cell walls. *Plant Physiol.* **53**, 728-731

WHITMORE, F.W. (1976) Binding of ferulic acid to the cell wall by peroxidases of *Pinus ellioti*. *Phytochem.* **15**, 375-378

WILHELM, W.W., NELSON, C.J. (1978) Leaf growth, leaf ageing and photosynthetic rate in tall fescue genotypes. *Crop Sci.* **18**, 769-772

WILSON, L.G., FRY, S.C. (1986) Extensin - a major cell wall glycoprotein. *Plant Cell Environ.* 9, 239-260

WOOD, A., PALEG, L.G. (1972) The influence of gibberellic acid on the permeability of model membrane systems. *Plant Physiol.* **50**, 103-108

WOOD, A., PALEG, L.G. (1974) Alteration of liposomal membrane fluidity by gibberellic acid. *Aust. J. Plant Physiol.* **1**, 31-40

WOODWARD, F.I., FRIEND, A.D. (1988) Controlled environment studies on the temperature response of leaf extension in species of *Poa* with diverse altitude ranges. *J. Exp. Bot.* **39**, 411-420

WORLAND, A.J. (1986) Gibberellin sensitive dwarfing genes in southern European wheats. *Euphytica* **35**, 857-866

YAMAMOTA, R., SHINOZAKI, K., MASUDA, Y. (1970) Stress relaxation properties of plant cell walls with special reference to auxin action. *Plant Cell Physiol.* **11**, 947-956

YEN, S.T., CARTER, O.G. (1972) The effect of seed pretreatment with gibberellic acid on germination and early establishment of grain sorghum. *Aust. Agric. Anim. Husb.* **12**, 653-661

YEOMAN, M.M., BROWN, R. (1971) Effects of mechanical stress on the plane of cell division in developing callus cultures. *Ann. Bot.* **35**, 1101-1112

YORK, W.S., DARVILL, A.G., ALBERSHEIM, P. (1984) Inhibition of 2,4-dichlorophenoxyacetic acid stimulated elongation in pea stem segments by a xyloglucan oligosaccharide. *Plant Physiol.* **75**, 295-297

YOUSSEFIAN, S. (1986) The development and pleiotropic effects of GA-insensitive dwarfing genes in wheat. *PhD* thesis, University of Cambridge

YOUSSEFIAN, S., KIRBY, E.J.M., GALE, M.D. (1992a) Pleiotropic effects of the GA-insensitive *Rht* dwarfing genes in wheat. 1. Effects on development of the ear, stem and leaves. *Field Crops Res.* **28**, 179-190

YOUSSEFIAN, S., KIRBY, E.J.M., GALE, M.D. (1992b) Pleiotropic effects of the GA-insensitive *Rht* dwarfing genes in wheat. 2. Effects on leaf, stem, ear and floret growth. *Field Crops Res.* **28**, 191-210

ZACCAI, M., PINTHUS, M.J., LEVY, A.A. (1987) The effect of the *Rht1* gene on grain protein content in tetraploid wheat *Triticum turgidum*. *J. Cer. Sci.* **6**, 27-32

ZACK, C.D., LOY, J.B. (1984) Comparative effects of gibberellin and benzyladenine on dry matter partitioning and osmotic and water potentials in seedling organs of dwarf watermelons. *J. Plant Growth Reg.* **3**, 65-73

ZOBEL, A. (1985) The internode of *Sambucus racemosa* L. originates from a single cell layer. *Ann. Bot.* **56**, 105-107

APPENDIX

Table A.1 *Analysis of variance showing effect of genotype and internode number on the length and width of developing internodes. (DF = degrees of freedom, SS = sum of squares, MS = sum of squares, F = F value, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. Graph gives letter reference to the graph shown in Fig. 3. 5, Chapter 3 to which the statistical analysis relates.*

Graph	Source	DF	SS	MS	F	Sig
Internode length						
(4a)	Genotype	1	112	112	0.11	n.s.
	Internode n°	3	11677	3892	3.93	n.s.
	Error	3	2965	988		
	Total	7	14755			
(6a)	Genotype	1	67650	67650	8.02	*
	Internode n°	5	1151080	230216	27.30	**
	Error	5	412162	8432		
	Total	11	1260892			
(9a)	Genotype	1	722	722	0.07	n.s.
	Internode n°	8	1152556	144069	15.69	**
	Error	8	73420	9177		
	Total	17	1226698			
Internode width						
(4b)	Genotype	1	450	450	5.55	n.s.
	Internode n°	3	166594	55531	68.56	**
	Error	3	243	81		
	Total	7	167287			
(6b)	Genotype	1	9861	9861	1.89	n.s.
	Internode n°	5	492255	98451	18.88	**
	Error	5	26060	5212		
	Total	11	528175			
(9b)	Genotype	1	264	264	0.20	n.s.
	Internode n°	8	5429434	678679	519.66	***
	Error	8	10450	1306		
	Total	17	5440149			

Table A.2 *Analysis of variance showing effect of genotype and time from sowing on internode longitudinal sectional area. (Int = internode number, DF = degrees of freedom, SS = sum of squares, MS = sum of squares, F = F value, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. Numbers refer to internode numbers shown in Fig. 3.6, Chapter 3 to which the statistical analysis relates.*

Int	Source	DF	SS	MS	F	Sig
1	Genotype	1	0.015	0.015	2.63	n.s.
	Time	6	0.421	0.070	12.28	*
	Error	6	0.034	0.0057		
	Total	13	0.047			
2	Genotype	1	0.00009	0.00009	0.023	n.s.
	Time	6	0.409	0.0683	17.69	**
	Error	6	0.0231	0.00386		
	Total	13	0.433			
3	Genotype	1	0.0012	0.0012	0.387	n.s.
	Time	6	0.358	0.0597	19.25	**
	Error	6	0.018	0.0031		
	Total	13	0.378			
4	Genotype	1	0.00483	0.0048	0.857	n.s.
	Time	6	0.0587	0.0097	1.732	n.s.
	Error	6	0.0336	0.0056		
	Total	13	0.0971			
5	Genotype	1	0.000483	0.00048	1.20	n.s.
	Time	6	0.0115	0.00192	4.80	n.s.
	Error	6	0.00240	0.0004		
	Total	13	0.0144			
6	Genotype	1	1.00×10^{-7}	1.00×10^{-7}	8.4×10^{-3}	n.s.
	Time	4	2.84×10^{-4}	7.10×10^{-5}	5.96	n.s.
	Error	4	4.77×10^{-5}	1.19×10^{-5}		
	Total	9	3.31×10^{-4}			
7	Genotype	1	9.66×10^{-7}	9.66×10^{-7}	0.46	n.s.
	Time	3	2.59×10^{-5}	8.63×10^{-6}	4.12	n.s.
	Error	3	6.27×10^{-6}	2.09×10^{-6}		
	Total	7	3.31×10^{-5}			
8	Genotype	1	8.16×10^{-8}	8.16×10^{-8}	0.069	n.s.
	Time	2	1.82×10^{-6}	9.11×10^{-7}	0.772	n.s.
	Error	2	2.36×10^{-6}	1.18×10^{-6}		
	Total	5	5.26×10^{-6}			
9	Genotype	1	1.43×10^{-8}	1.43×10^{-8}	1.44	n.s.
	Time	1	1.44×10^{-7}	1.44×10^{-7}	14.5	n.s.
	Error	1	9.90×10^{-9}	9.90×10^{-9}		
	Total	3	1.68×10^{-7}			

Table A.3 *Analysis of variance showing effect of genotype and internode number on cell number. (DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F value, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. Graph and letter correspond to graph in Fig. 3.7, Chapter 3 to which statistical analysis relates.*

Graph	Source	DF	SS	MS	F	Sig
Internode length						
(4a)	Genotype	1	2.53	2.53	2.94	n.s.
	Internode nº	3	31.34	10.44	12.14	*
	Error	3	2.59	0.86		
	Total	7	34.47			
(6a)	Genotype	1	31.69	31.69	8.56	*
	Internode nº	5	417.00	83.40	22.54	**
	Error	5	18.48	3.70		
	Total	11	467.16			
(9a)	Genotype	1	0.68	0.68	0.26	n.s.
	Internode nº	8	3016.8	377.10	147.30	***
	Error	8	20.44	2.56		
	Total	17	3037.90			
Internode width						
(4b)	Genotype	1	0.02	0.02	0.01	n.s.
	Internode nº	8	547.89	182.63	133.30	***
	Error	8	4.11	1.37		
	Total	17	552.01			
(6b)	Genotype	1	18.00	18.00	1.59	n.s.
	Internode nº	5	1927.00	385.55	34.11	**
	Error	5	56.52	11.30		
	Total	11	2002.20			
(9b)	Genotype	1	33.3	33.33	1.96	n.s.
	Internode nº	8	6919.40	864.91	50.87	***
	Error	8	135.83	17.00		
	Total	17	7088.60			

Table A.4 *Analysis of variance showing effects of genotype and internode number on cell length (a) and cell width (b). (DF = degrees of freedom, SS = sum of squares, MS = mean square, F = F value for statistical tables, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. Graph and letter give reference to graph shown opposite in Fig. 3.8, Chapter 3 to which statistical analysis relates.*

Graph	Source	DF	SS	MS	F	Sig
Internode call length						
(4a)	Genotype	1	0.84	0.84	5.25	n.s.
	Internode n°	3	8.65	2.88	18.00	*
	Error	3	0.49	0.16		
	Total	7	9.99			
(6a)	Genotype	1	38.88	38.88	5.36	n.s.
	Internode n°	5	165.03	33.01	4.55	n.s.
	Error	5	36.27	7.25		
	Total	11	240.18			
(9a)	Genotype	1	52.70	52.70	5.74	*
	Internode n°	8	85.94	10.73	1.17	n.s.
	Error	8	73.4	9.17		
	Total	17	211.95			
Internode cell width						
(4b)	Genotype	1	3.64	3.64	4.91	n.s.
	Internode n°	3	43.29	14.43	19.50	*
	Error	3	2.02	0.74		
	Total	7	49.14			
(6b)	Genotype	1	34.00	34.00	13.44	*
	Internode n°	5	66.7	13.33	5.27	n.s.
	Error	5	12.67	2.53		
	Total	11	113.33			
(9b)	Genotype	1	32.00	32.00	18.82	**
	Internode n°	8	443.71	55.46	32.63	***
	Error	8	13.63	1.70		
	Total	17	489.34			

Table A.5 *Analysis of variance showing effect of genotype and internode age (time from sowing) on internode length. (Int = internode number, DF = degrees of freedom, SS = sum of squares, MS = sum of squares, F = F value, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. INT = to internode on graph shown in Fig. 3.9, Chapter 3 to which the statistical analysis relates.*

Int	Source	DF	SS	MS	F	Sig
1	Genotype	1	0.400	0.400	9.75	*
	Time	8	14.110	1.764	43.02	***
	Error	8	0.330	0.041		
	Total	17	14.842			
2	Genotype	1	0.1922	0.192	6.19	*
	Time	8	13.988	1.748	56.38	***
	Error	8	0.247	0.031		
	Total	17	14.428			
3	Genotype	1	0.298	0.298	11.87	**
	Time	8	16.656	2.082	82.49	***
	Error	8	0.200	0.0251		
	Total	17	17.155			
4	Genotype	1	0.326	0.362	5.32	*
	Time	8	15.752	1.969	28.95	***
	Error	8	0.546	0.068		
	Total	17	16.624			
5	Genotype	1	0.306	0.306	3.43	n.s.
	Time	8	8.836	1.105	12.41	**
	Error	8	0.713	0.089		
	Total	17	9.856			
6	Genotype	1	0.110	0.110	2.34	n.s.
	Time	8	3.254	0.406	8.63	*
	Error	8	0.378	0.047		
	Total	17	3.743			
7	Genotype	1	0.086	0.086	6.14	*
	Time	8	1.220	0.153	10.92	*
	Error	8	0.115	0.014		
	Total	17	1.423			
8	Genotype	1	0.00369	0.00369	3.76	n.s.
	Time	8	0.383	0.00478	4.87	n.s.
	Error	8	0.00784	0.000981		
	Total	17	0.492			
9	Genotype	1	2.14×10^{-4}	2.14×10^{-4}	1.76	n.s.
	Time	8	9.02×10^{-2}	1.12×10^{-2}	92.56	***
	Error	8	9.68×10^{-4}	1.21×10^{-4}		
	Total	17	9.14×10^{-2}			

Table A.6 *Analysis of variance showing effect of genotype and internode age (time from sowing) on internode length. (Int = internode number, DF = degrees of freedom, SS = sum of squares, MS = sum of squares, F = F value, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. INT = to internode on graph Fig. 3.15 to which the statistical analysis relates.*
Internode length data taken 1-15 d after sowing. Summary results presented in Table 3.10, Chapter 3.

Int	Source	DF	SS	MS	F	Sig
1	Genotype	1	0.00058	0.00058	2.761	n.s.
	Time	6	1.41078	0.235	1119.040	***
	Error	6	0.00127	0.00021		
	Total	13	1.41263			
2	Genotype	1	0.00030	0.00030	0.372	n.s.
	Time	6	0.9361	0.15602	192.88	***
	Error	6	0.0048	0.00081		
	Total	13	0.9413			
3	Genotype	1	0.00048	0.00048	1.48	n.s.
	Time	6	0.4980	0.08300	256.17	***
	Error	6	0.00194	0.000324		
	Total	13	0.5005			
4	Genotype	1	0.000098	0.000098	0.36	n.s.
	Time	6	0.32694	0.05440	201.48	***
	Error	6	0.001643	0.00027		
	Total	13	0.32819			
5	Genotype	1	0.00075	0.000758	0.205	n.s.
	Time	6	0.12424	0.020729	5.61	*
	Error	6	0.02217	0.00369		
	Total	13	0.12735			
6	Genotype	1	0.00017	0.000172	1.35	n.s.
	Time	6	0.06321	0.01053	82.91	***
	Error	6	0.00075	0.000127		
	Total	13	0.06414			
7	Genotype	1	0.000912	0.000912	5.15	n.s.
	Time	6	0.03297	0.00549	30.01	***
	Error	6	0.00106	0.000177		
	Total	13	0.03494			
8	Genotype	1	0.000087	0.000087	2.50	n.s.
	Time	6	0.01735	0.00289	83.04	***
	Error	6	0.000209	0.0000348		
	Total	13	0.017646			
9	Genotype	1	0.0000643	0.000064	0.99	n.s.
	Time	6	0.003471	0.000578	8.98	**
	Error	6	0.000385	0.0000643		
	Total	13	0.00392			

Table A.7 *Analysis of variance showing effect of genotype and internode age (time from sowing) on internode length. (Int = internode number, DF = degrees of freedom, SS = sum of squares, MS = sum of squares, F = F value, Sig = significance, n.s. = not significant, */**/*** = P < 0.05/0.01/0.001 respectively. INT = to internode on graph shown in Fig. 3.15 of Chapter 3, section 3.3 to which the statistical analysis relates. Internode data taken 16-63 d after sowing. Summary results presented in Table 3.10.*

Int	Source	DF	SS	MS	F	Sig
1	Genotype	1	0.9516	0.9516	29.73	**
	Time	6	0.9435	0.1572	4.91	*
	Error	6	0.1919	0.0320		
	Total	13	2.0870			
2	Genotype	1	0.4791	0.4971	8.25	*
	Time	6	1.9533	0.3265	5.42	*
	Error	6	0.3610	0.0602		
	Total	13	2.7992			
3	Genotype	1	0.7269	0.7269	34.94	**
	Time	6	4.5026	0.7504	36.07	***
	Error	6	0.1251	0.0208		
	Total	13	5.3545			
4	Genotype	1	0.6776	0.6776	9.92	*
	Time	6	4.9198	0.8200	12.00	**
	Error	6	0.4096	0.0683		
	Total	13	6.0070			
5	Genotype	1	0.811	0.811	7.05	*
	Time	6	4.272	0.712	6.19	*
	Error	6	0.693	0.115		
	Total	13	5.776			
6	Genotype	1	0.3333	0.3333	4.64	n.s.
	Time	6	1.9163	0.3194	4.45	*
	Error	6	0.4299	0.0717		
	Total	13	2.6795			
7	Genotype	1	0.1170	0.1170	7.26	*
	Time	6	0.5283	0.0880	5.46	*
	Error	6	0.0969	0.0161		
	Total	13	0.7422			
8	Genotype	1	0.01272	0.01272	8.83	*
	Time	6	0.09292	0.01549	10.75	**
	Error	6	0.00866	0.00144		
	Total	13	0.11430			
9	Genotype	1	0.000424	0.000424	3.26	n.s.
	Time	6	0.024141	0.00403	31.00	***
	Error	6	0.000781	0.000130		
	Total	13	0.025345			

Table A.8 *Equations of linear regression lines fitted to L2 extension data presented in sections 4.3.1 and 4.3.3 of Chapter 4. Data of Fig. 4.2 & 4.12*

Genotype Temp (°C)	L2 description	Regression equation	Correlation coefficient
rht3 20	L2 length	y = 31.9x - 173.7	0.995
Rht3	"	y = 18.8x - 95.4	0.995
rht3 20	L2 surface area	y = 104.0x - 744.9	0.999
Rht3	"	y = 68.5x - 494.8	0.998
rht3 20	L2 dry weight	y = 3.38x - 24.8	0.992
Rht3	"	y = 2.27x - 16.3	0.989
rht3 10	L2 length	y = 15.49x - 193.7	0.998
Rht3	"	y = 11.99x - 140.0	0.998
rht3 10	L2 surface area	y = 44.5x - 680.5	0.998
Rht3	"	y = 38.1x - 599.1	0.995
rht3 10	L2 dry weight	y = 1.99x - 30.5	0.995
Rht3	"	y = 1.39x - 20.0	0.994