

Genotype By Environment Interaction in Shoot Branching

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Abstract

Plant development is highly plastic, allowing plants to adapt to constant changes in environmental conditions. An excellent example of developmental plasticity is shoot branching. The final architecture of the shoot system is determined by the integration of environmental cues such as light and nutrients with endogenous cues. In this thesis the effect of Nitrogen (N) availability on Arabidopsis shoot branching was used as a model to investigate plant developmental plasticity. In particular, natural variation in shoot branching response to N supply was investigated using a set of multi parent advanced generation inter cross (MAGIC) lines (Kover et al., 2009).

Correlations between traits in a selected group of MAGIC lines revealed several interesting correlations, characterising two strategies for N response. One strategy involved flowering early, maintaining branch numbers of low N, and minimal shift in resource allocation to roots. This was associated with good seed yield and yield retention on low N. An alternative strategy involves late flowering, high branching on high N but low branching on low N, (i.e. high branching plasticity), and a substantial increase in root fraction on Low N. This was associated with high seed yields on high N, but poor yield retention on low N.

The molecular basis for these different strategies are currently unknown, but it seems likely that plant hormones are involved. Analysis of bud activation on isolated nodal stem segments provided strong evidence that the regulation of branching by N availability requires strigolactone (SL), and that strigolactone acts by increasing the competition between buds. There was some evidence of strigolactone resistance in a low plasticity MAGIC line.

Shoot system architecture is a key factor underlying crop yield, and yield stability under low N input is an agricultural priority. Therefore, in parallel the branching responses of a set of *Brassica rapa* lines to N limitation were determined. Results highlight many conserved features between Arabidopsis and Brassica, as well as some differences. These comparisons should aid breeding for shoot system architectures that can deliver improved yield under low N.

Table of Contents

Abstract	2
Table of Contents	3
List of Tables	10
List of Figures	11
Acknowledgements	16
Author's declaration.....	17
Abbreviations.....	18
Chapter 1 Introduction	21
1.1 General Introduction	22
1.2 Shoot system architecture	24
1.2.1 Branching as an agronomic trait	28
1.3 Factors affecting regulation of shoot branching.....	29
1.3.1 Hormonal regulation of shoot branching	29
1.3.2 Environmental regulation of shoot branching	38
1.3.2.1 Light quality	38
1.3.2.2 Nutrients.....	39
1.4 Role of Nitrogen	40
1.4.1 N uptake and assimilation.....	40
1.4.2 Nitrate transporters	41
1.4.3 N remobilization	41
1.4.4 Nitrogen and yield.....	42
1.4.5 N status sensing	43
1.4.6 Alterations in shoot-root ratios in response to N availability.....	43
1.4.7 Shoot-root signalling	45
1.5 Interaction between Nitrate and hormones.....	45
1.5.1 Auxin.....	45
1.5.2 Cytokinins (CK).....	47

1.5.3 Strigolactones (SL)	47
1.6 Natural allelic variation and shoot branching plasticity	49
1.6.1 Tools for exploiting natural variation in shoot branching	49
1.7 Importance of genotype by environment interactions	50
1.8 Brassica.....	51
1.9 Aims and layout of thesis.....	52
1.10 Published work	53
Chapter 2 Materials and Methods.....	54
2.1 Chemicals.....	55
2.2 Plant culture media.....	55
2.2.1 Variations in plant culture.....	55
2.3 Arabidopsis plant material	56
2.4 Plant hormones	56
2.5 General growth conditions for Arabidopsis	56
2.6 Growth conditions for studying nitrate effects on Arabidopsis	57
2.7 Arabidopsis decapitation assay	57
2.8 Nitrate response phenotypic analysis	58
2.9 Seed yield and Seed viability analysis.....	58
2.9.1 Quantification of total seed yield	58
2.9.2 Quantification of single seed weight	58
2.9.3 Quantification of seed viability	59
2.10 Nitrate analysis.....	59
2.10.1 Extraction.....	59
2.10.2 Determination of Nitrate concentration.....	59
2.11 Arabidopsis bud assays.....	60
2.12 <i>Brassica rapa</i> plant material	61
2.13 <i>Brassica rapa</i> general plant growth conditions	61
2.14 <i>B. rapa</i> plant growth conditions for nitrate studies.....	61

2.15 <i>B. rapa</i> decapitation assays	61
2.16 <i>B. rapa</i> seed sterilization	62
2.17 <i>B. rapa</i> split plate assay	62
2.18 <i>B. rapa</i> hormone response assays	62
2.19 <i>B. rapa</i> hypocotyl elongation, root length and light assays	63
2.20 <i>B. rapa</i> grafting	63
2.21 <i>B. rapa</i> TILLING	64
2.22 Statistical analysis	64
Chapter 3 MAGIC lines as a tool to study variation in shoot branching response to Nitrate	65
3.1 Terminology used in this chapter	66
3.2 Introduction	67
3.2.1 MAGIC lines	67
3.3 Background studies and aims	67
3.4 Characterisation of the basic effects of Nitrate limitation on branching in <i>Arabidopsis</i> accession ‘Columbia’	70
3.5 Characterisation of MAGIC lines – An Overview	74
3.6 Effect of N limitation on 15 MAGIC lines	78
3.7 Detailed N response studies with selected MAGIC lines	81
3.7.1 Flowering time	83
3.7.2 Branching related traits	84
3.7.2.1 Effect of N limitation on branching	84
3.7.2.2 Branching plasticity	84
3.7.2.3 Decapitation response to N limitation	87
3.7.3 Fitness related traits	89
3.7.3.1 Seed yield and yield retention on low N	89
3.7.3.2 Seed Viability	91
3.7.3.3 Single seed weight	91

3.7.3.4 Residual shoot dry matter (RSDM).....	92
3.7.3.5 Harvest Index (HI)	94
3.7.3.6 Shoot – root partitioning	95
3.7.4 Shoot and seed Nitrate concentrations.....	97
3.7.5 ANOVA	101
3.7.6 Relationship between different traits.....	106
3.7.6.1 Relationship between shoot branching traits.....	108
3.7.6.2 Relationship between branching and fitness traits	109
3.7.6.3 Relationship between flowering time and other traits	113
3.7.6.4 Relationship between fitness related traits	116
3.7.6.5 Summary of correlations between traits	117
3.8 Discussion	118
3.8.1 Major variation in branch number and its response to N correlates with flowering time.....	118
3.8.2 Plasticity in shoot branching could be seasonally regulated	120
3.8.3 Effect of seasonality on plasticity of other traits	121
3.8.4 Nitrate has no strong effect on flowering time.....	122
3.8.5 Branch loss of high plasticity lines under N limitation is partially overcome by decapitation	123
3.8.6 High N is crucial for higher yields but yield retention on low N is different for different lines	123
3.8.7 Single seed weight was unaffected by N limitation	125
3.8.8 Harvest index is affected by N supply.....	126
3.8.9 Root proportion is affected by N	127
3.8.10 Similar Nitrate levels in shoots and seeds in high plasticity lines regardless of the N supply.	127
3.8.11 Strategies adapted by different lines.....	128
3.8.12 Causal links between correlations	130
3.9 Summary.....	130

Chapter 4 Hormone response studies on high and Low N.....	132
4.1 Introduction.....	133
4.2 Background and aims.....	134
4.3 Plant growth and experimental set up	136
4.4 Assays to characterise bud SL responses.....	137
4.4.1 Single node assays of Columbia, <i>max2</i> and <i>max4</i>	137
4.4.2 Two node assays of Columbia, <i>max2</i> and <i>max4</i>	138
4.4.3 Further analysis of Columbia bud behaviour.....	141
4.5 Hormone response assays on high and low N	143
4.5.1 Single and two node assays of Columbia on high and low N.....	144
4.5.2 Comparison of Columbia, <i>max2</i> and <i>max4</i> two bud assays on high and low N.....	146
4.5.3 Single node assays of Columbia with shift in N status	150
4.5.4 Two node assays of Columbia with shift in N supply	152
4.5.5 One node assays with increased stem length.....	155
4.6 Hormonal assays with MAGIC lines	158
4.6.1 Single node assays on high and low N	158
4.6.2 Two node assays on high and low N	160
4.6.3 Two node assays of MAGIC lines with shift in N supply	163
4.6.4 Three node assays of MAGIC lines	166
4.7 Discussion	168
4.7.1 Mechanism of action of GR24 in regulating bud activation and elongation	168
4.7.1.1 SL is not a straightforward bud inhibitor	169
4.7.1.2 SL tends to enhance competition between buds such that only one remains active	169
4.7.1.3 SL does not play a role in deciding which bud activates	170
4.7.1.4 SL has no role in bud elongation	171

4.7.1.5 SL delays bud activation in the two node system consistent with enhanced competition	172
4.7.2 Mechanism of action of N deprivation in inhibiting buds	172
4.7.2.1 SL synthesis is upregulated by low N	172
4.7.2.2 The behaviour of Col buds on low N and with GR24 treatment is similar	173
4.7.3 Response to shift in N supply	174
4.7.4 The MAGIC lines 552 and 471 differ in their N and GR24 responses	174
4.7.4.1 MAGIC line 552	174
4.7.4.2 MAGIC line 471	175
4.7.5 Summary	176
Chapter 5 N and hormone response studies in <i>Brassica rapa</i>	177
5.1 Introduction.....	178
5.2 Background and aims.....	178
5.3 R-o-18 morphology.....	179
5.4 N response studies.....	181
5.4.1 Characterisation of the basic effects of Nitrate limitation in <i>Brassica rapa</i> line R-o-18	181
5.4.2 N response studies in different lines of <i>Brassica rapa</i>	182
5.4.3 Decapitation response of different lines on N sufficient and N limited conditions.....	184
5.5 Preliminary characterisation of line 8.....	189
5.5.1 Morphological abnormalities in line 8.....	189
5.5.2 Preliminary experiments in an attempt to understand the cause of the phenotypes	191
5.6 Grafting attempts in <i>Brassica rapa</i>	194
5.7 Hormonal assays in R-o-18.....	195
5.7.1 Split plate assays.....	195
5.7.2.1 One bud assays in <i>Brassica rapa</i>	198

5.7.2.2 Two bud assays in <i>Brassica rapa</i>	201
5.7.2.3 Effect of SLs on leaf senescence	204
5.8 Tilling in <i>Brassica rapa</i>	204
5.9 Discussion	215
5.9.1 Architecture and basic N response of <i>Brassica rapa</i> line R-o-18 is similar to Arabidopsis.....	215
5.9.2 EMS mutagenised lines differ in their N response	216
5.9.3 Decapitation response of <i>Brassica rapa</i>	216
5.9.4 Hormonal response of <i>Brassica rapa</i>	217
5.9.5 SL and leaf senescence in <i>Brassica rapa</i>	219
5.9.6 Response of mutant line 8 to nutrients and hormones.....	219
5.9.7 SL signalling mutant in <i>Brassica rapa</i>	220
5.10 Summary	221
Chapter 6 General discussion.....	222
6.1 N affects branching in Arabidopsis and Brassica	223
6.2 The effect of N on branching is likely to be mediated by hormones	223
6.2.1 A model of SLs on high Vs low N.....	224
6.3 High branching correlates with high plasticity in the MAGIC lines	227
6.4 The mechanisms underlying MAGIC line strategies are unknown.	227
6.5 Unresolved aspects of the mechanism of SL action.....	228
6.6 Understanding alternative strategies for adaptation to N limitation may be agriculturally useful.....	230
6.7 Hormone studies in different plant species can contribute to understanding of different roles of hormones.....	230
6.8 Future prospects	232
Appendix 1 - 19 Parental lines and their geographical location	233
Appendix 2 - MAX2 genomic sequence.....	234
References	235

List of Tables

Table 3.1	Summary of experiments carried out with seasons and lines included in each experiment.	76
Table 3.2	Branching, plasticity and flowering times.	77
Table 3.3	Analysis of variance of eight traits for genotypes of <i>Arabidopsis thaliana</i> grown in different N environments.	102
Table 3.4	Analysis of variance of eight traits for genotypes of <i>Arabidopsis thaliana</i> grown on high N and low N in two seasons.	104
Table 3.5	Summary of correlations between traits on high N and low N.	107
Table 5.1	Bud outgrowth from R-o-18 plants decapitated at node 4.	185

List of Figures

Figure 1.1	An Arabidopsis plant with many branches and one with no branches.	23
Figure 1.2	Schematic representation of the shoot system comprising of main stem, branches and leaves.	26
Figure 1.3	Schematic representation of body plans.	27
Figure 1.4	Apical dominance and regulation of bud outgrowth.	28
Figure 1.5	Standing genetic variation drives morphological change in maize domestication.	29
Figure 1.6	Cloned genes in the SL pathway.	32
Figure 1.7	The strigolactone pathway.	33
Figure 1.8	Main hormones regulating shoot branching.	38
Figure 1.9	Schematic representation of fate of nitrogen.	42
Figure 1.10	Construction of a MAGIC population.	50
Figure 1.11	Yield trend of Oil seed rape in recent years.	52
Figure 3.1	Scatter plots showing relationship branch numbers and flowering time among the 400 MAGIC lines.	69
Figure 3.2	Scatter plots showing relationship branch numbers and flowering time (less than 30 days) among the 400 MAGIC lines.	69
Figure 3.3	Arabidopsis accession Columbia plants (5 weeks old).	71
Figure 3.4	Mean data of three most apical branches of Columbia.	73
Figure 3.5	Mean length of buds from the three most apical leaf bearing nodes of Columbia.	74
Figure 3.6	a) Mean total plant height (mm), b) Mean FT of MAGIC lines.	79
Figure 3.7	a) Mean total number of branches, b) Branching plasticity.	80
Figure 3.8	Phenotype of selected lines grown in greenhouse.	82
Figure 3.9	Mean FT of different lines grown in a) Summer, b) Autumn.	83
Figure 3.10	Mean total number of branches of different lines a) Summer b) Autumn.	86
Figure 3.11	Mean plasticity of different lines grown in a) Summer and b) Autumn.	86
Figure 3.12	Response of different lines to decapitation on high or low Nitrate.	88
Figure 3.13	Mean seed yield of different lines a) Summer, b) Autumn.	90
Figure 3.14	Yield retained on low N (%) for different lines a) Summer b) Autumn.	90
Figure 3.15	Seed viability (%) for different lines a) Summer b) Autumn.	91

Figure 3.16	Single seed weight (mg) for different lines on high (9mM) or low (1.8mM) Nitrate grown in a) Summer b) Autumn.	93
Figure 3.17	Mean RSDM (g) for different lines a) Summer b) Autumn.	93
Figure 3.18	Harvest index (%) for different lines a) Summer b) Autumn.	94
Figure 3.19	Mean shoot fresh weight of different lines at bolting.	96
Figure 3.20	Root proportion (%) for different lines from a) dry weights at the end of the experiment, b) fresh weights at bolting.	96
Figure 3.21	Nitrate concentrations of residual shoot dry matter (RSDM) for different lines grown in a) Summer b) Autumn.	98
Figure 3.22	Nitrate concentration of dry seeds for different lines grown in a) Summer b) Autumn.	98
Figure 3.23	Concentration of Nitrate of shoot dry matter for different lines.	100
Figure 3.24	Schematic representation of ANOVA with data for eight traits.	102
Figure 3.25	Schematic representation of ANOVA with data obtained from different lines in summer and autumn for of eight traits.	105
Figure 3.26	Scatter plot showing the relationship between plasticity and branch numbers on high and low N.	108
Figure 3.27	Scatter plot showing relationship between branch numbers and fitness traits.	110
Figure 3.28	Scatter plots showing relationship between a) branch numbers and harvest index, b) Plasticity and HI.	112
Figure 3.29	Scatter plots showing relationship between a) Branch numbers and FT on high and low N. b) Plasticity and FT.	114
Figure 3.30	Scatter plots showing relationship between FT and fitness traits.	115
Figure 3.31	Scatter plot showing relationship between RSDM and seed yield on high and low N.	116
Figure 3.32	Scatter plot showing relationship between RSDM and harvest index on high and low N.	117
Figure 3.33	Schematic representation of significant trait correlations on high and low N.	118
Figure 4.1	Schematic representation of two node assay set up.	136
Figure 4.2	Col, <i>max2</i> and <i>max4</i> plants at the stage chosen for excision of stem segments for bud assays.	137
Figure 4.3	Bud length (mm) of Col, <i>max2</i> and <i>max4</i> buds in one node assays with or without GR24 (1µM).	138
Figure 4.4	Explants where both buds grow out and explants where one bud dominates.	139
Figure 4.5	Bud lengths (mm) of a) Col, b) <i>max2</i> and c) <i>max4</i> in two node assays with or without GR24 (1µM).	139

Figure 4.6	Mean Relative growth index of a) Col, b) <i>max2</i> and c) <i>max4</i> .	140
Figure 4.7	Summary of bud lengths (mm) of Col.	142
Figure 4.8	Bud lengths of different assay set ups on day 4.	143
Figure 4.9	Excised Arabidopsis buds at the stage taken for a) one node, b) two node and c) three node assay set up.	143
Figure 4.10	Bud lengths (mm) of a) Col in one node assays.	144
Figure 4.11	Bud lengths (mm) of Col in two node assays.	145
Figure 4.12	Mean Relative growth index (RGI) of Col in two node assays.	145
Figure 4.13	Bud elongation of control and treated buds.	146
Figure 4.14	Bud lengths (mm) of Col on a) high N b) low N, <i>max2</i> on c) high N d) low N and <i>max4</i> on e) high N f) low N in two node assays.	147
Figure 4.15	Mean Bud length of a) Col on high N b) Col on low N c) <i>max2</i> on high N d) <i>max2</i> on low N , e) <i>max4</i> on high N f) <i>max4</i> on low N.	149
Figure 4.16	Mean Relative growth index of a) Col on high N b) Col on low N c) <i>max2</i> on high N d) <i>max2</i> on low N and e) <i>max4</i> on high N f) <i>max4</i> on low N.	149
Figure 4.17	Bud lengths (mm) of Col single bud on a) high N b) low N, c) high to low N d) low to high N in one node assays.	150
Figure 4.18	Mean number of days taken by Columbia buds to reach 3mm with different N treatments.	152
Figure 4.19	Bud lengths (mm) of Col on a) high N b) low N, c) high to low N d) low to high N in two node assays with or without GR24.	153
Figure 4.20	Mean relative growth index (RGI) of Col on a) high N b) low N, c) high to low N d) low to high N in two node assays.	154
Figure 4.21	Bud lengths (mm) of Col – Comparison on high N.	156
Figure 4.22	Time taken by buds to reach 3mm on high N.	156
Figure 4.23	Bud lengths (mm) of Col – Comparison on low N.	157
Figure 4.24	Time taken by buds to reach 3mm on low N.	157
Figure 4.25	Bud lengths (mm) of single buds of Col.	159
Figure 4.26	Mean number of days taken by Col, 552 and 471 buds to reach 3mm on high N and low N.	159
Figure 4.27	Bud length (mm) of Col, MAGIC line 552 and MAGIC line 471.	161
Figure 4.28	Mean Relative growth index of Col, MAGIC line 552 and MAGIC line 471 on high N.	162
Figure 4.29	Bud length (mm), of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and shift from high to low N.	164
Figure 4.30	Relative Growth Index (RGI) of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and shift from high to low N.	165

Figure 4.31	Bud length (mm) of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and on low N (d, e, f) in three node assays.	167
Figure 5.1	Relationship between different Brassica species depicted as the "Triangle of U".	178
Figure 5.2	Four week old R-o-18 seedlings.	180
Figure 5.3	Six week old R-o-18 seedlings on high and low N.	181
Figure 5.4	Mean total plant height, number of branches, number of leaf-bearing nodes, number of pods of R-o-18.	182
Figure 5.5	Mean total plant height (cm) of mature plants of different <i>Brassica rapa</i> lines.	183
Figure 5.6	Mean total number of first order branches of mature plants of different <i>Brassica rapa</i> lines.	183
Figure 5.7	R-o-18 plants showing bud outgrowth at ten days following decapitation.	185
Figure 5.8	Mean branch lengths of two most apical first order branches in intact and decapitated plants of R-o-18.	186
Figure 5.9	Phenotypes of a) line 8, b) line 10, c) wild-type R-o-18 and d) line 5 two weeks after decapitation on high N.	187
Figure 5.10	Response of different lines to decapitation on high N.	187
Figure 5.11	Phenotype of a) line 8, b) line 10, c) Wild-type R-o-18 and d) line 5 two weeks after decapitation on low N.	188
Figure 5.12	Response of different lines to decapitation on low N. Plants were grown in greenhouse in continuous long day conditions.	188
Figure 5.13	Comparison of line R-o-18 and line 8 a) Mean total plant height, b) mean total number of branches, c) branching, d) higher order branching.	190
Figure 5.14	Comparison of flowers and seeds of R-o-18 and line 8.	191
Figure 5.15	Hypocotyl lengths of line R-o-18 and line 8.	192
Figure 5.16	Primary root lengths of line R-o-18 and line 8.	193
Figure 5.17	Phenotype of dark grown seedlings of R-o-18 and line 8.	193
Figure 5.18	Phenotype of seedlings of R-o-18 and line 8 grown in red light.	193
Figure 5.19	Phenotype of grafts between different <i>B. rapa</i> lines.	195
Figure 5.20	Split plate assays of isolated nodes of R-o-18 between agar Blocks.	196
Figure 5.21	Mean bud length of single nodes of R-o-18 on high N.	197
Figure 5.22	Mean bud length of single nodes of R-o-18 on low N.	197
Figure 5.23	<i>B. rapa</i> one bud assay set up, <i>B. rapa</i> two bud assay set up.	198
Figure 5.24	Bud lengths (mm) of R-o-18 single bud.	200

Figure 5.25	Mean bud length (mm).of R-o-18 and line 8.	202
Figure 5.26	Mean Relative growth index of R-o-18 and line 8.	203
Figure 5.27	Senescence of leaves.	204
Figure 5.28	Annotated sequence of <i>max2</i> .	206
Figure 5.29	Alignment of <i>max2</i> protein sequences.	207, 208
Figure 5.30	Mutation report of TILLING.	210
Figure 5.31	Seedling phenotype of R-o-18, line 8 and <i>max2</i> .	212
Figure 5.32	Branching phenotype of R-o-18, line 8 and <i>max2</i> .	213
Figure 5.33	Phenotype of reference line R-o-18, line 8 and possible <i>max2</i> segregants of an individual <i>max2</i> bushy plant.	214
Figure 5.34	Developmental defects in selected <i>max2</i> plants	215
Figure 6.1	Schematic representation of hormone transport	226

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Author's declaration

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Abbreviations

ANOVA – analysis of variance test

AMPRIL – Arabidopsis multiparent recombinant inbred line

ARF – auxin response factor

ARR – Arabidopsis response factor

ATS – *Arabidopsis thaliana* salts

AXR# – AUXIN RESISTANT

BLAST – basic local alignment search tool

BRC# – BRANCHED gene

CCD – carotenoid cleaving dioxygenase

cHATS – constitutive high affinity transport system

CK – cytokinins

CUC# – CUP-SHAPED COTYLEDON gene

CYP – cytochrome P450 haem-thiolate protein

CZ – central zone

D# – Rice DWARF gene

DAD – Petunia DECREASED APICAL DOMINANCE gene

DNA – deoxyribonucleic acid

DPA – days post anthesis

EMS – ethyl methane sulphonate

FC# – FINE CULM gene

FT – flowering time

GWA – Genome wide association

GEI – Genotype by environment interaction

HATS – high affinity transport systems

HI – harvest index

IAA – indole-3-acetic acid

iHATS – inducible high affinity transport system

IPT# – ISOPENTENYLTRANSFERASE gene

kb – kilo base pair of nucleic acid

LATS – low affinity transport system

LAX1 – LAX PANICLE1 gene

MAGIC – multiparent advanced generation intercross

MAX – MORE AXILLARY GROWTH

Mb – million base pair of nucleic acid

MOC1 – MONOCULM1 gene

N – Nitrogen

NAA – β -naphthoxyacetic acid

NCBI – National Centre for Biotechnology Information (Bethesda, USA)

NR – Nitrate reductase

NRT# or CHL# – nitrate transporter family

OC – organising centre

ORF – open reading frame

OSR – oil seed rape

P – phosphate

PAT – polar auxin transport

PhyB – PHYTOCHROME B

PIN – PIN-FORMED protein

PZ – peripheral zone

QTL – quantitative trait locus/loci

R: FR – red: far red

RAM – root apical meristem

RGI – relative growth index

RILS – recombinant inbred lines

RMS# – *RAMOSUS* gene

RNA – ribonucleic acid

rpm – rotations per minute

RSDM – residual shoot dry matter

SAM – shoot apical meristem

SAS – shade avoidance syndrome

SL(s) – strigolactone-related hormone(s)

SUNN – *SUPER NUMERIC NODULE* gene

TA – transit amplifying cell

TB1 – *TEOSINTE BRANCHED1* gene

TCP1 – TEOSINTE BRANCHED1 /CYCLOIDEA/PROLIFERATING CELL FACTOR

TF – transcription factor

TILLING – Targeting Induced Local Lesions IN Genomes

Chapter 1

Introduction

***“It is not the strongest of the species that survives, nor the most intelligent,
but the one most responsive to change”***

- Charles Darwin

1.1 General Introduction

All living organisms including plants and animals constantly interact with their environments. Among these, plants have a remarkable capacity to modulate development in response to different environmental cues (Sonia E, 2010). Plants respond to various environmental cues in a very versatile manner and many of these plant specific responses can be considered to be based on two aspects – lack of a central nervous system and immobility. Among the myriad of plant responses to various environmental cues, changes in shoot branching patterns leading to numerous architectural forms can be easily visualised in the diverse plant forms seen in nature.

The adaptive ability of plants to cope with diverse environmental conditions encountered throughout their life time is heavily dependent on phenotypic plasticity. Phenotypic plasticity is the property of a plant by which one genotype can have different phenotypes depending on the environment in which it is growing or to which it is exposed (Pigliucci, 2001). Plants exhibit plasticity in a range of post-embryonic developmental aspects. These different aspects are under genetic control but at the same time influenced by the environment. The constraints of the sessile, rooted nature may have contributed to the need for greater plasticity exhibited by plants in response to the environment.

Networks of interacting plant hormones are major players among the various long distance signals in processing and transmitting information systemically throughout plants. In addition to managing information within plants, these regulatory systems must be able constantly to monitor the environment in which the plant is growing, convey the information throughout the plant and integrate this information locally and at the whole plant level, in order to elicit an appropriate response to a particular cue. For example, a plant experiencing an environmental cue such as drought should make a relatively quick response about how to balance root and shoot growth accordingly, and whether to invest in making new leaves and seeds quickly at the expense of senescing older leaves.

Shoot branching is a major determinant of plant architecture (McSteen and Leyser, 2005). Apart from its architectural role in 'shaping the plant' (Agusti and

Greb, 2012) shoot branching is important from the point of many different aspects of plant development (Ward and Leyser, 2004). Branching affects light harvesting and determines light interception, which affects a plant's photosynthetic performance. The total number of branches produced by a plant and their placement affects the distribution of leaves, thus influencing light acquisition for example, by affecting the degree of self-shading or shading by other plants. Shoot system architecture also affects synchronisation of flowering and seed set by plants, and it can influence seed dispersal. The number of shoot branches produced by a plant can affect shoot biomass accumulation and seed yield and consequently affects the harvest index of plants (Huang et al., 2012). Thus shoot branching is important from an agricultural point of view as well (Wang and Li, 2006).

Shoot branching is an excellent example of plant developmental plasticity. A single genotype can make a large number of branches resulting in a bushy plant or it can stay as a single primary shoot with no branches at all (figure 1.1). This flexibility contributes to the enormous diversity in the shape and form of plants found in nature varying from tall oak trees with branches stretching out in all directions to coconut trees which usually lack branches altogether.



Figure 1.1 An Arabidopsis plant with many branches and one with no branches

In this context, it is very interesting as well as important to understand how information from various environmental cues are integrated into the developmental programme of plants. A wealth of knowledge has accumulated slowly but steadily over the years about different separate aspects of plant development. Apart from areas such as environmental control of flowering transition and effect of light on early seedling development, comparatively less is known about how plant development is driven forward by interactions between genotype and environment. There are substantial gaps in this area and equally in linking the physiological and biochemical aspects at the whole plant level.

It is well established that shoot branching is influenced by genetic factors, exogenous environmental factors, as well endogenous hormonal signals. Although the study of shoot branching has gained momentum over the past decade there are still unanswered questions about shoot branching and its regulation, especially how shoot branching is influenced by various environmental cues. This study focuses on shoot branching and its regulation by Nitrogen (N) limitation as an environmental cue, in an attempt to understand the interactions between genotype and environment more effectively.

The first part of this introduction will give an overview of the shoot branching process and factors affecting shoot branching. The second part will outline role of Nitrogen, with particular focus on interaction between Nitrate and hormones. The final section will cover natural variation in shoot branching and various tools available to study this.

1.2 Shoot system architecture

Just as for any other multicellular organism, plants start life as a single cell. Development in animals follow a more rigid programme where as the flexibility in plant development is evident after embryogenesis. Unlike many animals which broadly speaking stick on to the embryonic body plan, the body plan of plants are extensively elaborated post-embryonically.

Post embryonic development in plants take place through the activity of meristems. Meristems consist of multipotent cells that can divide rapidly and they

drive the iterative growth of new modules continuing throughout the life cycle of plants. During embryogenesis, plants develop a basic apical- basal body axis with shoot apical meristem (SAM) and root apical meristem (RAM) at opposite ends. During post embryonic development the SAM gives rise to all the above ground organs including the primary shoot, and the RAM gives rise to below ground root system. The final form of a plant is dependent on the activity of meristems (active or inactive). Secondary shoot apical meristems are formed from a group of cells in the leaf axils by the action of a number of transcription factors (Schmitz and Theres, 2005). SAM is organized into different regions including a central zone (CZ) consisting of stem cells, a peripheral zone (PZ) surrounding the CZ containing more rapidly dividing transit amplifying (TA) cells and an organizing centre (OC) which acts as a stem cell niche (Skylar and Wu, 2011). Several genes which are regulators of axillary meristem development have been characterised in different plant species. For example, *CUP-SHAPED COTYLEDON (CUC)* genes play a major role in initiation of axillary meristems in *Arabidopsis* (Takada et al., 2001). Shoot branches can initiate from secondary shoot meristems in the axil of leaves. These meristems have the same developmental potential as the primary shoot apical meristem. But in most cases when the plant is intact, the axillary meristems are less active than the primary shoot apical meristem and often their growth arrests, resulting in a small axillary bud, which does not grow out.

The development of the shoot system is modular in nature and the entire shoot system is an iteration of a series of modules or phytomers consisting of a leaf, a segment of stem, a node where a leaf joins the stem and one or more axillary meristems (AM) at the leaf axil (McSteen and Leyser, 2005). These phytomers are the fundamental units of the plant shoot system. Plant growth can be determinate or indeterminate depending on whether the SAMs continue to produce a series of phytomers continuously, or terminate after producing a limited number of these units. Both leaves and branches join the main stem at the node and the region of stem between two nodes is referred to as the internode (figure 1.2). A bud is a shoot apical meristem surrounded by small unexpanded leaves and can be in the axil of the leaf (axillary buds) or at the apex (apical bud).

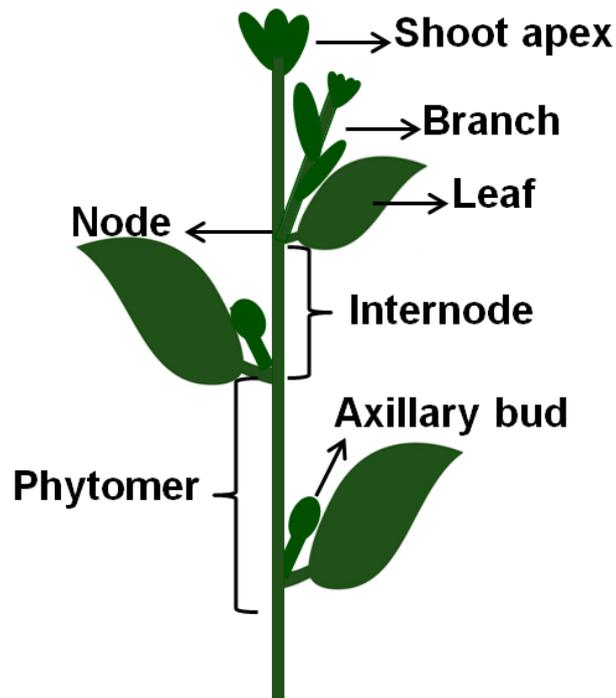


Figure 1.2 Schematic representation of the shoot system comprising of main stem, branches and leaves.

The body plan of animals is usually invariant and genetically determined. Animals cope with changing environments by changing their behaviour, for example moving away from any adverse environments. However, the body plan of plants is much more variable and can be altered to suit the environment in which they are growing. Leyser compared the environmentally regulated development of new plant growth axes as being functionally equivalent to environmentally regulated animal behaviour (Leyser, 2009b).

I will use bud growth and activity as an example. Every plant species has a specific or characteristic pattern of bud outgrowth resulting in different body plans and growth habits (McSteen and Leyser, 2005) (figure 1.3). For example, in *Arabidopsis*, there is a basal to apical sequence of bud growth during vegetative growth which shifts to apical to basal gradient after flowering (Hempel and Feldman, 1994, Stirnberg et al., 1999). In addition to the different body plans of different species, there is difference in the fate of phytomers depending on their position along the shoot and with the different developmental growth phases.

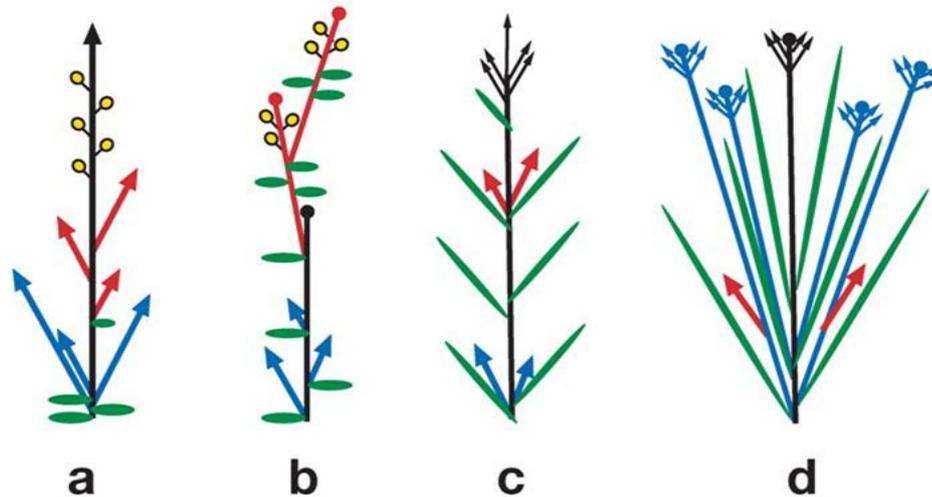


Figure 1.3 Schematic representation of body plans of (a) Arabidopsis, (b) tomato, (c) maize, and (d) rice. Arrows represent indeterminate meristems and circles represent determinate meristems. The black lines indicate the primary axis. The green lines indicate leaves. The blue lines indicate the most basal-arising branches. The red lines indicate axillary shoots arising later during development. The yellow circles represent flowers. Maize and rice also produce lateral branches in the inflorescence indicated by the black or blue lines with smaller arrowheads. Figure adapted from Mcsteeen and Leyser, 2005.

The activity of axillary buds in a plant defines the final number of shoot branches produced by any plant species (Domagalska and Leyser, 2011). The decision of axillary buds to grow out to form branches or to remain dormant as a bud is dependent on several factors which include external environmental, and the internal developmental and physiological status of the plant. An intricate network of hormonal signals which integrate information from different sources, including local signals, convey this information to the axillary bud which makes an appropriate decision based on the information (Leyser, 2009a).

One of the extensively studied examples of an environmental factor influencing the activation of axillary buds and involvement of hormonal signals is the removal of the primary shoot apex for instance by herbivory or pruning. This releases apical dominance, which is the dominance of the shoot apex over the subtending axillary buds (Cline, 1997). When the primary shoot apex is removed or damaged, dormant axillary buds in the subtending leaf axils start growing out. The ideal candidate for mediating the inhibition of the outgrowth of axillary buds by the shoot apex is the hormone auxin, as it is synthesized in the shoot apex and is transported basipetally down the stem. Indeed Thimann and Skoog

demonstrated as early as 1933 that when the hormone auxin was applied to the decapitated primary shoot stump, activation of the axillary buds was prevented (Thimann and Skoog, 1933) and since then auxin has been implicated as the axillary bud growth inhibiting substance (figure 1.4).

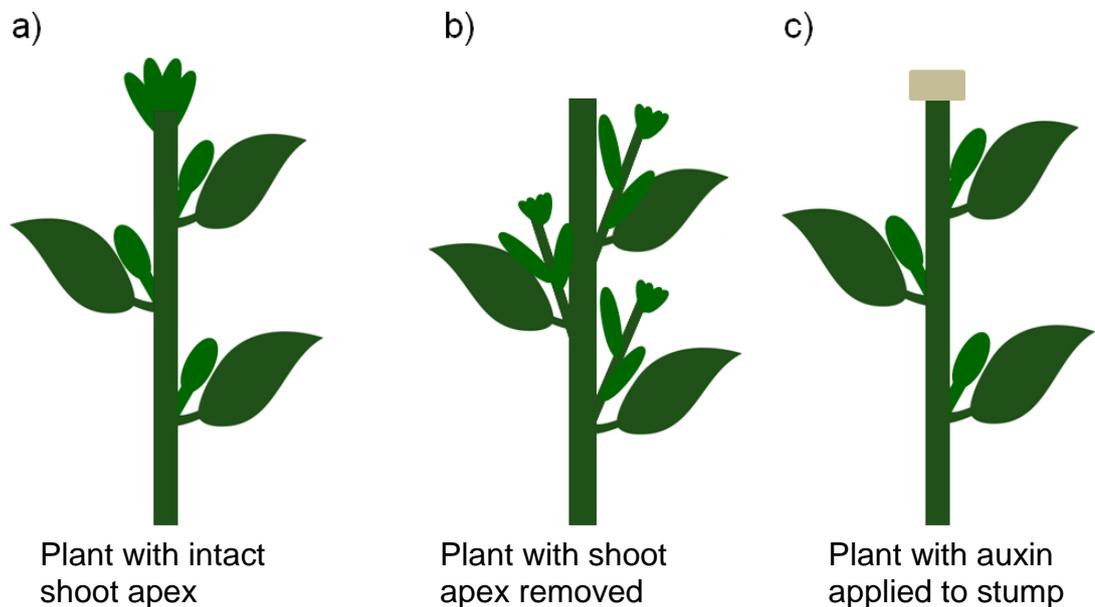


Figure 1.4 Apical dominance and regulation of bud outgrowth. A) If there is an intact shoot apex, bud outgrowth is inhibited b) Removal of the apex results in outgrowth of dormant buds in subtending leaf axils c) Application of auxin to the apical stump inhibits bud outgrowth.

1.2.1 Branching as an agronomic trait

The importance of branching in agriculture is evident from the changes in shoot branching patterns that have been selected during domestication of crop plants. In nature, most of the plants have the ability to modulate their branching pattern in response to the fluctuations in environment. However, domestication and deliberate selection for specific characters have led to more rigid architectural traits (Doust and Kellogg, 2006). Most domesticated cereal crops such as wheat, oat, barley etc have fewer branches from vegetative nodes, and a higher order of branching in their inflorescences (Doust, 2007). This may have been selected because as a result branching, seed production and maturation happen over a restricted period of time, which is advantageous for harvesting and avoiding yield loss. An excellent example is that of reduced branching in modern domesticated maize, when compared to its wild ancestor teosinte (Tsiantis, 2011). The growth habit of teosinte allows for a prolonged period of seed production, even when the inflorescences on the initial branches have matured. Harvest Index (HI) the ratio

of grain yield to total plant mass is generally lower on branchy plants, so relatively high density planting of unbranched plants is likely to yield more than lower density branchy plants. But in nature, competing with neighbours by branching and taking their light is likely a more successful strategy. This example of Maize (figure 1.5) emphasizes the importance of studying key architectural traits like shoot branching from a crop point of view.

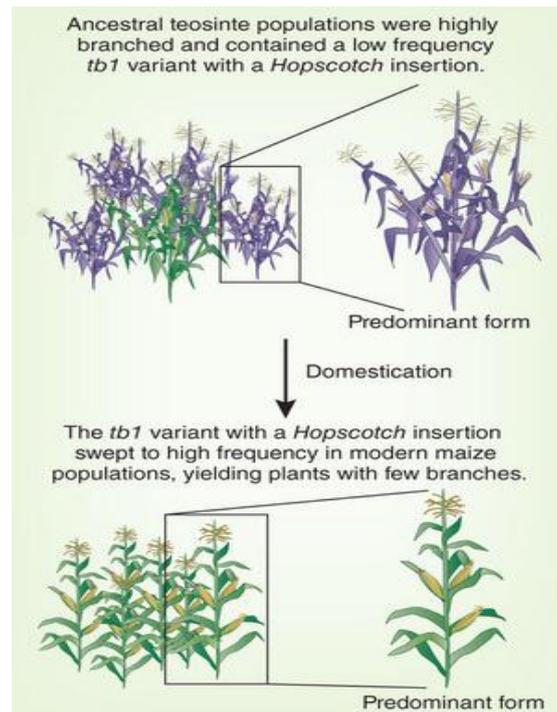


Figure 1.5 Standing genetic variation drives morphological change in maize domestication (Adapted from Tsiantis, 2011).

1.3 Factors affecting regulation of shoot branching

As described, shoot branching is regulated by a complex interplay between three main players namely genetic, environmental and hormonal factors. In this section each of these factors are described briefly.

1.3.1 Hormonal regulation of shoot branching

Involvement of mobile hormonal signals in regulating axillary bud growth has been known for a long time. After the formation of an axillary bud, its activity is under the control of a hormone signalling network (Domagalska and Leyser, 2011). Interactions of plant hormones have a major effect on shoot system architecture. Among these, the most studied one is auxin, which affects not only

bud growth but all aspects of plant development. Leyser summed up the role of auxin in a statement “what does it do? Everything!” (Leyser, 2001) and the study of shoot branching has revolved mainly around auxin ever since the role of this hormone was confirmed in 1933.

Auxin is synthesised in the shoot apex, principally in young expanding leaves (Ljung et al., 2001) and is transported basipetally down the shoot in the polar auxin transport (PAT) stream. One of the earliest studies on apical dominance and auxin came from a system developed by Snow with two branched pea and bean (Snow, 1931). In his experiments where the primary shoot was decapitated just above the cotyledons, buds in the axils of both cotyledons activated. These buds grew continuously but in most cases growth of one bud dominated and leading eventually to the arrest in growth of the other bud. When the dominant bud was removed the arrested shoot reactivated. He concluded that an inhibitory signal must be coming downwards from the dominant shoot and at the same time another inhibitory signal must be going up the arrested shoot. Several studies from thereon have shed light into why auxin transport is always basipetal and this was supported by the identification of auxin efflux carriers. Auxin is actively transported out of plant cells through the action of the PIN-FORMED (PIN) family of auxin efflux carriers which contribute to the directionality of the auxin transport (Paponov et al., 2005). In plant stems, PIN1 proteins are localised basally (in the root-ward direction) in the xylem parenchyma cells (Gälweiler et al., 1998). Although understanding of auxin transport and its involvement in plant growth and environmental responses is increasing, information is lacking on several aspects of the regulatory mechanism as highlighted in a review by Peer and colleagues (Peer et al., 2011).

When auxin is applied directly to buds, bud outgrowth is not prevented. This coupled with the strict basipetal direction of auxin transport indicated the inability of apically-derived auxin to bring about direct inhibition of bud outgrowth. More evidence for this came from studies with radiolabelled auxin. In decapitated plants when the shoot apex is replaced by radiolabelled auxin, label cannot be detected in buds, even when the buds are inhibited (Morris, 1977). All these led to the proposal that auxin inhibits bud outgrowth indirectly and suggested the involvement of a second messenger.

Cytokinin was identified as the best candidate for this role, as its direct application promoted axillary bud outgrowth (Sachs and Thimann, 1967). Cytokinin can enter the buds directly and is involved in promoting shoot branching (Cline, 1991). Cytokinin moves acropetally in the transpiration stream in the stem, through the xylem and is synthesised in shoots and roots (Nordström et al., 2004).

The possibility of a novel upwardly mobile hormone in addition to cytokinin in the regulation of shoot branching control and an indication of its carotenoid origin was evident in several studies from the Leyser group using *more axillary growth (max)* mutants in Arabidopsis (Sorefan et al., 2003, Booker et al., 2004, Booker et al., 2005, Stirnberg et al., 2002). Studies using shoot branching mutants from other species such as pea *ramosus (rms)* mutants (Foo et al., 2005, Beveridge et al., 1996, Beveridge et al., 2000, Morris et al., 2001), petunia *decreased apical dominance (dad)* mutants (Napoli et al., 1999, Snowden et al., 2005, Napoli, 1996) and rice *dwarf (d)* mutants (Ishikawa et al., 2005, Arite et al., 2007) also suggested that a mobile signal is transported acropetally, and is involved in regulating shoot branching. Identification of Strigolactones (SL) (or their derivatives) as this additional hormone in the control of shoot branching happened only in 2008, from studies in two different plant species- pea (Gomez-Roldan et al., 2008) and rice (Umehara et al., 2008). Like cytokinin, strigolactones are also transported in the transpiration stream through xylem (Kohlen et al., 2011).

The discovery of role of SLs as plant hormones and that of SL biosynthetic enzymes was mainly through the study of a class of genes from the above mentioned highly branched mutants in different plant species. These genes which are conserved in monocots and dicots are hypothesized to be involved in the biosynthesis or signalling of the branching inhibitor (Beveridge, 2006). Among the biosynthetic enzymes, three have been described in Arabidopsis (MAX1, MAX3 and MAX4). Among these, MAX3 and MAX4 acts in the chloroplast and encode carotenoid cleavage dioxygenases (CCD7 and CCD8 respectively) where as MAX1 encodes a cytochrome P450 (Booker et al., 2005, Sorefan et al., 2003, Booker et al., 2004). SL insensitivity of *max2* mutant and genetic evidence suggested that MAX2, an F-box leucine rich protein is involved in SL signalling

(Stirnberg et al., 2007, Umehara et al., 2008). Genes identified in other species involved in the SL pathway and their functions can be viewed in figure 1.6. As mentioned, SLs are derived from carotenoids through a pathway involving CCD7, CCD8 and D27 (an iron binding protein) and the first steps of biosynthesis occur in plastids. Recently Alder et al (2012) identified that D27 acts upstream of CCD7 and CCD8 to produce a compound, Carlactone which has SL like biological activities. Despite these findings, several steps in the SL pathway have not been completely elucidated (figure 1.7).

Locus name in				Mutant rescue by		Protein function
<i>Arabidopsis</i>	<i>Oryza</i>	<i>Petunia</i>	<i>Pisum</i>	-Grafting	-GR24	
<i>MAX3</i>	<i>D17/HTD1</i>		<i>RMS5</i>	Y	Y	B carotenoid cleavage (CCD7) / plastid
<i>MAX4</i>	<i>D10</i>	<i>DAD1</i>	<i>RMS1</i>	Y	Y	B carotenoid cleavage (CCD8) / plastid
	<i>D27</i>			n.d.	Y	B unknown Fe-containing protein / plastid
<i>MAX1</i>				Y	Y	B cytochrome P450
	<i>D14/HTD2/D88</i>			n.d.	N	? alpha/beta-fold hydrolase
<i>MAX2</i>	<i>D3</i>		<i>RMS4</i>	N	N	S F-box protein / nucleus

Figure 1.6 Cloned genes in the SL pathway and function of the proteins encoded (functional categories: B, SL biosynthesis; ?, unknown; S, SL signalling). N, no; n.d., not done; Y, yes (Stirnberg et al., 2010 and references therein).

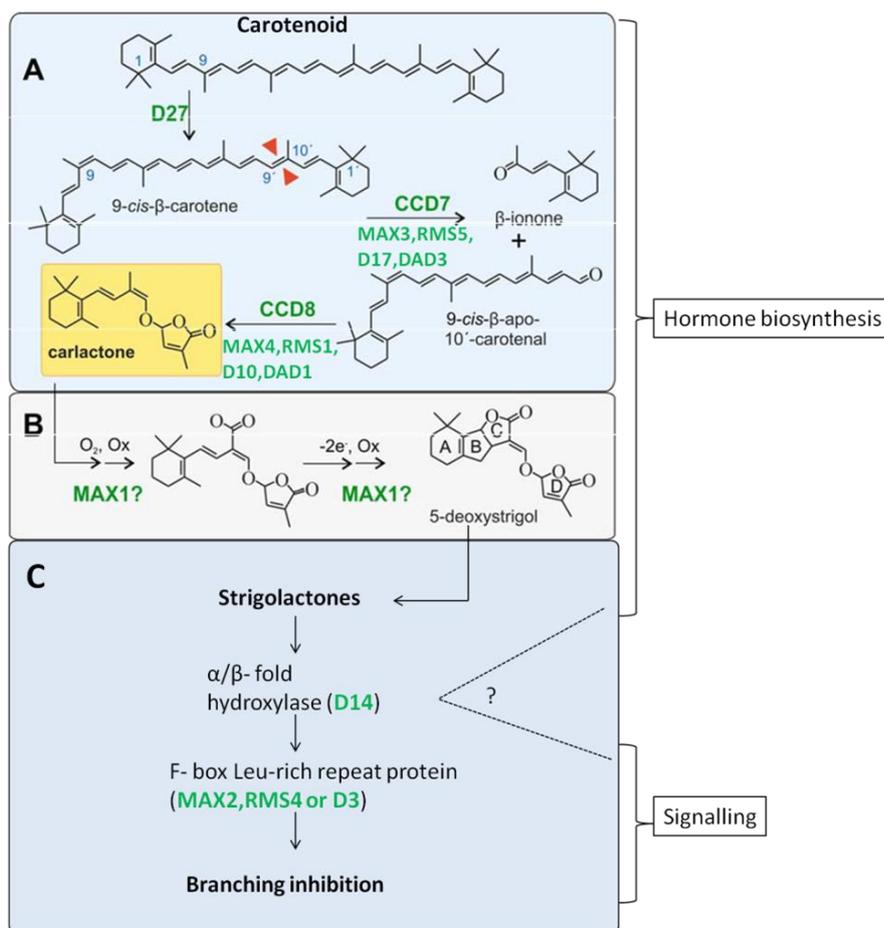


Figure 1.7 The strigolactone pathway (combined from Alder et al (2012) and Domagalska and Leyser (2011)). (A- Steps established by Alder et al, B- Steps proposed for continuation of the pathway, C- Components of the SL signalling pathway leading to branch inhibition.

Although the role of auxin in inhibiting bud outgrowth has been known for years the mechanism by which auxin brings about inhibition is a matter of ongoing debate. Two main, non-exclusive hypotheses have been proposed over the years to account for the indirect action of auxin in bud inhibition. The first one, referred to as the second messenger hypothesis is based on the likelihood of cytokinin and/or strigolactone acting as a second messenger as mentioned in the previous paragraph. According to second messenger hypothesis auxin regulates the production of this second messenger which can enter the buds directly and regulate bud activity (Snow, 1931, Sachs and Thimann, 1967). Consistent with the idea, CK application directly to buds promotes bud outgrowth and this bud outgrowth cannot be prevented even by application of apical auxin (Sachs and Thimann, 1967). Cytokinin synthesis in the main stem and roots is down regulated by auxin (Bangerth, 1994, Tanaka et al., 2006). The likelihood of antagonistic action of auxin and cytokinin in regulating bud outgrowth has been

supported by several experiments. One example in *Arabidopsis* includes CK supplied basally leading to bud outgrowth in excised *Arabidopsis* stem segments, despite being supplied with apical auxin (Chatfield et al., 2000b). Down regulation of CK synthesis by auxin in *Arabidopsis* was shown to be dependent on AUXIN RESISTANT1 (AXR1) mediated auxin signalling (Nordström et al., 2004). Thus the second messenger hypothesis suggests that the mechanism of action of auxin could be by down regulating CK synthesis and limiting CK availability to buds, thereby restricting bud outgrowth. Nevertheless, some physiological studies implicated a more local action of CK in bud outgrowth (Shimizu-Sato et al., 2009, Ferguson and Beveridge, 2009). Thus there is no conclusive evidence for CK as a second messenger.

Similarly, SLs have been proposed to act as second messengers. SL mutant buds in pea were shown to be resistant to exogenously supplied auxin applied on to the stumps of decapitated plants (Beveridge et al., 2000) indicating involvement of SL in auxin mediated bud inhibition. Similarly interactions between auxin and SL were evident from the resistance of buds to inhibition by auxin in the highly branched *Arabidopsis max* mutants (Sorefan et al., 2003, Bennett et al., 2006a). Auxin upregulated transcription of CCD7 and CCD8 was also demonstrated in different plant species with the likelihood of increased levels of SL (Sorefan et al., 2003, Johnson et al., 2006, Arite et al., 2007, Hayward et al., 2009). Furthermore, transcript levels of SL biosynthetic genes decreased considerably upon removal of auxin source by decapitation leading to decreased production of SLs (Johnson et al., 2006, Foo et al., 2005, Arite et al., 2007, Brewer et al., 2009). Based on these evidence a model was proposed suggesting that auxin upregulates SL synthesis and upwardly mobile SL moves into buds suppressing bud outgrowth directly (Brewer et al., 2009, Dun et al., 2009). Both in *Arabidopsis* and pea it has been shown that direct application of SLs to buds can inhibit their outgrowth which is consistent with the second messenger hypothesis (Gomez-Roldan et al., 2008, Brewer et al., 2009).

The second hypothesis by which auxin could inhibit activation of axillary buds is referred to as the auxin transport canalization-based hypothesis. Tsvi Sachs introduced the concept of canalization to denote the movement of auxin from a

source to sink being canalized into narrow cell files with a high capacity for highly polarised auxin transport (Sachs, 1981). Sachs' predictions were validated by Sauer et al who showed that PIN auxin transport proteins accumulated to high levels and in a highly polarised manner linking an auxin source to an auxin sink (Sauer et al., 2006). Involvement of auxin transport in controlling bud activation has been suggested by the observation of a strong correlation between bud outgrowth and canalized auxin export from the bud (Morris, 1977, Li and Bangerth, 1999).

Auxin transport canalization is closely associated with vascular development. The canalization theory was based entirely on observations of the differentiation of vascular strands linking auxin sources to auxin sinks. Studies by Sachs over the years demonstrated that an auxin source can trigger vascular strand development connecting the auxin source to an existing vascular strand only when the existing strand had a high sink strength for auxin (Sachs and Thimann, 1967, Sachs, 1981). When a bud is activated and the branch grows out, vascular connection between the growing branch and the main stem should be established. Differentiation of vasculature and connectivity is brought about by auxin transport canalization out of the young leaves in the axillary bud into the main stem. This is perfectly plausible as young leaves in the bud are good auxin sources and the polar auxin transport stream in the stem is potentially a good sink, as it transports auxin down the stem to roots (Leyser, 2009b).

A mechanistic explanation for the inhibition of bud activation by apical auxin, based on these ideas, was proposed by Bennett et al, involving competition for auxin transport pathways in the main stem (Bennett et al., 2006b). In this model it is assumed that for a bud to activate, auxin transport should be canalized out of the bud, into the main stem. In an intact plant, where the apical auxin source (primary shoot apex) is present, the sink strength of the stem is low and hence canalization and activation of buds will be prevented. When the shoot apex is removed, for example by decapitation, canalization of auxin from the bud to the stem occurs as the sink strength of the stem will be higher, resulting in bud outgrowth. Results from computational modelling studies also supported this mechanism (Prusinkiewicz et al., 2009). According to this model, apical auxin need not enter the bud to regulate its activity, but can regulate bud outgrowth

through competition between the axillary bud and main stem for the common auxin transport route, down the main stem. This model can also explain the bud inhibition when SLs are applied directly to buds. According to the canalization based model, the bud to which SL is applied becomes a poor competitor when compared to other buds and thereby its outgrowth is inhibited (Prusinkiewicz et al., 2009). Thus a major suggestion of auxin transport canalisation based model is that no second messenger is needed for bud regulation. However, as suggested by Domagalska et al (2011) both of these models could be active in different scenarios and are not mutually exclusive.

Three of the main hormones involved in shoot branching regulation described in this section interact with each other and studies on hormonal interactions in the control of shoot branching are increasing in recent years. These studies emphasize the level of interactions and involvement of interconnected feedback loops in maintaining the balance of the whole shoot branching system. As previously described, auxin downregulates CK synthesis through the auxin signalling pathway but additionally CK might activate buds through different modes of action like modulating auxin transport or by upregulating auxin biosynthesis locally in the bud (Müller and Leyser, 2011). Dun et al (2012) suggested recently that CK and SLs acted antagonistically in controlling bud outgrowth. Indication of interactions between auxin and SL came from *Arabidopsis max* mutants, which are defective in either SL synthesis or signalling. Buds of these mutants are resistant to auxin mediated bud inhibition and this is associated with increased auxin transport in the main stem (Bennett et al., 2006b). Expression of SL biosynthetic genes is upregulated by auxin (Hayward et al., 2009, Brewer et al., 2009). Although the mechanism of SL action is still under debate there is an increasing amount of strong evidence that SLs act by dampening auxin transport and by reducing PIN1 accumulation in xylem parenchyma cells (Crawford et al., 2010, Prusinkiewicz et al., 2009). Thus auxin and SL function in intimately connected feedback loops where auxin increases the expression of SL biosynthetic genes which leads to increased SL levels. This SL in turn leads to reduced auxin transport (Stirnberg et al., 2010, Domagalska and Leyser, 2011).

Local regulation of bud activity adds another level of complexity to the hormonal regulatory network in addition to the long distance signals. This regulation is also very important as it is known that different buds in the same plant can have different potentials to activate and this inherent capacity may affect their response to different signals. An example is the out of sequence bud activation in decapitated pea plants where the bud at node 2 activates, whereas buds at nodes one and three do not (Morris et al., 2005). Studies attempting to find genes whose expression correlates with bud activity have led to the identification of several members of TCP family of transcription factors (Leyser, 2009b). The best characterised among these genes are the maize *TEOSINTE BRANCHED 1 (TB1)* gene (Doebley et al., 1997) and closely related genes in other species- sorghum *TB1* (Kebrom et al., 2006), rice *FINE CULM1 (FC1)* (Takeda et al., 2003) and Arabidopsis *BRANCHED 1 (BRC1)* (Aguilar-Martínez et al., 2007). Over expression of these genes led to bud inhibition and it was shown that their expression is necessary to inhibit bud activation. These genes have been implicated in the integration of environmental and hormonal regulation (Durbak et al., 2012) of shoot branching as summarised in figure 1.8.

Cytokinin, the only hormone known to promote bud outgrowth is synthesised locally in the nodal stems and there is increased CK levels in axillary buds corresponding to bud activity (Turnbull et al., 1997) Auxin is known to regulate local CK biosynthesis (Tanaka et al., 2006, Turnbull et al., 1997). Moreover, direct application of CK to buds was shown to correlate with increased auxin levels in buds and increased auxin export from buds leading to their outgrowth (Li and Bangerth, 2003). This is consistent with the canalisation model as well. Very recently it was shown that pea *BRC1* may have a possible role in integrating CK signals and SL within the bud to regulate bud outgrowth (Braun et al., 2012).

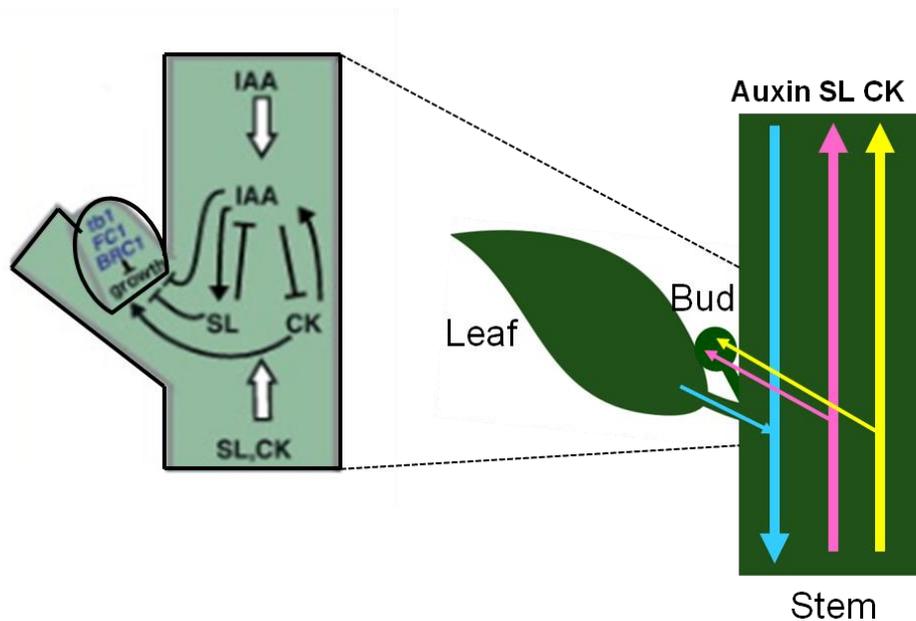


Figure 1.8 Main hormones regulating shoot branching.

a) The blue arrow represents auxin, which is transported down the stem and is exported from young expanding leaves into the stem. The pink arrow represents SL, which is transported upwards and into the buds. The yellow arrow represents cytokinins, which are transported up the stem and into the bud.

b) IAA (indole-3-acetic acid or auxin) transported basipetally inhibits the growth of axillary buds indirectly, while CK and SL travel acropetally in the stem and regulate axillary bud outgrowth acting locally in the bud and/or systemically through regulating auxin transport. Balanced levels of IAA, CK, and SL may be maintained by interactions between these hormones through feedback loops. The TCP family of transcription factors, including the maize *tb1*, rice *FC1*, and Arabidopsis *BRC1* genes suppress bud outgrowth downstream of multiple bud repressing signals. White arrows indicate direction of hormone transport. Black arrows indicate regulation. Genes in blue, function in downregulation. (Adapted from Durbak et al 2012).

1.3.2 Environmental regulation of shoot branching

The hormonal regulation of shoot branching, as elaborated above, has to interact with various environmental signals to co-ordinate the developmental programme of plants. Some of the environmental signals affecting shoot branching are discussed here.

1.3.2.1 Light quality

When plants are exposed to shading by another plant/plants for a prolonged period they respond by increased stem elongation, reduced branching and by flowering early. These responses, collectively referred to as the shade avoidance syndrome (SAS) are initiated by the environmental signal – light, mediated through PHYTOCHROME B (PhyB), a major shade sensor (Smith and Whitelam,

1997). The ratio of red to far red (R: FR) light perceived by phyB is reduced when plants are crowded or shaded, and SAS is considered as a plant adaptive response to reduced light quality caused by shading by another plant (Ballaré, 1999, Schmitt et al., 2003). Plant hormones have been implicated in shade avoidance with majority of evidence involving auxin. The vast majority of this work has been conducted on very young seedlings, using hypocotyl elongation as an indication of the SAS. However, less is known about the links between phytochrome mediated shade avoidance, plant hormones and branching. *Arabidopsis phyB* mutants are defective in detecting R: FR ratios and in high R: FR light these mutants have reduced branching where as *max2* mutants are branchy (Shen et al., 2007), suggesting involvement of SLs. Similar results have been reported in other species such as sorghum, where both loss of phyB and low R: FR ratios induced by shading have been reported to affect branching, and to lead to increased expression of *tb1* (Kebrom et al., 2006). In a recent study Finlayson et al (2010) suggested that phytochrome signals controlled shoot branching partly through modulating auxin physiology (Finlayson et al., 2010). His studies in *Arabidopsis* showed that *brc1* mutant was not responsive to shade, suggesting involvement of *BRC1* gene in bud inhibition mediated by PhyB. In this study, involvement of both auxin and SL signalling pathways and expression of both *BRC1* and *BRC2* were pointed out as requirements for phytochrome signals to control shoot architecture.

1.3.2.2 Nutrients

In addition to light quality mentioned above nutrient availability also regulates shoot branching. Every plant absorbs nutrients from soil allowing it to maintain and sustain its growth and development. Clearly, fluctuations in availability of nutrients in the soil require communication between the root and shoot system if nutrient absorption by roots and nutrient demand from the shoot system are to be balanced. There is mounting evidence that plant hormones function as long distance signalling molecules in communicating the nutrient status between root and shoot.

The essential nutrients that are necessary for plants to complete their life cycle include Nitrogen and Phosphorous, which form building blocks for several macromolecules and are mainly taken up from soil. Limitation of nutrients

occurring in soils could be due to several factors such as low accessibility, low soil nutrient concentrations, leaching, low solubility etc (Schachtman and Shin, 2007).

This study focuses on Nitrogen (N) availability as an important environmental input. Nitrogen is an important component of all living organisms, including plants. Due to their sessile nature plants adopt several strategies and mechanisms to cope with fluctuations in supply of this nutrient. The choice of N as a model environmental cue is based on several reasons, including its position as the most limiting nutrient affecting plant growth among all the mineral nutrients (Epstein, 2005), its importance from an agronomic point of view, ease of manipulating and the easily visible effect on shoot branching. N availability is a key regulator of plant architecture and both shoot and root system architecture is modulated according to the supply of N. Modern agriculture is heavily reliant on nitrogenous fertilizers and the increasing costs of N fertilizers, their high carbon footprint, and the inefficient fertilizer use of crop plants is a concern while attempting to maintain higher yields, without compromising seed quality. N deficiency leads to slow stunted growth, reduction in leaf size, reduction in number of branches and increase in root to shoot ratio.

1.4 Role of Nitrogen

Nitrogen is the most limiting mineral element required for plant growth. N is the building block of many components such amino acids and thus proteins, and nucleic acids. Plants require N more than other mineral nutrients. N from soil is taken up by plants commonly in the form of Nitrate (NO_3^-) but is present in various forms including organic forms (amino acids, urea) and inorganic forms (ammonia). Different plant species prefer different N sources, mainly either Nitrate or ammonium, but Nitrate is the major form present in aerobic soils. Use of N by plants is a multistep process including N uptake, assimilation, transport, and remobilisation. Brief descriptions of these are given here.

1.4.1 N uptake and assimilation

Nitrate in the soil solution is actively transported across the plasma membrane of root epidermal and cortical cells and this is driven by electrochemical gradients

(Forde, 2000). Once inside the roots, Nitrate could be a) reduced to nitrite by the enzyme Nitrate reductase and then to ammonium catalysed by nitrite reductase (Guerrero et al., 1981). Ammonium is then converted into amino acids by the action of glutamine synthetase and glutamate synthetase, b) effluxed back across the plasma membrane c) stored in the vacuole d) transported to shoots through the xylem vessels and assimilated in the shoots (Crawford and Glass, 1998).

1.4.2 Nitrate transporters

Nitrate levels in soil vary extensively and plants have evolved a matching range of active transport systems for uptake of Nitrate. Three different Nitrate uptake systems operate in higher plants which take part in uptake of Nitrate into roots (Forde, 2000). Of these, two are high affinity transport systems (HATS) which take up Nitrate at low concentrations (micromolar range) in the external medium. One of the HATS is known as the inducible high affinity transport system (iHATS) because of its strong induction in the presence of low external Nitrate supply. The second, HATS is constitutively expressed and is known as the constitutive high affinity transport system (cHATS) (reviewed in Forde and Clarkson, 1999). The third system is a low affinity transport system (LATS), which can exploit abundant soil Nitrate supplies (> 1mM).

Two gene families of Nitrate transporters exist in higher plants namely the NRT1 and NRT2 families which correspond to the LATS and HATS respectively (Tsay et al., 2007). Among these NRT1.1 (CHL1), NRT1.2, NRT2.1 and NRT2.2 facilitate Nitrate uptake; NRT1.4 is involved with Nitrate storage; NRT1.7 in Nitrate remobilisation in leaves and NRT1.6 in Nitrate supply to seeds (reviewed in Krouk et al., 2010). The only exception to the LATS classification of these transporters is NRT1.1, which acts as a dual affinity transporter (Liu et al., 1999).

1.4.3 N remobilization

N remobilisation is a key step during the growth and development of plants which partitions nitrogen between organs. When plants are in the vegetative phase both roots and leaves accumulate and assimilate Nitrate and after flowering, the nitrogen assimilated by the vegetative parts of the plant is remobilised to the

developing seed. At this stage both shoots and senescing leaves serve as source of Nitrate (Masclaux et al., 2001). Senescing leaves are an important nitrogen source that can be used during development of new leaves and seeds. Contribution of nitrogen uptake, assimilation and remobilisation (figure 1.9) varies among different plant species depending on several factors such as soil nitrogen availability, environmental conditions such as light, biotic and abiotic stress (Hirel et al., 2007).

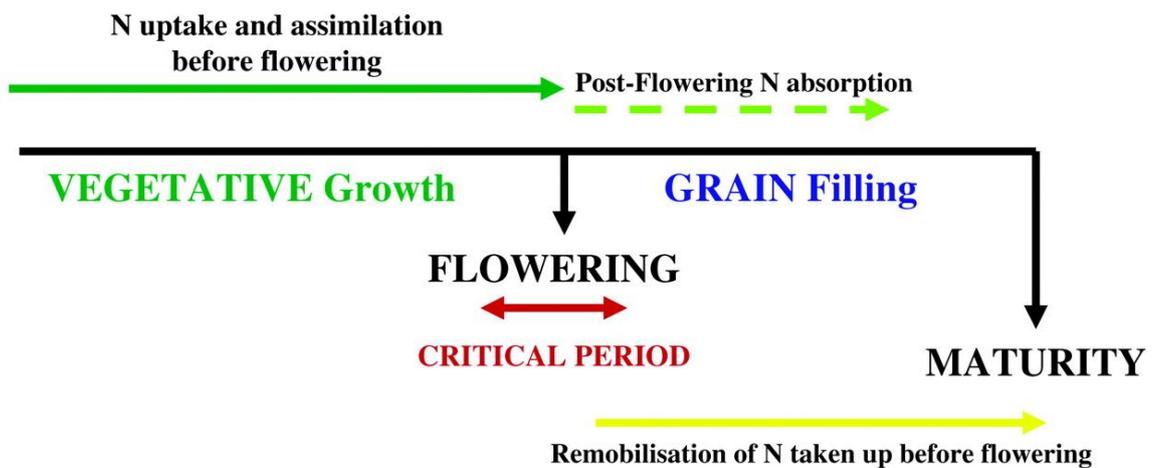


Figure 1.9 Schematic representation of fate of nitrogen during different phases of plant development (adapted from Hirel et al., 2007).

1.4.4 Nitrogen and yield

Nitrogen has a major impact on seed yield of plants. It has been shown in several crops, as well as in *Arabidopsis*, that a decrease in nitrogen availability affected both biomass accumulation and yield (Loudet et al., 2003a). In *Arabidopsis*, Lemaitre et al (2008) demonstrated that N limitation affects seed quality in addition to reducing yield and biomass (Lemaître et al., 2008). It is widely accepted that there is an urgent need to identify ways to reduce excessive use of nitrogenous fertilizers without compromising yield (Hirel et al., 2007). Degree of shoot branching, which is affected by N availability, in turn affects the harvest index. Harvest Index is used as a criterion for selection to improve crop yields (Sharma et al., 1991). There are contradictory reports about the effect of nitrogen on harvest index. The study by Lemaitre et al (2008) concluded that harvest index was lower when *Arabidopsis* plants were grown on high N (10mM Nitrate)

compared to limited N (2mM) but conversely another study have reported that harvest index was poorly affected by nitrogen nutrition (Masclaux-Daubresse and Chardon, 2011).

1.4.5 N status sensing

N uptake by the roots changes according to N availability and shoot and root architecture is modified accordingly suggesting that N status is sensed by the plant to feedback and regulate the process. At whole plant level, N sensing modulates shoot and root growth in response to fluctuations in availability of nitrate. As Nitrate is the main available form of N for plants which gets converted finally to amino acids, it was suggested that internal pools of amino acids within a plant, might provide a signal indicating N status which in turn regulates N uptake and assimilation (Lee and Rudge, 1986, Cooper and Clarkson, 1989). Consistent with this, measurements of plant tissues have shown that N status is reflected by changes in tissue pools of nitrogenous compounds including Nitrate and amino acids (Miller et al., 2008). It was shown that Nitrate triggered changes in gene expression in addition to changes in plant growth (Crawford and Glass, 1998, Stitt, 1999). Direct sensing of Nitrate and sensing of N status, can be distinguished using Nitrate reductase mutants. Scheible et al (1997) in his study with Nitrate reductase mutants demonstrated that Nitrate accumulating in shoots regulated shoot - root resource allocation and the involvement of long distance signals. Root growth in mutants with low Nitrate reductase activity was inhibited which resulted in high shoot to root ratios than wild type plants even though levels of amino acids and other metabolites were similar. Their split root experiments showed that the decreased root growth was due to accumulation of Nitrate in shoots (Scheible et al., 1997). Studies by Zhang and Forde (1998) also demonstrated that tissue nitrate levels are involved in Nitrate status sensing.

1.4.6 Alterations in shoot-root ratios in response to N availability

Plants adjust their shoot branching and shoot: root ratio according to N availability. High N shifts biomass partitioning from root to shoot resulting in a higher shoot: root ratio and plants grown on low N is reported to have a lower shoot: root ratio when compared to those grown on highly fertile environments (Hermans et al., 2006). Although plants exhibit phenotypic plasticity in response

to N availability very little is known about the genetic regulation of developmental responses to N (Jin et al., 2012).

Significant advances have been made in the understanding of modification of root system architecture according to N supply. Response of root system to N varies depending on the concentrations of Nitrate in the soil and the N status of the plant. When there is a high uniform concentration of N, lateral root formation is repressed (Zhang et al., 1999). When there is N starvation there is an increase in primary and lateral root growth relative to shoot growth, leading to a shift in shoot to root ratios. When a patch of high N is presented to an otherwise N starved plant, there is local stimulation of lateral root growth in the patch, and suppression outside the patch (Roycewicz and Malamy, 2012, Walch-Liu et al., 2006, Zhang and Forde, 2000).

One of the earliest examples of response of plant root system to N was from the experiments of Drew et al (Drew et al., 1973, Drew and Saker, 1975). They introduced the classic split root system, where roots of one plant were separated onto different Nitrate concentrations, and showed that when barley roots were grown in locally high concentrations of Nitrate there was an increase in lateral root growth specifically in the parts of the root exposed to high-N. Similar results were obtained in other species such as Tobacco (Scheible et al., 1997) and Arabidopsis (Zhang and Forde, 1998, Linkohr et al., 2002). Experiments with split roots also demonstrated the importance of shoot system in repressing root growth and the involvement of signalling pathways operating between roots and shoots (Walch-Liu et al., 2005).

These studies emphasize that co-ordination between root growth and shoot growth must be maintained to sustain plant development and changes in root architecture are likely to trigger changes in shoot system architecture, and vice versa. Compared to studies on root architecture, very little work has been done to study the effect of N on shoot architecture although these two processes are not independent.

1.4.7 Shoot-root signalling

Scheible et al (1997) demonstrated that Nitrate accumulating in shoots regulated shoot - root resource allocation and the involvement of long distance signals. His experiments proved that signalling between shoot and root system exist and this could be mediated by the shoot. Involvement of shoot- root signalling has been extensively studied in N fixing nodulation process. As a result of the symbiotic association between legume plants and rhizobial bacteria, specialised root organs called nodules develop, which assist in the fixation of atmospheric N in leguminous plants. Long distance signalling between roots and shoots is involved in this autoregulation process, where a root derived signal is transported to leaves which initiate nodulation related responses and in turn a shoot derived signal is transported back to the roots leading to inhibition of further nodule formation (Delves et al., 1986, Reid et al., 2011). Furthermore, it has been shown that several supernodulating mutants in legume species such as soybean are unable to suppress their nodulation in response to Nitrate, despite that presence of nitrate in the soil is known to restrict nodulation (Carroll et al., 1985). Shoot-root signalling has been implicated in this and this assumption was strengthened by grafting studies between mutants and wild type plants which showed that the supernodulation phenotype is controlled by the genotype of the shoot (van Noorden et al., 2006).

1.5 Interaction between Nitrate and hormones

As described above changes in N availability leads to a shift in shoot to root ratios which is clearly manifested in shoot and root branching. And there is growing evidence about the involvement of plant hormones in mediating responses to N availability. Examples of the best studied interactions are discussed below.

1.5.1 Auxin

As discussed above, studies by Scheible et al (1997) and the above mentioned nodulation studies proposed a shoot to root signal involved in the regulation of shoot to root ratio by N availability. Among the candidates for this long distance signal, auxin is prominent due to its basipetal transport and its role in regulation of both primary and lateral root growth (Bhalerao et al., 2002, Forde, 2002). There is

also evidence for the involvement of shoot derived auxin in N-status communication. Van Noorden et al (2006) showed that long distance auxin transport from shoots of supernodulating mutants is associated with a failure to reduce the amount of auxin loaded from shoot to root. Furthermore it was shown very recently that shoot-to-root auxin transport increased with shoot N concentration in the mutants, whereas the transport was negatively correlated in wild type plants. This study also suggested a role for *SUPER NUMERIC NODULE (SUNN)* gene in modulating long distance signalling and shoot to root auxin transport in response to N status (Jin et al., 2012).

Besides, adaptation of the root system to Nitrate availability was shown to involve AXR4 which is involved in auxin transport (Zhang et al., 1999). In addition, expression of one of the Nitrate transporters, NRT1.1, was shown to be strongly induced by auxin in both roots and shoots (Guo et al., 2002). Auxin response factors (ARF) and auxin receptor genes have been recently shown to be Nitrate inducible. Studies with N inducible auxin response factor, ARF8, and a microRNA (miR167a) that targets it, suggest that Nitrate regulated auxin signalling controls lateral root initiation (Gifford et al., 2008). Involvement of an auxin receptor gene, *AFB3*, and its targeting miR393 in regulation of root system architecture by Nitrate was shown recently in Arabidopsis (Vidal et al., 2010). A strong auxin- Nitrate link was provided when Krouk et al (2010) showed that NRT1.1 (*CHL1*) facilitated cell to cell auxin transport and that Nitrate could act as an inhibitor of this auxin transport. Their study showed that in low Nitrate conditions auxin accumulation at the root tip is prevented and there by lateral root growth is inhibited, where as in high Nitrate situations, Nitrate inhibits the auxin transport by NRT1.1 leading to auxin accumulation at the root tip and subsequent outgrowth of lateral roots (Krouk et al., 2010). Furthermore, evidence for the regulation of auxin levels in Arabidopsis roots depending on the nitrogen status of plants was confirmed by studies of Kiba et al (2011). They showed that when Arabidopsis seedlings grown on low N and high N were compared, seedlings grown on high N had lower levels of root auxin than those on low N. These studies give evidence for the involvement of auxin in Nitrate response and likely mechanisms connecting these two.

1.5.2 Cytokinins (CK)

CK is probably the most studied hormone for the involvement in plant responses to nutritional status. CK acts as a long distance signal in communicating the soil Nitrate availability to the shoots. Sakakibara et al (1998) reported a decrease in CK levels in maize roots when there was reduction in N supply (Sakakibara et al., 1998). Further evidence for a CK- N association came from the studies of Takei et al (2001) who suggested that CK played a role in transmitting information about N status from roots to shoots (Takei et al., 2001). His studies provided evidence for translocation of CK from root to shoot where it promotes bud outgrowth. Also, resupply of N to N-depleted maize plants led to an increase in concentration and the transport rate of CK in xylem sap. This model of CK-mediated N signalling was further validated by showing that levels of CK biosynthetic genes *ISOPENTENYLTRANSFERASE3 (IPT3)*, *CYP735a* and *IPT5* were upregulated in Arabidopsis roots by Nitrate (Takei et al., 2004, Wang et al., 2004, Miyawaki et al., 2004). Among these, the Nitrate inducible expression of *AtIPT3* was shown to be mediated in part by NRT1.1 the dual affinity Nitrate transporter (Wang et al., 2009). The expression of several ARABIDOPSIS RESPONSE REGULATOR (ARR) proteins involved in CK signalling was also increased upon Nitrate treatment (Scheible et al., 2004). In addition to this, involvement of CKs in co-ordinating shoot growth according to Nitrate availability was supported by other studies (Rahayu et al., 2005, Sakakibara et al., 2006).

Recently Kiba et al (2011) suggested the likelihood of CK playing a major role in regulating root system architecture in response to Nitrate availability based on the close correlation between CK content and Nitrate status (Kiba et al., 2011). He cited several examples where CKs negatively regulated other nutrient acquisition genes in Arabidopsis and rice. Additional roles for CK as a local signal in controlling local responses to Nitrate availability have been suggested by studies in Arabidopsis (Kiba et al., 2005, Miyawaki et al., 2004).

1.5.3 Strigolactones (SL)

Plants exude SLs into the soil in response to N deficiency. Several studies have demonstrated an elevation of SL levels in root exudates of plants that experience N or P starvation (Yoneyama et al., 2007a, Yoneyama et al., 2007b, Umehara et

al., 2008, Yoneyama et al., 2008). In particular, results from Yoneyama et al (2007) gave evidence that in sorghum plants SL secretion was promoted in conditions of N deficiency (Yoneyama et al., 2007b). Furthermore, importance of N availability in regulation of SL production, irrespective of the form of Nitrogen was also demonstrated by Yoneyama et al (2007a).

However, this response is not universal but may relate to the nutrient acquisition strategy of the plant. For example many, but not all, legume species exude SLs only in response to P deficiency and not N deficiency. The ability of leguminous plants to acquire N through their symbiotic association with rhizobial bacteria was cited as the reason for the lack of increase in SL exudation in leguminous plants (Yoneyama et al., 2007a). The hypothesis that response of different plant species to nutrient availability and SL exudation was based on the legume/non-legume distinction was investigated in a recent study with plants from four different families including legumes and non legumes (Yoneyama et al., 2012). Results of this study demonstrated that this hypothesis does not always hold true as among the studied plants, there were some exceptions. Both N and P deficiency enhanced SL production in some, which included a leguminous plant belonging to Fabaceae (Chinese milk vetch) whereas a non-leguminous plant, tomato which belongs to Solanaceae, did not respond to N deficiency. However, it might also relate to the relationship between P and N deficiencies, given an association in some species between N deficiency and P shoot levels. The study by Yoneyama et al (2011) indicated a relationship between reduced levels of P in shoots of plants grown under N deficiency and SL exudation suggesting that N deficiency might regulate P accumulation in shoots.

Umehara et al (2010) showed that rice tillering is affected by P deficiency and this is mediated by SLs. In Arabidopsis plants grown on P deficient conditions, SLs transported through xylem plays a major role in regulating shoot branching (Kohlen et al., 2011). They propose that modification of shoot architecture under P deficiency as an adaptive strategy to cope with growth limiting conditions (Kohlen et al., 2011, Umehara et al., 2010b). Nevertheless, root exudation might have nothing to do with endogenous or shoot SL. But there is some evidence that nutrient deficiency affects shoot SL too. For example, Umehara et al (2010)

showed that in rice plants, the transcript levels of SL related genes were higher in the shoots of P deficient plants than in P sufficient plants.

1.6 Natural allelic variation and shoot branching plasticity

Although it seems very likely that plant hormones have a role in shoot branching plasticity in response to N variation, it is difficult to study this using null alleles, because they simultaneously remove gene function and the ability to modulate it. So natural allelic variation offers a promising alternative to forward genetics studies. Furthermore, using classical forward genetics, it is difficult to screen directly for plasticity mutants, for example it is not possible to screen the same M2 plant in two different environments like high and low N.

1.6.1 Tools for exploiting natural variation in shoot branching

Dissecting variations in any complex genetic trait like shoot branching, and its environmental responsiveness needs mapping populations containing sufficient natural genetic variation and recombination to identify quantitative trait loci (QTLs). These mapping populations can be natural populations (Weigel and Mott, 2009) or synthetic populations of recombinant inbred lines (RILs) which are stable and can be used in multiple experiments (Huang et al., 2011). Both of these provide large, genetically diverse, collections of homozygous lines, which are stable and can be screened in multiple environmental conditions. However, RILs are randomised genetic combinations whereas natural accessions represent only those combinations that can compete in the wild.

Multiparent advanced generation intercross lines (MAGIC lines) are a recently developed resource with a large amount of genetic variation (figure 1.10), which Cavanagh et al (2008) described as a second generation mapping tool (Cavanagh et al., 2008). Such a RIL population has been developed in Arabidopsis (Kover et al., 2009). More recently, an Arabidopsis multiparent recombinant inbred line (AMPRIL) population was generated from eight founder accessions (Huang et al., 2011). Multiparent populations like these are being developed in several plant and animal species to allow better exploitation of available germplasm and provide additional resources for dissecting natural variation.

Multi-parent Advanced Generation Inter-Cross (MAGIC)

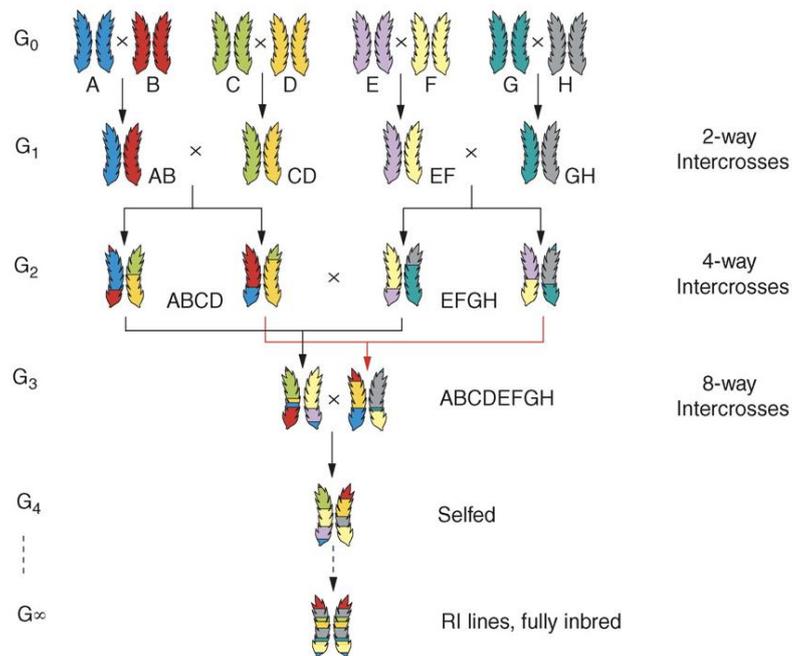


Figure 1.10 Construction of a MAGIC population. Founder or parental accessions (here eight, (A-H) are crossed to produce four hybrids. These then undergo intercrosses and the resulting population is self-fertilised to produce fully inbred recombinant lines (Figure from Cavanagh et al., 2008).

1.7 Importance of genotype by environment interactions

Genotype by environment interactions are often cited as a common source for phenotypic variation and such studies are documented in several species including humans and plants (Gerke et al., 2010). Genotype by environment interaction studies by several people have emphasised the importance of such interactions in regulating the vegetative and reproductive architecture of plants. However, the genetic basis of both GEI and plasticity which affects not only the morphological traits but also the life history and fitness traits is poorly understood (Ungerer, 2003). Genotype by environment interactions in shoot branching in response to the environmental cue, N has not been attempted to date to knowledge, rendering this GEI study particularly relevant in the understanding of shoot branching. Most of the traits associated with fitness of a plant such as seed yield and seed viability are important for persistence in nature. For example, modifying plant architecture leading to increase in crop yields is cited as the reason behind the success of green revolution. Therefore it can be assumed that

understanding the mechanism underlying plasticity in shoot branching will contribute to breeding of high yielding crops as well.

1.8 Brassica

Brassica crops share a common ancestry with Arabidopsis. Both Brassica and Arabidopsis are members of the diverse *Brassicaceae* family (known as the mustard or Cruciferae family), within which the Brassica and Arabidopsis genera diverged approximately 20 million years ago (Yang et al., 1999). The Brassica genus includes several agronomically important vegetables such as cabbage, broccoli, cauliflower as well as oil seed crops such as *Brassica napus*, *B. juncea* and *B. rapa*. Among these, oil seed rape (OSR) is a Brassica species that is heavily dependent on nitrogen for yield. Modern OSR can be traced back to medieval times and this crop was exploited for its oily seed by the Romans who used it to heat their bath houses. Although oilseed rape was barely known in UK until the 1970's, it has since then become a valuable crop to UK agriculture. It is the 3rd most important crop in UK and the global demand for this crop is increasing every year. It is well established that N plays an important role in the growth of OSR and it has been reported that when compared to cereals, N requirement per unit yield of OSR is much higher (Hocking and Stapper, 2001). OSR being heavily dependent on nitrogenous fertilizers, yield stability under low N input is an agricultural priority and it is known that OSR has a high capacity for Nitrate uptake from soils (Lainé et al., 1993). However a substantial proportion of the Nitrate taken up remains in the leaves and is lost when the leaves senesce and are shed (Malagoli et al., 2005). A striking statistic is that average OSR yield has remained relatively static over the years (figure 1.11). Compared to other crops, such as wheat where the yields have more than doubled (5t/ha to over 13t/ha) since 1960's, the commercial yield of OSR have hardly risen (2.5 t/ha to 3.2t/ha). The estimated yield potential of current varieties is up to 6.5t/ha with an ultimate potential of up to 9.2 tonnes/ha. Studies of yield analysis of OSR reported considerable potential for yield improvement (Diepenbrock, 2000).

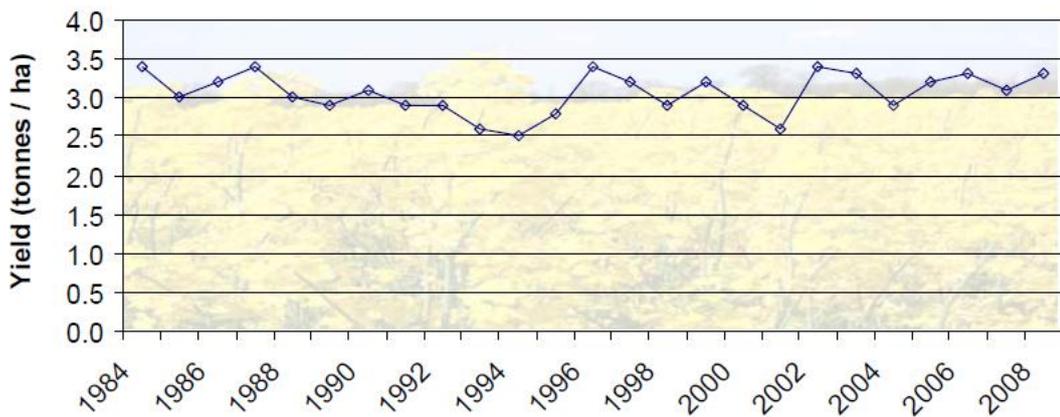


Figure 1.11 Yield trend of Oil seed rape in recent years (Twining and Clarke, 2009).

The architecture of OSR has not undergone any major modifications and is similar to how it was centuries ago. Crops such as wheat have been the focus of major breeding efforts for over 100 years, during which time dramatic alterations have been made to its architecture and yield. Unlike wheat, which grows upright, OSR has an indeterminate growth habit and when conditions are favourable makes many branches and keeps on growing upwards and outwards. Its inefficient canopy structure leads to shading by the late growing branches and pod filling on lower branches is affected. Increase in yield of cereals in the past decades have been attributed to a higher harvest index. The harvest index of OSR increased by only about 25-30% when compared to upto 45-50% in wheat (Dreccer et al., 2000, Diepenbrock, 2000). Furthermore, OSR plants can grow very tall and can be floppy, making harvest difficult. These considerations make shoot system architecture a key target for breeding for improved OSR yield.

Among the Brassica species grown for oil seed, *Brassica napus* and *Brassica rapa* are the two major cultivated species of OSR. *B. rapa* has a shorter growing period than *B. napus* making it the optimal choice for growers in many areas. In this thesis the term Brassica refers to *Brassica rapa* in particular, unless otherwise mentioned.

1.9 Aims and layout of thesis

The primary aim of this thesis is to contribute to the understanding of shoot branching plasticity in *Arabidopsis*, using N availability as a model environmental

input that affects branching. A resource to exploit natural allelic diversity in Arabidopsis, namely the Kover MAGIC lines, will be used to investigate the different strategies adopted by plants to cope with variations in Nitrate supply. Progress in this area will be described in Chapter 3. Chapter 4 describes work to investigate the role of the relatively new hormone-strigolactone, in shoot branching regulatory network and to understand how it regulates branching response to N supply. This is particularly interesting because it is known that SL biosynthesis is upregulated by nutrient deficiency. Chapter 5 describes preliminary attempts to translate the bioassays and established techniques in Arabidopsis to *Brassica rapa*. Results of individual Chapters will be discussed towards the end of each Chapter. In addition, the main conclusions will be brought together in a general discussion at the end.

1.10 Published work

Part of the work from chapter four involving single and two bud assays was published in the Development Journal in 2010.

CRAWFORD, S., SHINOHARA, N., SIEBERER, T., WILLIAMSON, L., GEORGE, G., HEPWORTH, J., MULLER, D., DOMAGALSKA, M. A. & LEYSER, O. 2010. Strigolactones enhance competition between shoot branches by dampening auxin transport. *Development*, 137, 2905-13.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma, UK unless otherwise mentioned.

2.2 Plant culture media

Arabidopsis thaliana salts (ATS) (Wilson et al., 1990) was used in all hormonal assays, experiments involving nitrate (modifications mentioned below) and all experiments were performed in sterile conditions, including glass jar assays and split plate assays. From here on wherever ATS is mentioned it denotes the basal media from the above reference, unless otherwise specified.

Reagent	Final concentration (mM)
KNO ₃	5
KH ₂ PO ₄ (buffered to pH 5.5 with K ₂ HPO ₄)	2.5
MgSO ₄	2
Ca(NO ₃) ₂	2
Fe-EDTA	2.5

Micronutrients:

- A. 70mM H₃BO₃
- B. 14mM MnCl₂,
- C. 0.5mM CuSO₄
- D. 1mM ZnSO₄,
- E. 0.2mM NaMoO₄,
- F. 10mM NaCl,
- G. 0.01mM CoCl₂

2.2.1 Variations in plant culture

When plants were grown on low (1.8mM) nitrate, 5mM KNO₃ was replaced by 1mM KNO₃ and 4mM KCl and 2mM Ca(NO₃)₂ was replaced by 0.4mM Ca(NO₃)₂ and 1.6mM CaCl₂.

(Sucrose – 1% (w/v) and agar – 0.8% (w/v) were added wherever solid medium was used, unless otherwise mentioned).

2.3 Arabidopsis plant material

Col-0, bulked from Leyser lab seed stock

max2-1, bulked from Leyser lab seed stock (Stirnberg et al., 2002)

max4-1, bulked from Leyser lab seed stock (Sorefan et al., 2003)

MAGIC lines (Kover et al., 2009), bulked from Leyser lab seed stock

2.4 Plant hormones

Dilutions from the stocks of hormones below were used in the assays described in chapters 4 and 5. Synthetic hormone analogues of SL (GR24) and auxin (NAA) were used in all the assays.

- 1) GR24, from LeadGen Labs LLC (equal mix of diastereomers) was dissolved in acetone and stocks were stored at - 80°C.
- 2) NAA (1-naphthaleneacetic acid) was dissolved in ethanol and stocks were stored at -20°C.

All control treatments in hormonal assays were performed with the carrier alone.

2.5 General growth conditions for Arabidopsis

All plants were grown either in the green house or a growth room under conditions described below.

Arabidopsis plants were grown in P40 trays (seed trays with forty 16cm² square compartments from Cookson Plantpak, Maldon, UK) on F2 compost treated with Intercept 70 WG (both Levington Horticulture, Ipswich, UK). Seeds were sown into these trays and stratified at 4°C for 2 days before transferring to greenhouses having natural day light supplemented with artificial light to provide long day (16 hours light) conditions at ~150µmol photons m⁻²s⁻¹. Temperature in

the greenhouses ranged between 15-24°C. Seed bulking for all the experiments in this thesis was done on F2 compost as mentioned above.

In experiments conducted in growth rooms (as mentioned with individual experiments hereafter), plants or explants were maintained in the following standard conditions: 16 hours light, 8 hours dark, temperatures 19-22°C day, 18-20°C night, light intensity $\sim 60-100 \mu\text{mol m}^{-2}\text{s}^{-1}$ unless otherwise specified.

2.6 Growth conditions for studying nitrate effects on Arabidopsis

For all nitrate studies, plants were grown on a 1:1 mixture by volume of sand (Hepworth Minerals & Chemicals, Cheshire) and Terragreen (Oil-Dri UK Ltd, Cambridgeshire) in pots of 2" diameter. Pots were filled using a measuring pot to ensure that all pots had the same volume of the mixture. Filled pots were treated with pesticide Intercept (Levington Horticulture, Ipswich) and left to dry before sowing. Plants were fed with measured amounts (25ml/pot first application and 10 ml/pot thereafter) of appropriate liquid ATS solution (either 9mM or 1.8mM Nitrate) on a weekly basis. Plants were watered daily except on the day of nutrient application.

Seeds were imbibed on moistened filter paper in Petri dishes at 4⁰ C for three days, prior to sowing. A moistened tooth pick was used to transfer seeds onto the surface of pots pre-fed with appropriate nutrient solution. Three seeds per pot were sown maintaining even spacing. Trays with pots were covered with lids and watering was done by misting, until seedling growth was fully established. Then the vents on the lids were gradually opened and seedlings were thinned to one plant per pot. Plant pots were randomised in each tray so that every line was represented in each tray. The plant trays were also moved around every week.

2.7 Arabidopsis decapitation assay

For decapitation assays in long days, plants were sown on 50% sand and 50% Terragreen mixture as described above and grown in long-day conditions (20 – 24⁰ C day / 15⁰ C night temperatures, 16 hour light and 8 hour dark photoperiod, light intensity of 120-150 $\mu\text{molm}^2 \text{s}^{-1}$). Decapitation was performed when the

primary bolt was ~ 10cm long (Greb et al., 2003). Rosette branches were scored ten days after decapitation.

2.8 Nitrate response phenotypic analysis

Dates of germination and bolting were recorded for each plant. Bolting time was calculated as the number of days from germination to bolting. The number of cauline and rosette branches (longer than 5mm) were counted when the plant had two filled siliques. This defined developmental stage was chosen to ensure that plants of similar developmental age were compared. Plant height was measured as the height of the primary inflorescence. Each plant was carefully bagged when the first silique was ripe and ready to shatter. Watering and feeding nutrient solution were discontinued 2-3 weeks after bagging. Plants were then left to dry and harvested. Care was taken to collect any rosette leaves left outside the bags while harvesting plants. Wherever root samples were collected, plant pots were lowered into a plastic container with water, and once soaked, roots were gently separated from the sand and Terragreen. Harvested plants were left to dry at room temperature for 4 weeks before seeds, residual shoot dry matter and roots were separated manually. For germination assays, seeds were after ripened for a month at room temperature.

2.9 Seed yield and Seed viability analysis

2.9.1 Quantification of total seed yield

Total seeds collected from each plant were stored at room temperature and weighed on a Sartorius fine balance. Residual shoot dry matter was weighed on a Mettler toledo AB 204 balance, having cut the dry branches into small sized pieces.

2.9.2 Quantification of single seed weight

Two methods were used for single seed weight. 1) 100 seeds/ plant were counted manually and weighed on a Sartorius fine balance and weight of a single seed was calculated. 2) A fixed amount of seed was weighed (2.5mg) and spread out evenly on moistened filter paper. The spread seed were scanned on a flat

bed scanner (HP Scanjet 5300C) and total numbers of seeds were counted manually. Single seed weight was calculated from this.

2.9.3 Quantification of seed viability

100 seeds of each plant were evenly placed with a moistened toothpick on two layers of filter paper moistened with 1.5ml of distilled water in Petri plates and the plates were closed with micropore tape to prevent drying. These plates were wrapped with aluminium foil and stratified at 4°C in a cold room for three days prior to moving them to a growth room with long day conditions (20°C day / 15°C night temperatures, 16 hour light- 8 hour dark photoperiod, light intensity of 120-150 $\mu\text{molm}^2 \text{ s}^{-1}$). Plates were spread out and their moisture checked daily, and 0.5ml of distilled water was added if required. Seeds were scored on day 5 for germination, defined as the emergence of the radicle.

2.10 Nitrate analysis

Nitrate analysis was performed according to the procedure in Cataldo et al (1975).

2.10.1 Extraction

Residual shoot dry matter (RSDM) was dried in an oven and ground to a fine powder using a Retch mixer mill MM301 at 24 vibrations/sec for 3 minutes or until a fine powder was obtained. Dry seeds were ground in the same way. Approximately 5mg of powdered sample (RSDM or seeds) was weighed into a 1.5ml pre-weighed microcentrifuge tube and the weight was noted. 500 μl of deionized water at a temperature of about 90°C was added to this and mixed thoroughly. Tubes were placed in a water bath (80°C) and heated for 30 minutes with regular manual mixing. Tubes were allowed to cool to room temperature and centrifuged at 4500rpm in a microcentrifuge for 5 minutes. The supernatant (containing the extractable nitrate) was transferred to a new pre-weighed tube.

2.10.2 Determination of Nitrate concentration

Reagent A- 5% w/v salicylic acid - 1g of salicylic acid dissolved in 20ml 96% sulphuric acid prepared fresh every time.

Reagent B- 2 M NaOH

Standard solutions- Sodium Nitrate in deionized water in a range of: 0, 0.1, 0.2, 0.5, 1, 2, 4, 6, 8, 10 and 12mM of nitrate.

40 μ l of reagent A was added to 10 μ l standard or extract in a 1.5ml microcentrifuge tube and mixed well on a vortex mixer. This was followed by centrifugation in a microfuge for 2 minutes at high speed. The mixture was left for 20 minutes at room temperature. 1ml of reagent B was added and the contents were mixed well by vortexing. The mixture was cooled down to room temperature. The yellow colour which developed was stable for 48 hours. 300 μ l of sample, standard or blank was added to a 96 well plate. Three replicates for each sample can be done from the extract in the microcentrifuge tube. The absorbance was measured on a microplate reader at 410nm.

2.11 Arabidopsis bud assays

Bud hormone response assays were performed as described by Ongaro et al (2008) with some modifications. Arabidopsis plants grown on either F2 compost or on sand and Terragreen mixture were selected for the assay when they had just bolted (approximately 3 weeks old). The whole bolting stem was excised from the plants with a scalpel. For single bud assays the stem was trimmed to leave a single node with ~ 1cm of stem below the node, and the shoot apex was removed just above the node. At day 0, the size of buds used in the assays was not greater than 2.5mm. For two node assays, the shoot apex was removed just above the upper of the two nodes and stem was trimmed so that there was approximately 1cm below the bottom node. The stem segments were immediately transferred into 1.5ml microcentrifuge tubes (through holes on the lid) containing appropriate ATS solutions with or without the appropriate concentration of synthetic SL, GR24 or 100% acetone (as carrier control) as mentioned in specific experiments. Tubes were arranged on racks inside trays with propagator lids. Layers of moistened filter paper were laid out at the bottom of the trays to maintain humidity. In contrast to Ongaro et al (2008), cotton wool was not used in the microcentrifuge tubes, nor was the decapitated stump sealed with lanolin paste, as we found that omission of these procedures did not affect

the results. Trays were transferred to controlled growth rooms maintained at the following conditions: 16 hours light, 8 hours dark, temperatures 19-22°C day, 18-20°C night, light intensity $\sim 60\text{-}100\mu\text{mol m}^{-2}\text{s}^{-1}$. Bud lengths were measured daily for 7 days using a ruler and tube racks were randomised every day after measurement. ATS solutions in the 1.5ml microcentrifuge tubes were replenished when required.

2.12 *Brassica rapa* plant material

All seeds including *B. rapa* line R-o-18 and other lines from the EMS mutagenised populations were sourced from Dr Lars Ostergaard (John Innes Centre, Norwich, UK). The only exception is seeds of *B. rapa max2* families from TILLING, which were obtained from Dr Fran Robson (RevGen, JIC, UK).

2.13 *Brassica rapa* general plant growth conditions

B. rapa plants were grown on F2 compost treated with Intercept (both Levington Horticulture, Ipswich, UK) in 7" pots. Plants were watered daily and fertilised with Phostrogen All Purpose Plant Food (Bayer Garden, Bayer AG, Germany), once a week. Plants grown in the green house were maintained under long day conditions as described in section 2.5.

2.14 *B. rapa* plant growth conditions for nitrate studies

For all studies involving nitrate, *B. rapa* plants were grown as described above for Arabidopsis except that 4" diameter pots were used and fed with 250ml/pot for the first application and 100ml/pot thereafter of appropriate liquid ATS solution twice a week. The pots were randomised once a week.

2.15 *B. rapa* decapitation assays

B. rapa plants were grown in greenhouse conditions as in the nitrate experiments one per pot. Plants were decapitated immediately after they bolted (emergence of inflorescence buds among the leaves). At this stage different lines differed in the number of nodes and fully expanded leaves produced. Decapitation involved removal of the shoot tip just below the topmost unexpanded leaf. Lanolin was applied to the cut stump using a toothpick. At the stage chosen for decapitation,

there were no visible buds on any of the lines. Nodes were counted acropetally, with the cotyledonary node designated as n0. All plants selected had at least seven fully expanded leaves and were approximately 20 days old.

2.16 *B. rapa* seed sterilization

B. rapa seeds for sterile culture were sterilized as follows. Up to 50 seeds in a 1.5ml microcentrifuge tube were shaken in 1ml of 10% NaClO (Chlorine bleach) with 0.01% Triton-1000X (Sigma Aldrich Corporation) for 5 minutes. The bleach was removed and replaced by 1ml of 70% ethanol and shaken for 3 minutes. After removing the ethanol, seeds were rinsed with sterile distilled water four times. Seeds were sown onto ATS agar, with or without hormones as needed, in Petri plates or glass jars (Weck, Canning).

2.17 *B. rapa* split plate assay

Split plate assays in *B. rapa* were performed in a similar way to Arabidopsis split plate assays (Chatfield et al., 2000a). Plates for split plate assays were prepared by pouring 50ml ATS agar, either with high (9mM) or low (1.8mM) N into 10cm square Petri dishes. A central strip of ATS agar (1.2cm wide) was removed using a sterile scalpel, thereby creating two agar blocks with a gap in the middle. Synthetic auxin (0.5 μ M NAA) or an equivalent volume of ethanol was supplied via the apical agar block. Synthetic SL (1 μ M GR24) or an equivalent volume of acetone was supplied via the basal agar block. *B. rapa* seedlings were grown in sterile jars (Weck Canning) for three weeks. When taken for split plate assays plants had at least five fully expanded leaves. Single nodal segments were excised and inserted between the two agar blocks. Plates were grown vertically in racks in long day growth rooms (see above for conditions). Bud lengths were measured daily for eight days.

2.18 *B. rapa* hormone response assays

Four week old *B. rapa* plants were selected for hormone response assays. *B. rapa* stem segments bearing a single node (one node assays) or two nodes (two node assays) were excised using a sharp scalpel and inserted into 15ml falcon tubes containing high or low N ATS solutions with or without GR24. A SL treatment of 1 μ M GR24 or an acetone carrier control was used in all bud assays

unless otherwise specified. The stem above the top node was trimmed leaving between 0.5-1cm. Lanolin (Sigma) was applied to the decapitated stump using a sharpened cocktail stick. An auxin treatment of 1 μ M NAA or an ethanol control was applied in the lanolin. No buds were visible to the naked eye at excision. Falcon tubes were placed on racks and kept in a growth room, at standard growth room conditions on a tray lined with wet paper, which was covered with a propagator lid for single node assays, or with a humidity chamber made from transparent plastic bags. Bud lengths were measured daily for 10 days.

2.19 *B. rapa* hypocotyl elongation, root length and light assays

B. rapa seeds were sown onto ATS agar media in Petri plates, and stratified at 4°C for three days. Plates were then incubated vertically on racks in long day growth rooms at a light intensity of $\sim 150\mu\text{mol m}^{-2}\text{s}^{-1}$ provided by white fluorescent tubes. Plates were randomised every day, after measuring hypocotyl and root lengths with a ruler. Red light was provided by using a red filter sheet (Lee filters).

2. 20 *B. rapa* grafting

B. rapa seeds were sown under sterile conditions in large glass (Weck, Canning) jars (1L), stratified for three days at 4°C, and transferred to growth rooms for four weeks. Whole plants with roots embedded in agar were then transferred into sterile Petri dishes (Nunc[®], 140mm x 20mm) and wedge grafting was performed similar to the technique described for *Arabidopsis* (Turnbull et al., 2002). Scion and rootstock were joined in sterile conditions and a tight fit of the graft region was ensured by wrapping it in micropore tape. Grafted plants were carefully transferred back to the jars taking care not to disturb the graft junctions. These plants were maintained in the glass jars in the growth rooms for two more weeks. Before transferring them to soil, their roots were washed to remove any traces of agar. Plants were maintained under high humidity conditions for another two weeks by covering with a plastic bag. Successfully grafted plants were grown to maturity and seeds were collected.

2.21 *B. rapa* TILLING

All steps including primer design for TILLING in *B. rapa* for *max2* were carried out by the TILLING platform at the John Innes Centre, UK available through RevGen (<http://revgenuk.jic.ac.uk/>).

2.22 Statistical analysis

Student's t-test (2 tailed) was performed for comparison of two sample means ($P < 0.05$, Chapters 3, 5). Scatter plots analysis in Chapter 3 was performed on the means of each line on high and low N separately to determine the relationship between traits and to compare different lines. For each line, the results of two independent experiments were included in the scatter plot. Microsoft Excel was used for both these analyses. Statistical analysis of data (Chapter 3) was performed by analysis of variance using SPSS software (SPSS Inc, version 19). Pearson correlation analysis (Chapter 3) was performed on the mean data from each line on high and low N.

Chapter 3

MAGIC lines as a tool to study variation in shoot branching response to Nitrate

3.1 Terminology used in this chapter

High N - 9mM Nitrate

Low N - 1.8mM Nitrate

Total branch number - Number of rosette branches plus cauline branches > 1cm.

Plasticity – Difference in branch number between two different environmental conditions, typically the number of branches on high N minus the number of branches on low N.

Bolting –Transition from vegetative to reproductive growth, scored as emergence of the inflorescence apical bud among the rosette leaves

Flowering time – Number of days from germination to bolting

Seed yield – Total seed dry weight (g) per plant at completion of life cycle

Single seed weight – Dry weight of single seed (mg)

Total plant biomass- Weight of whole plant dry matter (g) including stem, leaves, seeds and roots

Above ground dry matter biomass – Weight of shoot dry matter (g) above ground including stem, leaves and seeds

Residual shoot dry matter (RSDM) biomass – Weight of shoot dry matter (g) above ground including stem, leaves and silique valves after separation of seeds

Root proportion – Proportion of the total plant biomass made up of roots

Harvest index (HI) – Proportion of the above ground dry matter made up of seed

Seed viability – Germination percentage (%) on water

Shoot Nitrate- Nitrate content expressed as mmol NO_3/g of residual shoot dry matter

Seed Nitrate – Nitrate content expressed as mmol NO_3/g of seeds

3.2 Introduction

3.2.1 MAGIC lines

As mentioned in the introduction, Kover et al created a set of 700 *Arabidopsis thaliana* MAGIC lines (Kover et al., 2009). They were produced by randomly inter-mating 19 natural accessions for four generations and then inbreeding for 6 generations. The genome of each of these MAGIC lines is a mosaic of the 19 parental accessions.

These lines are nearly homozygous and form a stable panel of recombinant inbred lines (RILs) that do not require repeated genotyping in each QTL study. Replicates of each line can be grown in different environments, data for many phenotypic traits can be accumulated, and studies of trait correlations, genotype by environmental interactions and phenotypic plasticity are feasible. The set of 400 of these MAGIC lines currently used in Leyser lab thus provides an excellent resource, with 400 different homozygous lines, each of which has a different combination of alleles of the shoot branching regulatory network and can be used to measure any trait of interest in diverse environments.

3.3 Background studies and aims

A N response study using 400 MAGIC lines was carried out in the summer of 2008 (S. Ward, L. Williamson unpublished). This study revealed interesting correlations between branching and the response of branching to N limitation. Lines with a high number of branches on high N responded strongly to N limitation, whereas lines with a medium number of branches were relatively unaffected (figure 3.1 a, b).

Across the whole dataset, there were no strong correlations between branch numbers and flowering time (FT) (figure 3.1 c, d). However, among the earlier flowering lines, some correlations were evident (figure 3.1 c, d). For example, plotting just those lines that flower before 30 days showed that branch number on both high N and low N correlated with plasticity, but in opposite directions, positively and negatively respectively (figure 3.2 a, b). In addition, on low N, there

was a strong negative correlation between total branch number and FT (figure 3.2 c, d).

Evidence of extensive genetic variation in branching and plasticity among the set of 400 MAGIC lines and the observation of interesting trait correlations provide a platform to understand plasticity in shoot branching, test different hypothesis with a limited number of selected lines, and to generate new hypothesis based on the results.

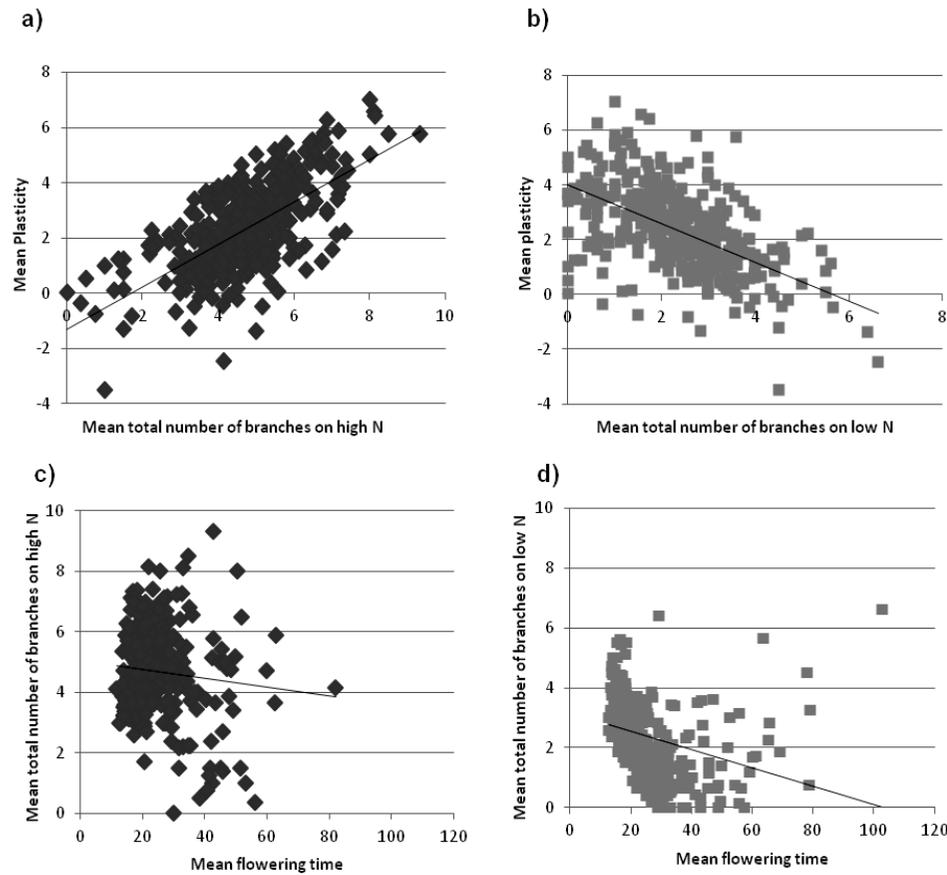


Figure 3.1 Scatter plots showing relationship between a) Branch numbers on high N and branching plasticity, b) Branch numbers on low N and branching plasticity, c) Branch number on high N and flowering time and d) Branch number on low N and flowering time among the 400 MAGIC lines. The solid lines in the scatter plot are the linear regression lines.

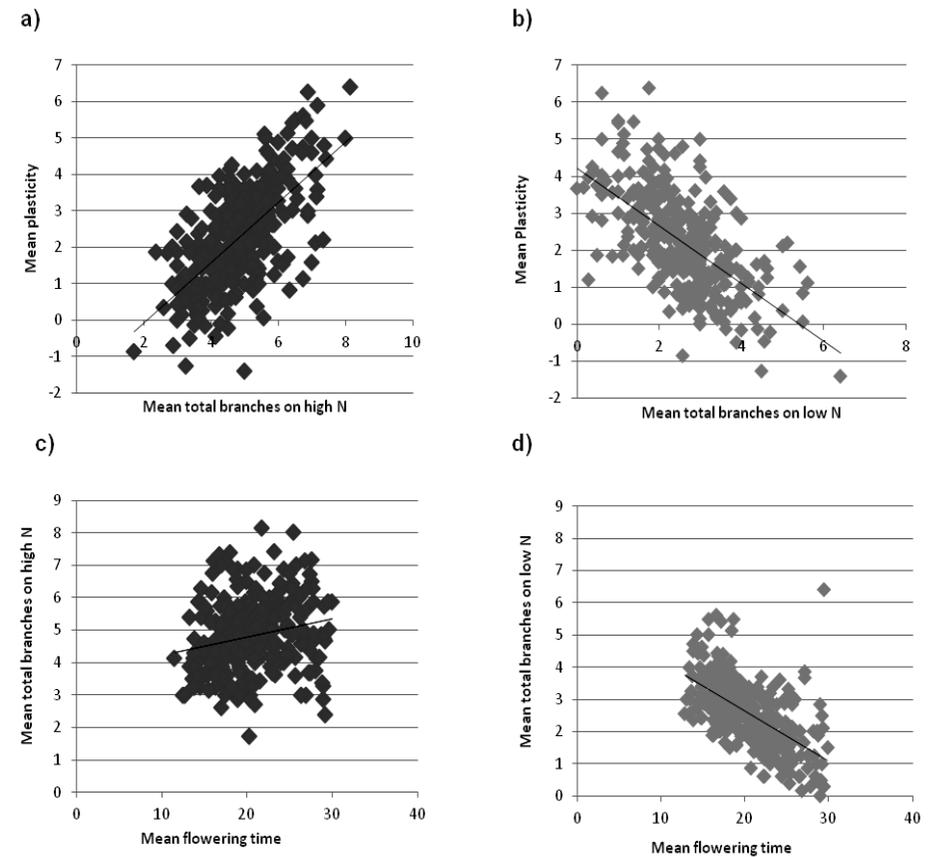


Figure 3.2 Scatter plots showing relationship between a) Branch numbers on high N and branching plasticity, b) Branch numbers on low N and branching plasticity, c) Branch number on high N and flowering time and d) Branch number on low N and flowering time among the MAGIC lines with flowering time below 30 days. The solid lines in the scatter plot are the linear regression lines.

This chapter presents a detailed investigation of branching, branching plasticity and fitness related traits of selected MAGIC lines when subjected to variation in an environmental input – Nitrate availability. Knowledge about correlations obtained from baseline experiment was used to inform the selection of a small set of MAGIC lines that would allow these correlations to be explored in more detail, as well as to investigate links between different related traits.

In particular, some of the questions addressed in this chapter are:

What is the range of branching and branching plasticity in the selected MAGIC lines?

How reproducible is branching plasticity in these lines?

Are there any correlations between branching traits? Are these correlations similar on high and low N?

Are there any correlations between branching traits and fitness and flowering time traits? Are these correlations similar on high and low N?

Are the fitness related traits correlated among themselves?

From a breeder's point of view, which branching behaviour is desirable for maximum or reliable yield or maximum biomass on high and low N?

3.4 Characterisation of the basic effects of Nitrate limitation on branching in Arabidopsis accession 'Columbia'

A system to grow Arabidopsis plants by feeding with controlled amounts of Nitrate had been established using the Columbia accession in the Leyser lab (Chapter 2, page 57). This was initially developed during studies aimed at assessing whether various mutants had an altered response to N (Willett, 2005).

Dose response studies have been performed using different levels of Nitrate (9mM, 7.75mM, 4.5mM, 2.25mM and 1.8mM) and previous work have shown that 9mM Nitrate was N sufficient whereas a Nitrate concentration of 1.8mM was limiting and could cause branching inhibition (Willett, 2005). Figure 3.3 shows typical 5-week old Arabidopsis plants grown in these conditions.

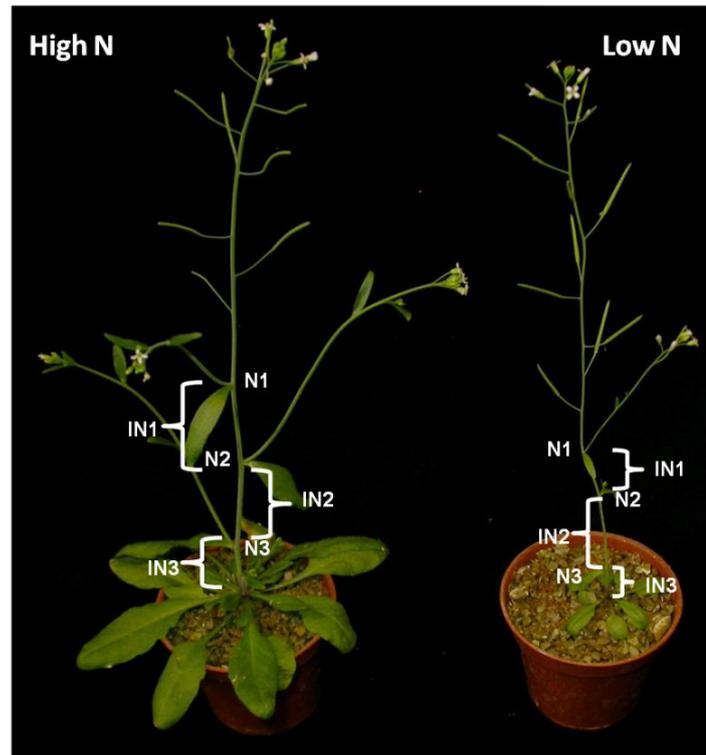


Figure 3.3 Arabidopsis accession Columbia plants (5 weeks old) grown in a greenhouse on 50% sand and 50% Terragreen mixture under high (9mM) and low (1.8mM) Nitrate supply. Cauline nodes (N) and internodes (IN) on the primary inflorescence of each plant were numbered successively in an apical to basal direction with N1 as the uppermost node and IN1 as the uppermost vegetative internode.

Previous studies have shown that, in long day conditions, bud activation in Arabidopsis normally follows a basipetal sequence after floral transition (Stirnberg et al., 1999, Hempel and Feldman, 1994). It has also been reported that this basipetal direction of bud activation was unaffected by N limitation, although the sequence terminates early on low N (O. Leyser, personal communication). However, a detailed quantitative analysis of the effects of N limitation on the sequence of bud activation has not been carried out.

In the current study, experiments were performed with Columbia to obtain quantitative data about the effect of N availability on branch lengths. Plants were grown on high (9mM) and low N (1.8mM) as described above and measurements of height, number of branches, branch lengths and internode lengths were made when plants were 5 weeks old. In these experiments, as previously observed, plants responded to N limitation by reduction in plant height, reduction in number of branches and overall reduction in plant growth (figures 3.3, 3.4 a, b). Measurement of the lengths of the three most apical branches on the primary inflorescence on high and low N showed that there was considerable reduction in branch lengths on low N for all the three branches (figure 3.4 c). The mean total length of successive branches from the uppermost node N1 downwards, showed a 2 fold (N1), 4 fold (N2) and 4.5 fold (N3) reduction respectively on low N (figure 3.4 c).

All three internodes were slightly shorter on low N compared with high N (figure 3.5 d). All three branches were longer on high N. Furthermore, while on high N branch lengths increased basipetally, on low N the reverse was true.

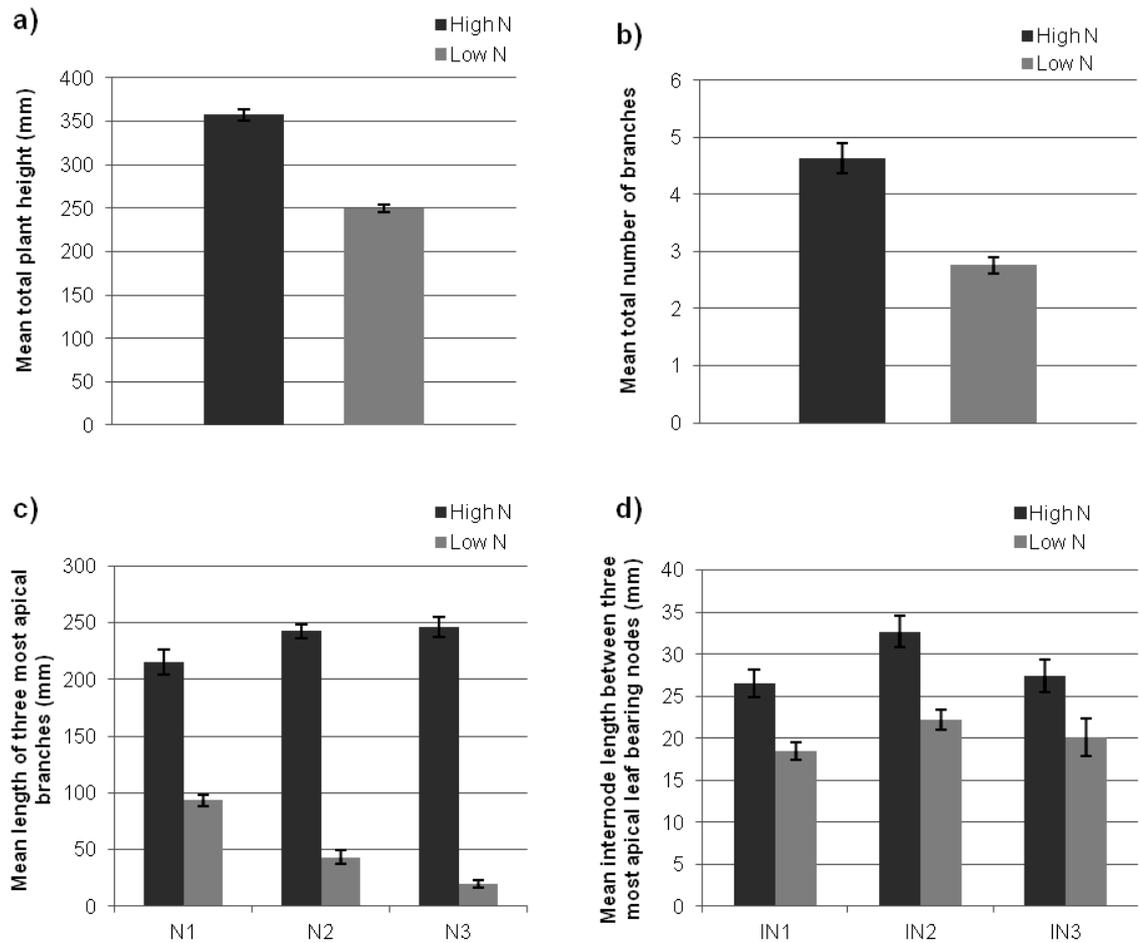


Figure 3.4 a) Mean total plant height (mm), b) Mean total number of branches, c) Mean lengths (mm) of three most apical branches (N1 - uppermost node), d) Mean lengths (mm) of the three most apical leaf bearing internodes (IN1- uppermost internode) of 5-week-old Columbia plants grown in a green house on 50% sand and 50% Terragreen mixture under high (9mM) and low (1.8mM) Nitrate supply. Measurements were made when flowering of the primary shoot was complete. Values are means \pm SE of 20 plants.

To obtain quantitative data on bud length progression over time, measurements were made at anthesis (opening of first flower), 2 days, 6 days and 11 days post anthesis (DPA). The elongation of buds was very rapid on high N, with buds apparently activating simultaneously, and lengths reaching up to 150 mm or more by 11 DPA (figure 3.5 a). By 11 DPA, bud lengths followed an acropetal gradient on high N with the most basal branch being the longest. On low N, all three buds appear to begin elongating near – simultaneously, as on high N (compare 0 days with 2 days). However, outgrowth then proceeds in a basipetal sequence, with the top most branch being longer than the middle one, while there was little further elongation of the bottom bud (figure 3.5 b).

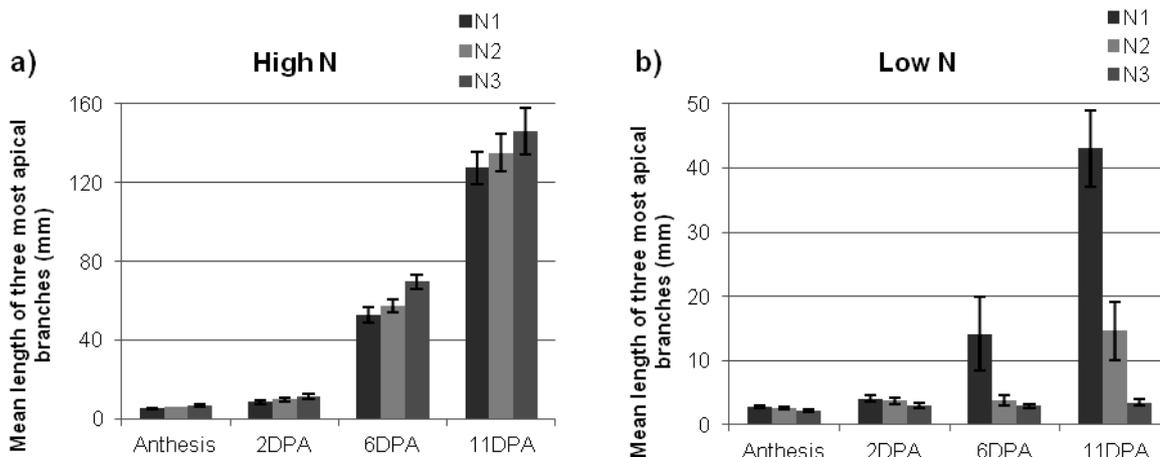


Figure 3.5 Mean length of buds from the three most apical leaf bearing nodes of Columbia on high and low N at 2, 6 and 11 days post anthesis (DPA) where N1 is the uppermost node. Values are means \pm SE of 15-20 plants.

3.5 Characterisation of MAGIC lines – An Overview

Experiments performed on the reference line, Columbia on high and low N showed that N limitation led to a reduction in plant height, branch numbers, branch lengths and internode lengths. The results of the baseline branching experiment with the set of 400 lines (L. Williamson, S. Ward, personal communication) showed that majority of lines behaved qualitatively similar, but quantitatively different from Columbia.

For a pilot study, fifteen MAGIC lines, with a combination of traits representative of the diversity in branch numbers and branching response to N were selected from among the set of 400 lines. Detailed analysis of these 15 lines gave a better understanding of the extent of, and relationship between variation in branch numbers, branching plasticity and flowering time (days from germination to bolting) in response to N limitation. These results directed the choice of a subset of lines for more detailed and labour-intensive studies of fitness traits.

Based on the pilot study using 15 lines, four lines were chosen for further characterisation. The MAX pathway have been suggested to be involved in mediating branching responses to N limitation (Willett, 2005) and hence the strigolactone deficient branchy mutant *max4* was included in my studies to compare the responses with its wild-type line, Col (which is also among the 19 parental accessions of the MAGIC lines). In later experiments two more MAGIC lines, 281 (earlier flowering) and 75 (later flowering) were added to allow better assessment of the correlations with flowering time.

It was suggested by my preliminary studies, and confirmed through repetition during my subsequent analyses, that there was seasonal variation in the traits measured and therefore it was decided to present the data sets with both branching and fitness related measurements from two seasons. Table 3.1 shows the different traits studied and the season in which the experiments were performed.

Data from winter and autumn were comparable and so results in this part of Chapter 3 are presented as a comparison of data collected over two seasons – summer 2009 (includes MAGIC lines 11, 552, 25, 471 and Columbia) and autumn 2010 (includes MAGIC lines 11, 552, 25, 471, 75, 281, Columbia and *max4*). A table summarising the branch numbers on high and low N, plasticity and flowering times of the lines studied, across all the experiments performed, are included for reference (table 3.2).

Results from the initial experiments gave suggestions of correlations between some of the traits and about the need to test them more systematically. Analysis of variance was therefore carried out for these traits to assess the relationships between them. These results are presented towards the end of this Chapter.

	Winter 2009	Summer 2009	Winter 2010	Autumn 2010	Winter 2011	Summer 2011
Lines included	25,45,73,110,163,184,219,223,459,471,552,574,631,665,732	11,552,Col,25,471, axr-1/max-4	11,552,Col,25,471, axr-1/max-4	11,281,552,Col, max4,25, 75, 471	11,281,552,Col, max4,25, 75, 471	11,281,552,Col, max4,25, 75, 471
Branching						
Plasticity						
Flowering time						
Decapitation						
Fitness related traits						
Nitrate measurements						

Table 3.1 Summary of experiments carried out, season in which they were carried out and lines included in each experiment. Bars represent the measurements included.

Background exp-summer 2008 (Williamson et al unpublished)									First experiment- winter 2009									summer 2009								
	11	25	471	552	Col	max4	75	281		11	25	471	552	Co l	max4	75	281		11	25	471	552	Col	max4	75	281
Branches on High N	6.8	4.4	8.1	4.5			7.2	7.4	Branches on High N		4.7	8.3	6.5					Branches on High N	5.7	4.7	8.3	4.9	4.5			
Branches on Low N	5.6	0.9	1.8	4.7			3.6	2.6	Branches on Low N		0.4	2.6	3.3					Branches on Low N	4.1	1.3	1.9	4.6	2.5			
Plasticity (Branches lost on low N)	1.1	3.6	6.4	-0.2			3.6	4.8	Plasticity (Branches lost on low N)		4.3	5.8	3.2					Plasticity (Branches lost on low N)	1.6	3.4	6.5	0.3	1.9			
Flowering time (High N)	16	21	22	16			17	18	Flowering time (High N)		35.3	36.2	24.9					Flowering time (High N)	16.3	23.9	25.9	17.3	18.5			

winter 2010									autumn 2010									winter 2011								
	11	25	471	552	Col	max4	75	281		11	25	471	552	Co l	max4	75	281		11	25	471	552	Col	max4	75	281
Branches on High N	5.2	6.3	7.9	5.7	4.9	10.1			Branches on High N	4.9	4.7	7.8	5.1	4.6	8.3	4.6	3.7	Branches on High N	4.6	4.7	9.0	4.7	4.9	9.6	6.0	5.0
Branches on Low N	5.3	0.9	0.6	3.0	2.0	6.9			Branches on Low N	3.9	1.8	0.7	3.6	2.5	6.2	2.3	2.6	Branches on Low N	3.4	1.6	2.3	2.3	2.8	5.4	3.0	2.8
Plasticity (Branches lost on low N)	-0.1	5.5	7.3	2.7	2.9	3.17			Plasticity (Branches lost on low N)	1.0	2.4	7.1	1.5	2.1	2.1	2.3	1.1	Plasticity (Branches lost on low N)	1.2	3.0	6.8	2.4	2.1	4.2	3.0	2.2
Flowering time (High N)	17	36	34	24	25	25			Flowering time (High N)	19.3	35.6	36.1	24.8	26	25	32	22.6	Flowering time (High N)	19.9	35.5	35.5	25.0	25.8	24.2	31.6	22.6

Table 3.2 Branching, plasticity and flowering times of the lines studied across the experiments.

3.6 Effect of N limitation on 15 MAGIC lines

Examples of high branching, high plasticity lines (471, 223); and medium branching, low plasticity lines (45,110, 631, 665) were included among the total of fifteen lines. Very late or very early flowering lines were excluded from this pilot study, which was carried out in winter 2009.

In contrast to the Columbia accession described above, not all the MAGIC lines responded to N deprivation with a reduction in plant height (figures 3.6 a). Among the selected set of fifteen, there were several lines (45,110, 552, 631, 665) that maintained similar plant heights on both high and low N. On the other hand, the set also included lines, such as 163, 219 and 459, which exhibited a more extreme reduction in plant height than the Columbia accession when N was limited. Despite the exclusion of extremely early or late-flowering lines from this study there was still (about 18 days) variation in flowering time between the earliest and latest flowering lines (figure 3.6 b). There was no significant effect of N on flowering time (figure 3.6 b, ANOVA- $P > 0.05$, table 3.3).

Figure 3.8 shows the extent of variation in branch number in selected MAGIC lines in response to N limitation. Branch number varied significantly between the MAGIC lines and between nutrient treatments (figure 3.7 a, ANOVA- $P < 0.0001$, table 3.3). Given sufficient N, most of the lines produced more branches than when N was limited. Low N limited bud activation to a few apical nodes in most lines (figure 3.9).

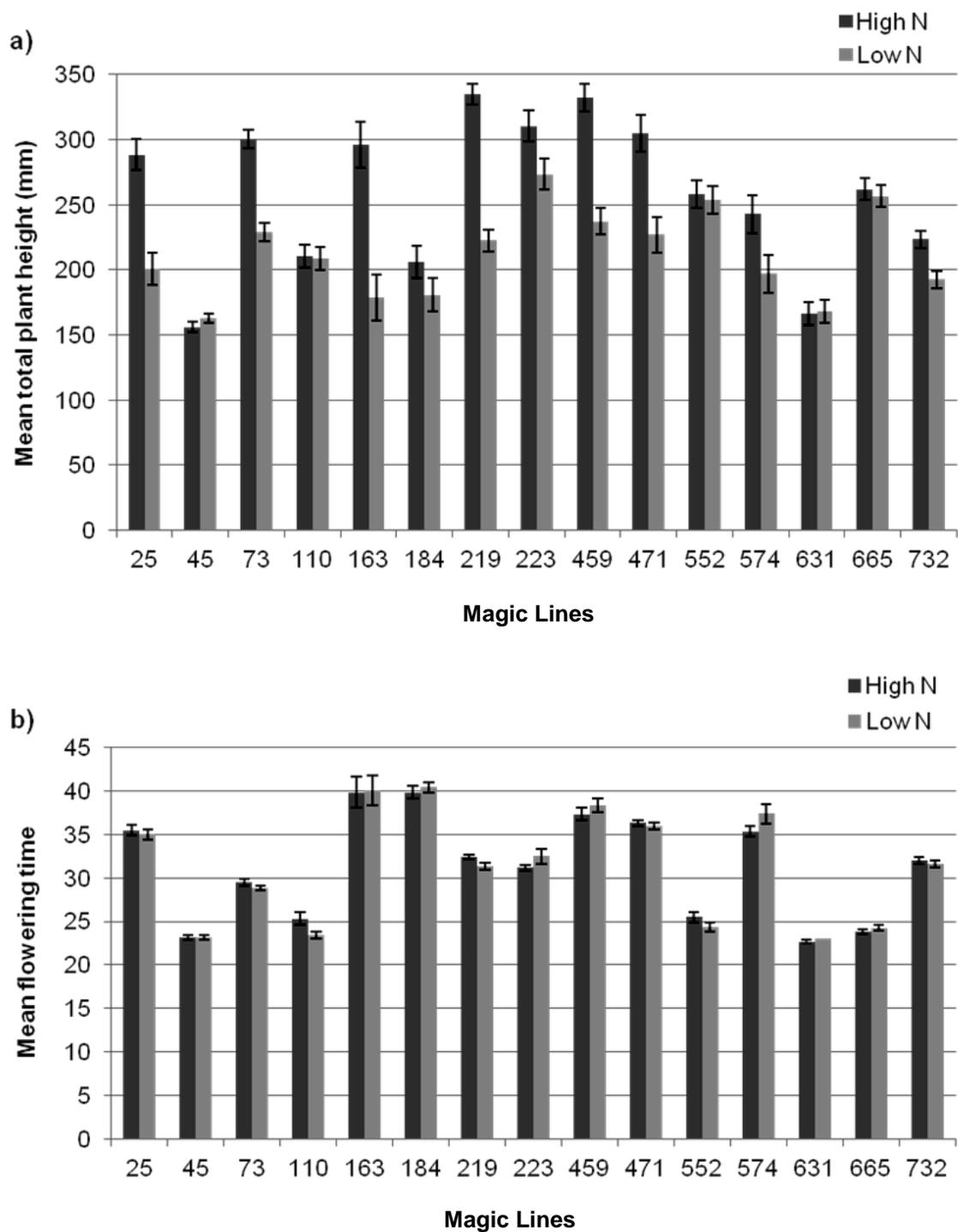


Figure 3.6 a) Mean total plant height (mm) and b) Mean flowering time (Flowering time was calculated as days from germination to bolting) of different MAGIC lines. Values are means \pm SE of 12 plants.

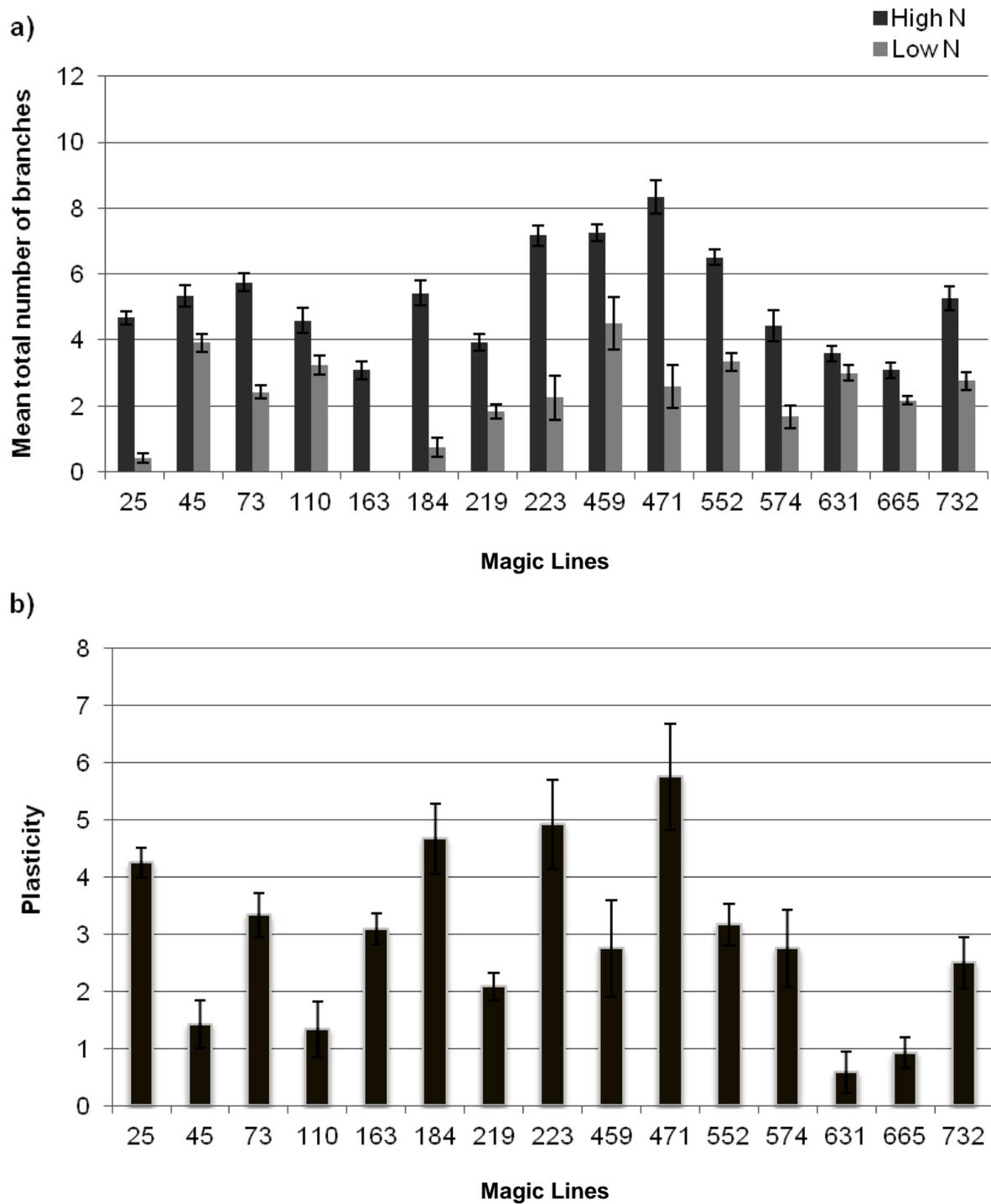


Figure 3.7 a) Mean total number of branches of selected MAGIC lines. Branches were counted at the two filled silique stage, b) Branching plasticity of different MAGIC lines. Values are means \pm SE of 12 plants.

Plasticity in shoot branching is defined in this chapter as the difference between the number of branches on high N and that on low N. Plasticity in branch number varied among the MAGIC lines from 0.6 ± 0.38 in line 631 to 5.8 ± 0.93 in the line 471 (figure 3.7 b). The range in plasticity among the fifteen lines showed that each of these lines had a distinct response to the same factor-Nitrate availability.

When the branching plasticity results of this pilot experiment with 15 MAGIC lines were compared to the branching plasticity of these lines in the previous baseline experiment (carried out in summer 2008) some variability was observed for some lines (table 3.2). For example, line 552 which had a low plasticity in the summer 2008 experiment (mean plasticity of -0.2), had high plasticity (mean plasticity of 3.2) in the pilot experiment (figure 3.7 a, b). This could indicate that branching plasticity in response to N also depends on environmental factors other than N alone, and it was interesting that this occurred only for certain genotypes.

3.7 Detailed N response studies with selected MAGIC lines

A limited number of lines covering a spectrum of trait combinations with regard to branching, branching plasticity and flowering time were selected for a detailed characterisation. These included three MAGIC lines 25, 471 and 552 from among the 15 lines included in the pilot study and another MAGIC line 11, from the original set of 400 lines (figure 3.8 shows phenotype of selected lines). Among these lines, 25 and 471 were reliable in that the branch number and branching plasticity were similar in the baseline experiment (summer 2008) and in the pilot study (Winter 2009) and both these lines flowered later than Columbia in both studies. Line 552 flowered earlier than Columbia and this line was chosen because 552 had very low branching plasticity in the baseline experiment (summer 2008), but exhibited higher branching plasticity, when grown in winter (winter 2009). Because line 552 was not reliable, another low plasticity line 11, which flowered even earlier than 552, was also included. Two more lines, 281 (chosen for flowering early and having low plasticity) and 75

(later flowering and having high plasticity) were included with other MAGIC lines for detailed characterisation.

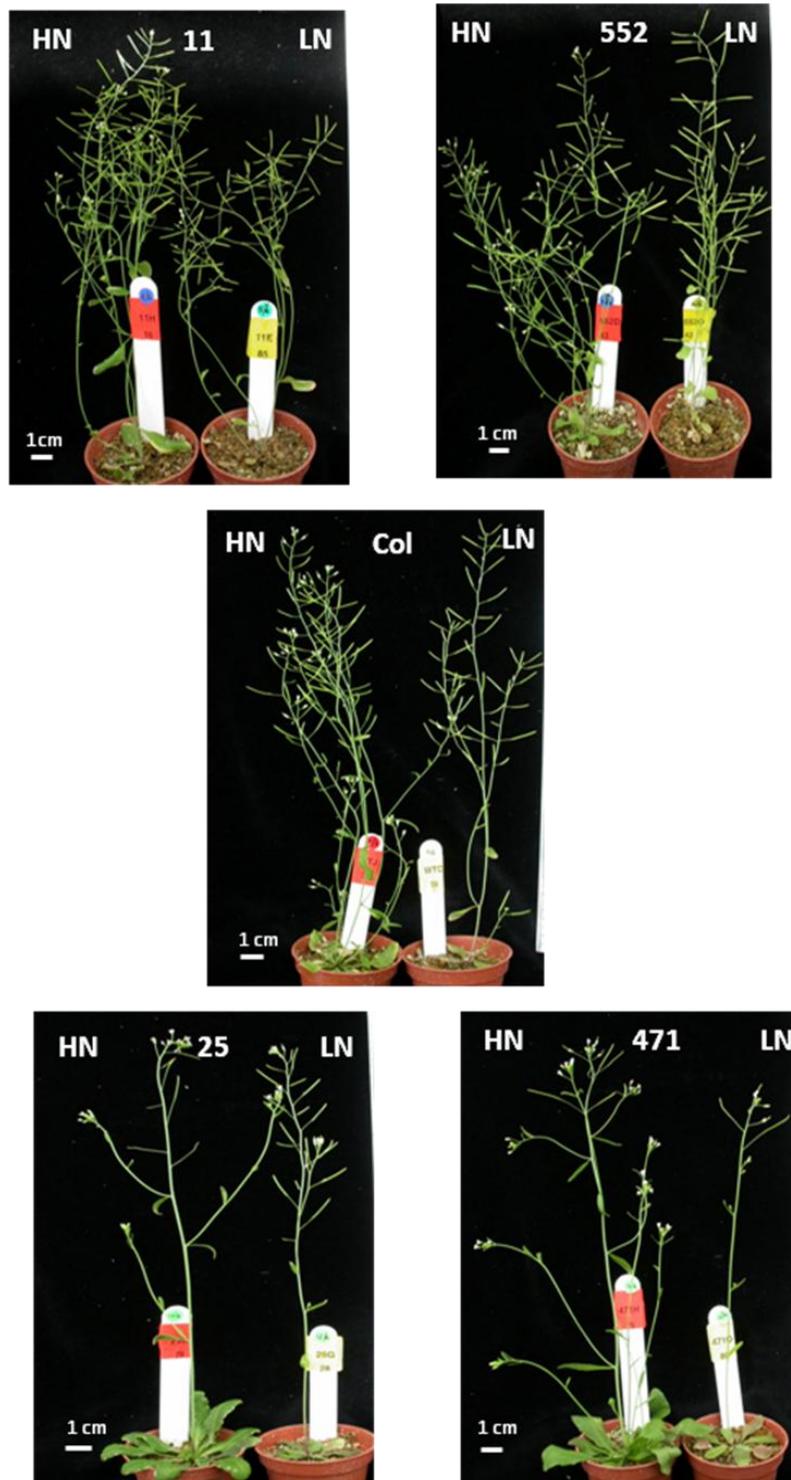


Figure 3.8 Phenotype of selected lines grown in greenhouse on 50% sand and 50% Terragreen mixture under high (9mM) and low (1.8mM) Nitrate supply at 5 weeks.

3.7.1 Flowering time

As described above, among the MAGIC lines studied, some flowered earlier than the reference line Columbia and some later. For ease of presenting data, the lines are grouped into a relatively earlier flowering group (green box) and a later flowering group (red box) in the figures here after. However, it should be noted that the total variation in flowering time between later and earlier flowering groups is only around an average of 7 (in summer) to 10 days (in autumn). All the lines in my study can be classified as ‘rapid cycling annuals’ or ‘fast cyclers’ which do not require vernalisation before flowering, unlike ‘winter annuals’ or ‘late flowerers’ which are late flowering natural accessions (Napp-Zinn, 1985). None of the lines studied showed any significant differences in flowering time between N sufficient and limited situations when grown in summer (figure 3.9 a). However four out of the six MAGIC lines - 11, 25, 75, 471, as well as Columbia and *max4*, flowered slightly earlier on low N when grown in autumn (figure 3.9 b).

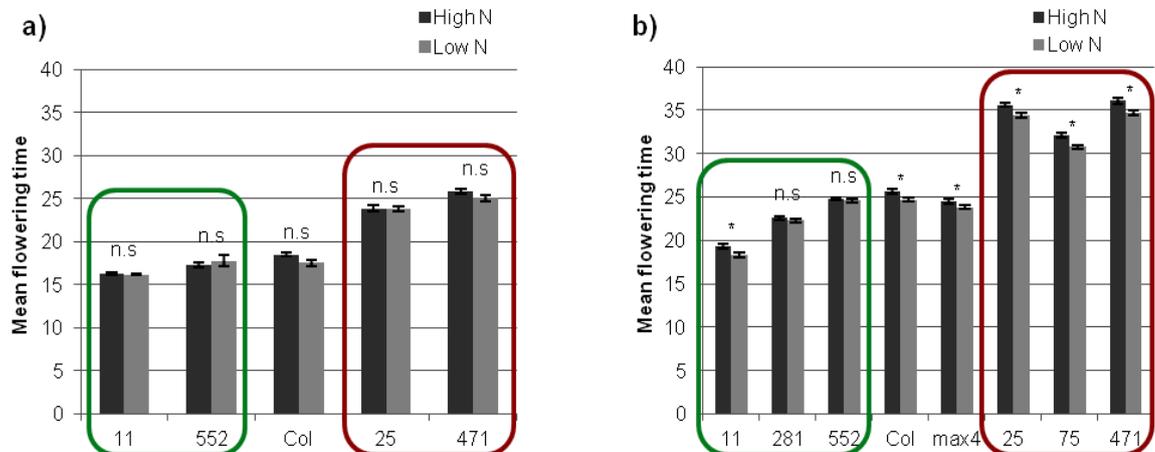


Figure 3.9 Mean flowering time (days) of different lines grown in a) Summer b) Autumn. Flowering time was calculated as days from germination to bolting. Values are means \pm SE of 8-15 plants. Asterisks indicate significant differences between high and low N treatments (Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; *, $P<0.0001$).**

3.7.2 Branching related traits

3.7.2.1 Effect of N limitation on branching

The results in figure 3.10 show that in summer, three out of four MAGIC lines (11, 25 and 471) and Columbia produced a significantly greater number of branches on high N compared to low N, whereas line 552 produced the same number of branches on both high and low N. In autumn, (figure 3.10 b) all the lines including 552 produced a significantly greater number of branches on high N.

A striking feature observed on low N was that lines 11 and 552 made many more branches than lines 25 and 471 in both experiments. It was interesting to note that the lines in the early flowering group (green box) tend to make more branches on low N than the lines in the late flowering group (red box).

The response of Columbia was similar in summer and autumn, in that it produced a similar number of branches on high N (summer = 4.2 ± 0.24 , autumn = 4.5 ± 0.18) and low N (summer = 2.6 ± 0.21 , autumn = 2.5 ± 0.18). Based on the hypothesis that the MAX pathway is involved in the branching response to low N (Willett, 2005), it can be expected that *max* mutants will be less sensitive to N limitation. Results of branching of the *max4* mutant on high and low N demonstrated that they were indeed less sensitive. *max4* mutant responded to N deprivation by reducing its branch number but still retained a large number of branches on low N, with the highest number of branches on low N among all the lines studied (figure 3.10 b).

3.7.2.2 Branching plasticity

Plasticity varied substantially among the lines (figure 3.11). Mean plasticity of the MAGIC lines showed that line 11 maintained consistently low plasticity, and lines 25 and 471 maintained consistently high plasticity, irrespective of the season (figure 3.11 a, b). Line 552, which had low plasticity in summer, became more plastic in autumn. From here on, lines 25, 75 and 471 are referred to as high plasticity lines and lines 11, 281 and 552 are referred to as low plasticity lines.

Columbia maintained similar levels of plasticity in both seasons (figure 3.11 a, b). Plasticity of the *max4* mutant was similar to that of Columbia (Plasticity of *max4* = 2 ± 0.54 and Columbia = 2 ± 0.27) although it should be noted that unlike Columbia, *max4* made many branches on both high and low N (figure 3.11 b).

The graphs of total branch numbers and plasticity together confirmed the observation that lines with low plasticity produced more branches on low N than those lines with high plasticity (figures 3.10 a, b, 3.11 a, b). Also it seems that among the lines studied, the early flowering group was generally less plastic, while the late flowering group was more plastic.

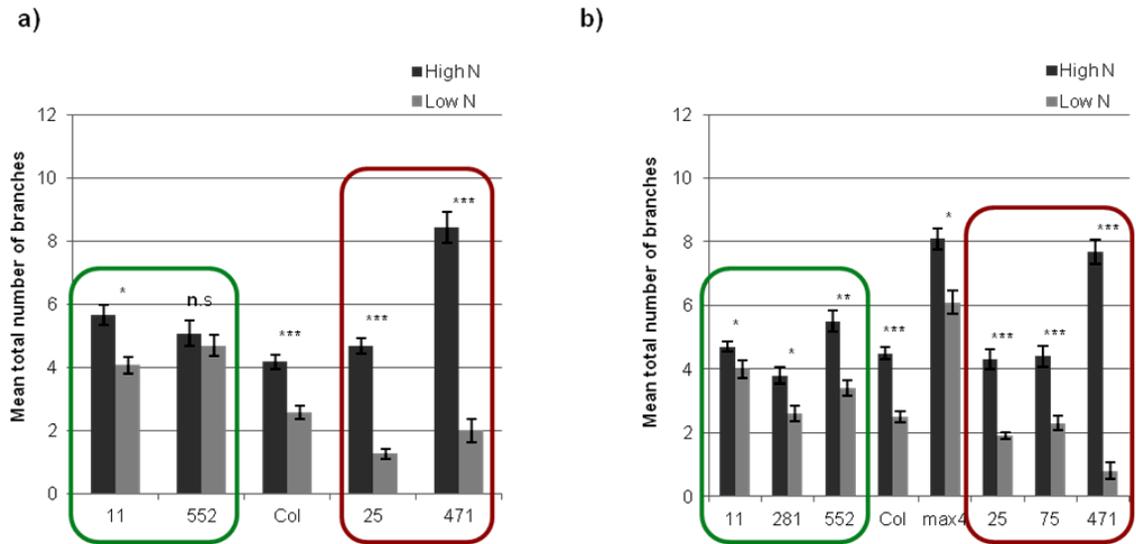


Figure 3.10. Mean total number of branches of different lines grown in a) Summer b) Autumn. Branches were counted at the two filled silique stage. Values are means \pm SE of 8-15 plants. Asterisks indicate significant differences between high and low N treatments (Student's t-test-n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

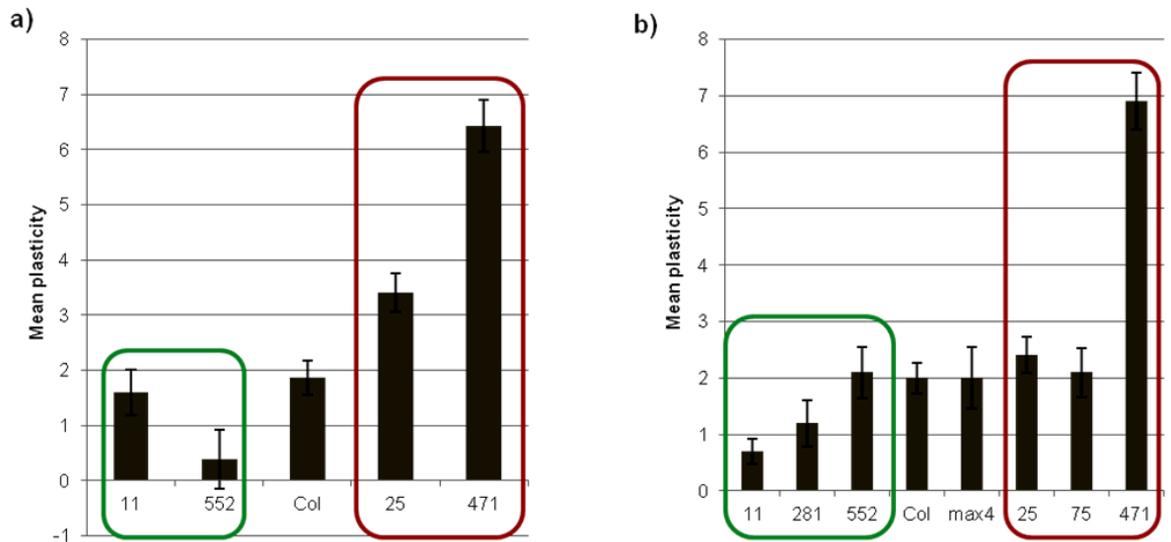


Figure 3.11 Mean plasticity of different lines grown in a) Summer and b) Autumn. Values are means \pm SE of 8-15 plants.

3.7.2.3 Decapitation response to N limitation

The response of branching to decapitation under N sufficiency and limitation of the selected MAGIC lines was studied to explore its relationship to plasticity in response to N supply. This experiment was conducted twice in winter (2010, 2011). The results were very similar in both experiments and the results of winter 2011 are presented.

The total number of branches produced by the lines with or without decapitation on high and low N can be seen in figures 3.12 a and b. All lines studied responded to decapitation by activating buds that would not otherwise have been activated; on both high and low N (figures 3.12 a, b). The low plasticity lines generally made fewer branches when decapitated than when they were intact (figure 3.12 a). The only exception was line 552 which made a similar number of branches whether decapitated or intact. The high plasticity lines on high N, also made fewer branches than intact plants, with the exception of line 25 where branch numbers were similar (figure 3.12 a). However, on low N (figure 3.12 b), the high plasticity lines (except line 75) made more branches than their intact controls, a phenomenon referred to as overcompensation (Belsky, 1986). This demonstrates that the low branch number of the high plasticity lines under N limitation can be substantially overcome by decapitation.

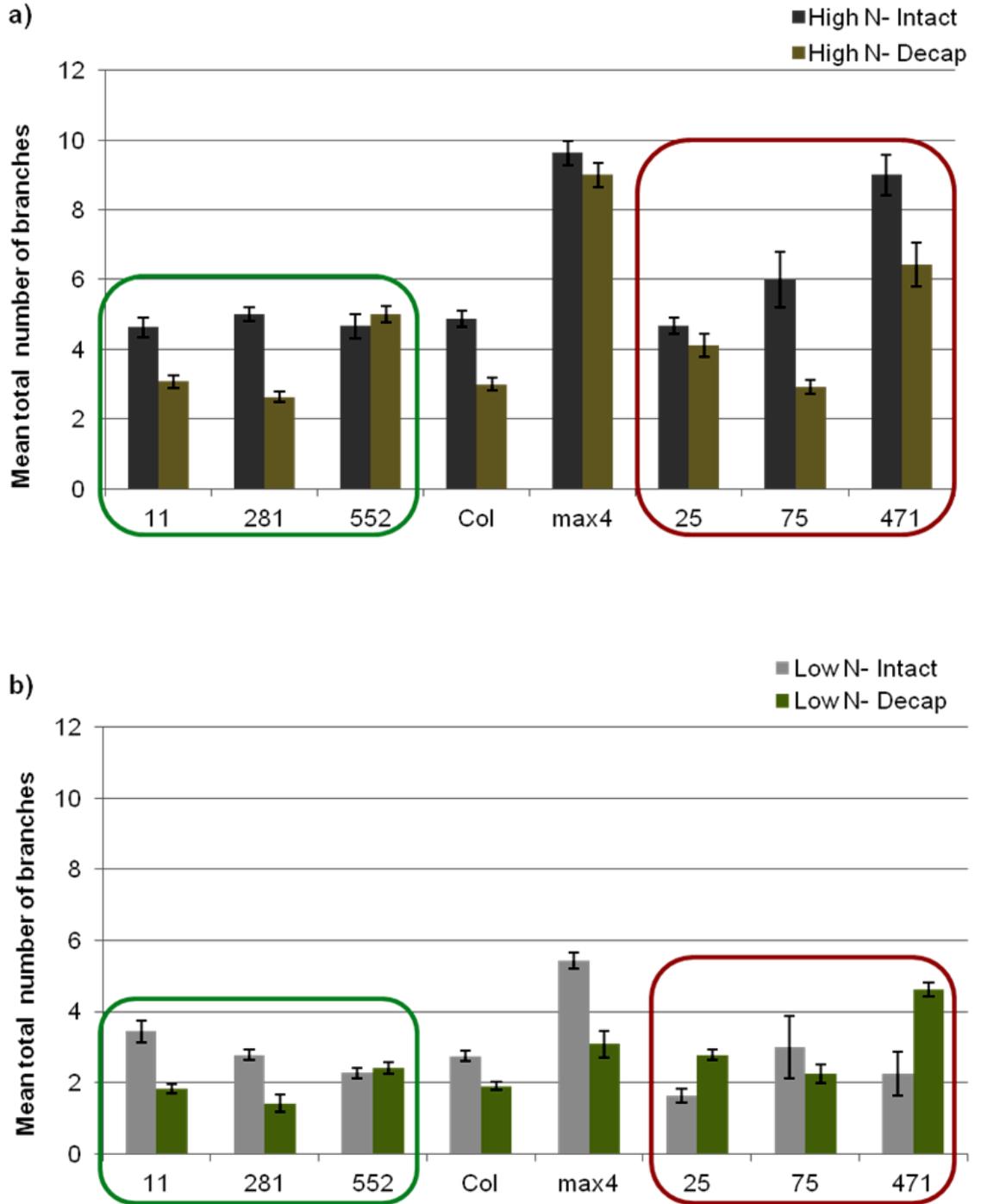


Figure 3.12 Response of different lines to decapitation on high (9mM) or low (1.8mM) Nitrate a) High N b) Low N. Branches were counted 10 days after decapitation. Total branches (rosette + cauline) of intact plants grown under the same condition were compared to rosette branches of decapitated plants. Values are means \pm SE of n=12.

3.7.3 Fitness related traits

3.7.3.1 Seed yield and yield retention on low N

It was not surprising that seed yield increased several fold on high N for all the lines and in both seasons (figure 3.13 a, b). However, the extent to which yield was higher was different among lines. The fold increase was higher for lines with high branching plasticity in both seasons (figure 3.13 a, b). An interesting result, which may be useful from a plant breeding point of view, was that the lines that were high yielding when there was sufficient N were not the ones with high yield when there was N limitation. Another interesting, though intuitive feature was that the lines with higher plasticity (25, 75 and 471) retained the least yield on low N, in both the seasons (figure 3.14 a, b). Line 11 consistently retained more seed yield and line 471 retained consistently less seed yield on low N in both seasons.

Although *max4* had far more branches on high and low N when compared to Col, the seed yield of *max4* on high and low N was comparable to that of Columbia. This demonstrated that more branches do not necessarily result in more seed. Yield retention on low N of Col and *max4* were also comparable (figure 3.14 b).

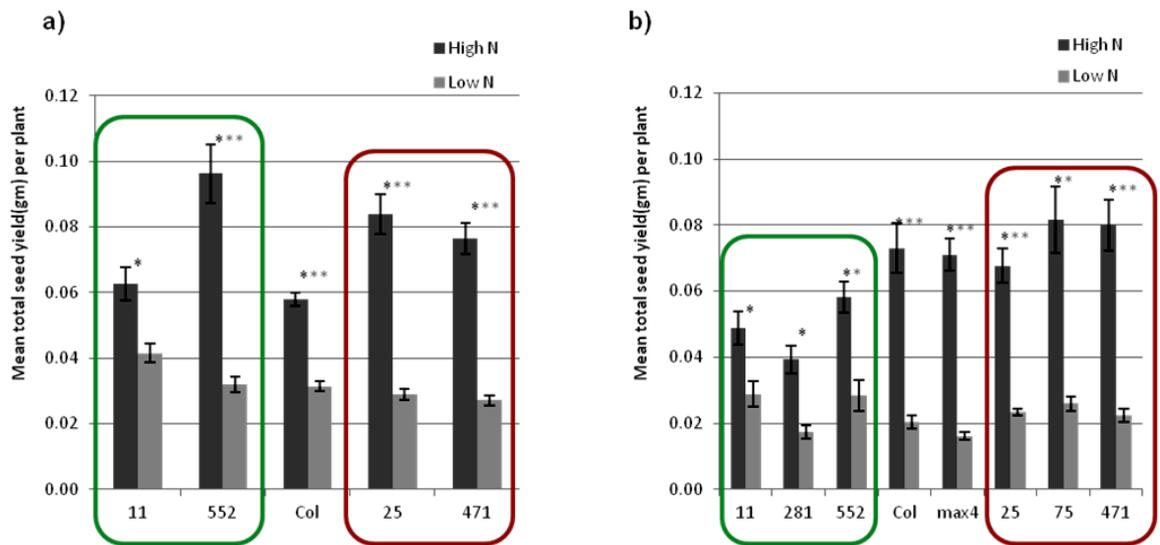


Figure 3.13 Mean seed yield of different lines grown in a) Summer b) Autumn. Values are means of \pm SE of 8-15 plants. Asterisks indicate significant differences between high and low N treatments (Student's t-test-n.s., not significant ($P>0.05$), * $P<0.05$, ** $P<0.01$, * $P<0.001$).**

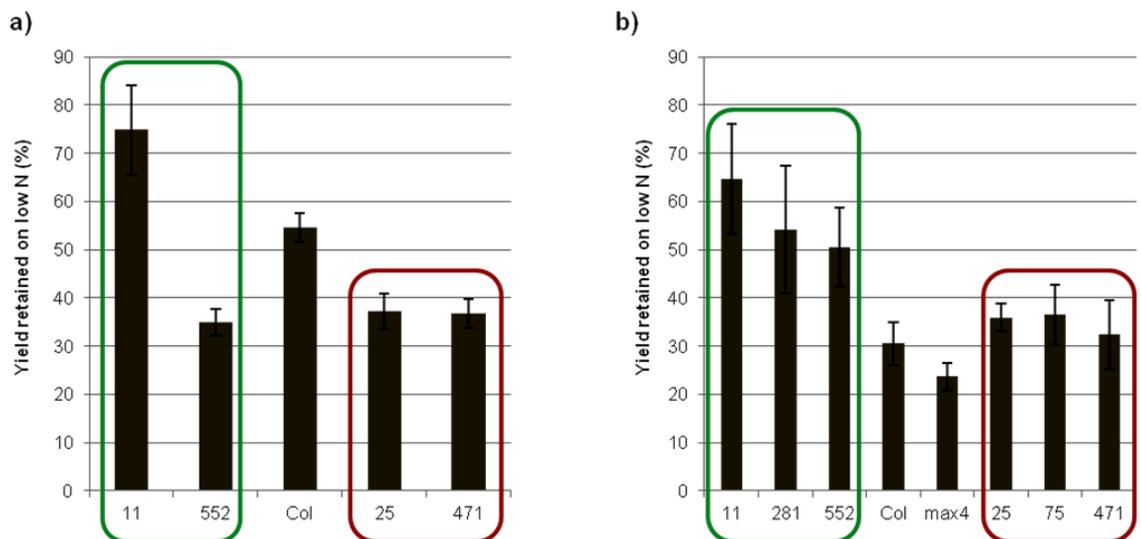


Figure 3.14 Yield retained on low N (%) for different lines grown in a) Summer b) Autumn. Values are means of \pm SE of 8-15 plants.

3.7.3.2 Seed Viability

Seed viability results showed variability among seasons and between lines, making them difficult to interpret (figure 3.15 a, b). The only line that showed consistently high and similar germination percentage on both high and low N and both seasons was the MAGIC line 11. In autumn, germination percentage was significantly higher on high N than low N for 6 out of 8 lines, which suggested that high Nitrate could perhaps have a positive effect on germination potential of seeds (figure 3.15 b). However, this was not observed in the summer experiment (figure 3.15 a).

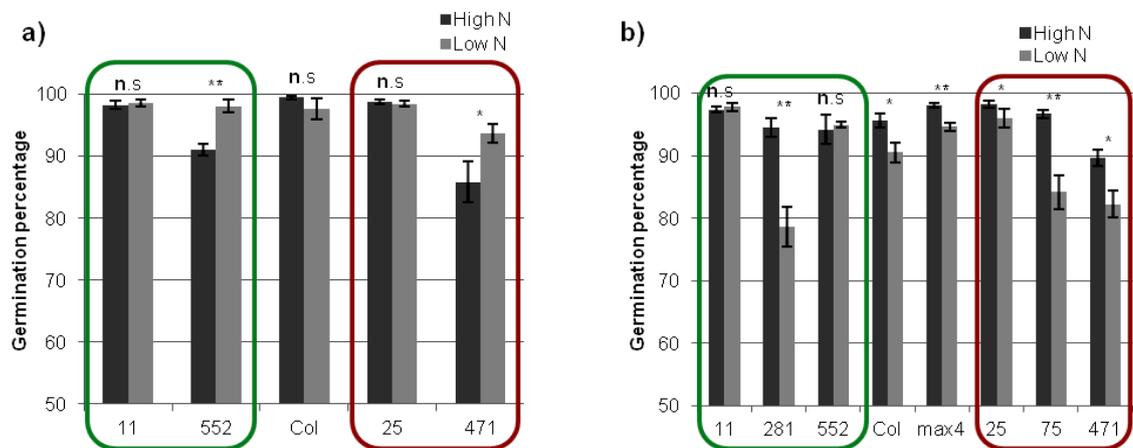


Figure 3.15 Seed viability (%) for different lines on high (9mM) or low (1.8mM) Nitrate grown in a) Summer b) Autumn. Seeds were collected from plants grown in greenhouse on 50% sand and 50% Terragreen mixture under high (9mM) or low (1.8mM) Nitrate supply. Values are means of percentage of seeds germinated on water \pm SE of 8-15 plants. Asterisks indicate significant differences between high and low N treatments (Student's t-test-n.s., not significant ($P>0.05$), *, $P<0.05$, ** $P<0.01$, *, $P<0.001$).**

3.7.3.3 Single seed weight

Dry weight of a single seed was not affected by N nutrition (ANOVA, $P>0.05$, table 3.3) for any of the lines in summer and the only line with significantly lower single seed weight on low N in autumn was MAGIC line 11 (figures 3.16 a, b). Considering that the difference in seed weight on high and low N was not significant for any of the other lines (7 out of 8), it could be reasonably concluded that single seed weight was unaffected by N limitation. It is interesting to note that the single seed weight was slightly lower for all lines and treatments

in autumn (single seed weight on high N ranged between 0.019 to 0.029 mg, on low N between 0.018 to 0.025mg) compared to summer (single seed weight on high N ranged between 0.029 to 0.032 mg, on low N between 0.028 to 0.03mg).

Single seed weight for *max4* was higher than Columbia on both high and low N and combined with the total seed yield data suggests that *max4* may be making fewer, but heavier seeds than Columbia (figures 3.13 a, b).

3.7.3.4 Residual shoot dry matter (RSDM)

Residual shoot dry matter (RSDM) was significantly reduced on low N for all the lines in both seasons (figures 3.17 a, b, ANOVA, $P < 0.0001$). RSDM was consistently high for high plasticity lines on high N in both seasons. All lines had comparable RSDM on low N in both seasons. *max4* had higher RSDM than Col on high N and lower RSDM than Col on low N.

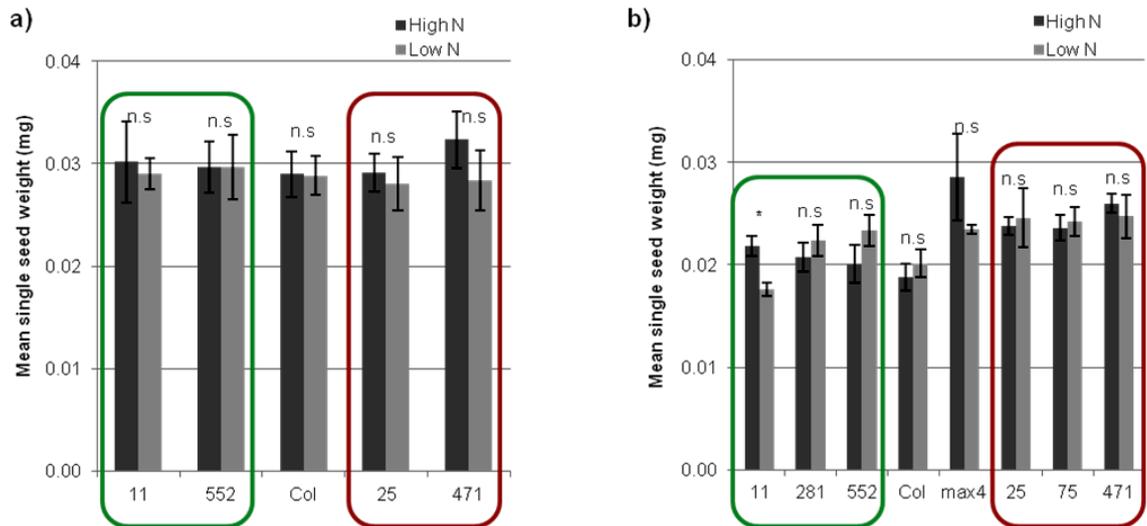


Figure 3.16 Single seed weight (mg) for different lines on high (9mM) or low (1.8mM) Nitrate supply grown in a) Summer b) Autumn. Values are means of single seed dry weights \pm SE of 6-10 plants. Asterisks indicate significant differences between high and low N treatments (Student's t-test-n.s., not significant ($P>0.05$), * $P<0.05$ ** $P<0.01$, ***, $P<0.001$).

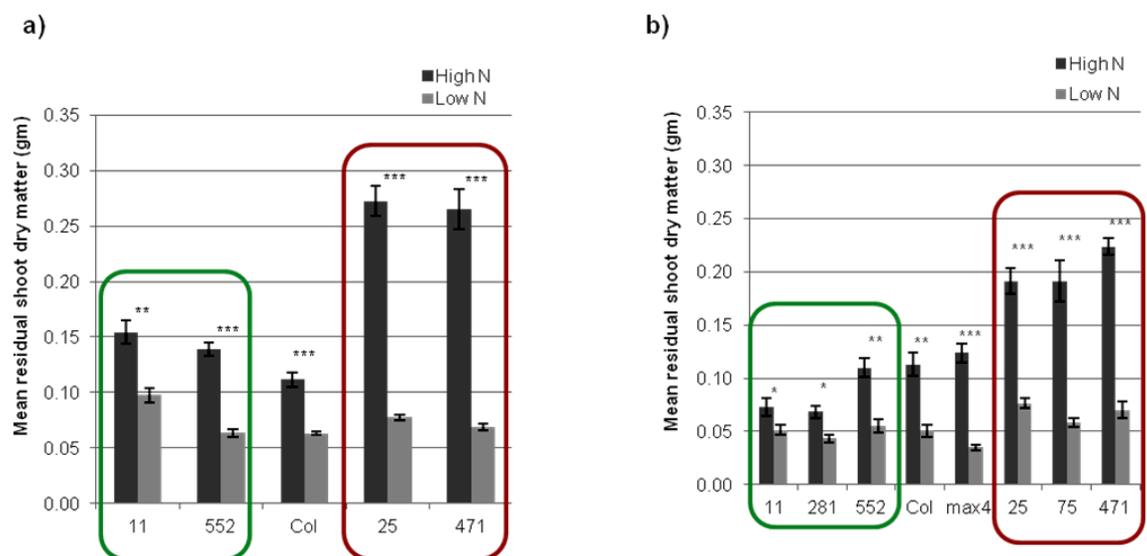


Figure 3.17 Mean residual shoot dry matter (g) for different lines grown in a) Summer b) Autumn. Values are means of single seed dry weights \pm SE of 8-15 plants. Asterisks indicate significant differences between high and low N treatments (Student's t-test-n.s., not significant ($P>0.05$), * $P<0.05$ ** $P<0.01$, ***, $P<0.001$).

3.7.3.5 Harvest Index (HI)

Harvest index (proportion of the above ground dry matter made up of seed) was consistently lower for the high plasticity lines on high N in both seasons (figures 3.18 a, b). In general, the high plasticity lines had a lower harvest indexes than the low plasticity lines, regardless of N availability. On high N, high plasticity lines allocated proportionately more biomass to RSDM and less to seed (figure 3.17 a). However, seed yield was high for these lines on high N, much higher than for the low plasticity lines (figures 3.16 a, b). Together these data suggests that when there was sufficient N, the strategy of high plasticity lines was to make proportionately more vegetative tissue and supporting the production of absolutely more but proportionately less seed biomass. These lines tended to invest proportionately more in seeds when N was limited in summer, but in autumn there was no significant difference in the seed proportion on high and low N (figures 3.18 a, b). Low plasticity lines seemed to adopt a different strategy. The seed proportion was generally higher on high N for these lines in both seasons, although this was only statistically significant for 552 in summer.

There was no significant difference in HI on high compared to low N for *max4*, whereas for Columbia, the HI was significantly lower on low N, but only in autumn (figures 3.18 a, b).

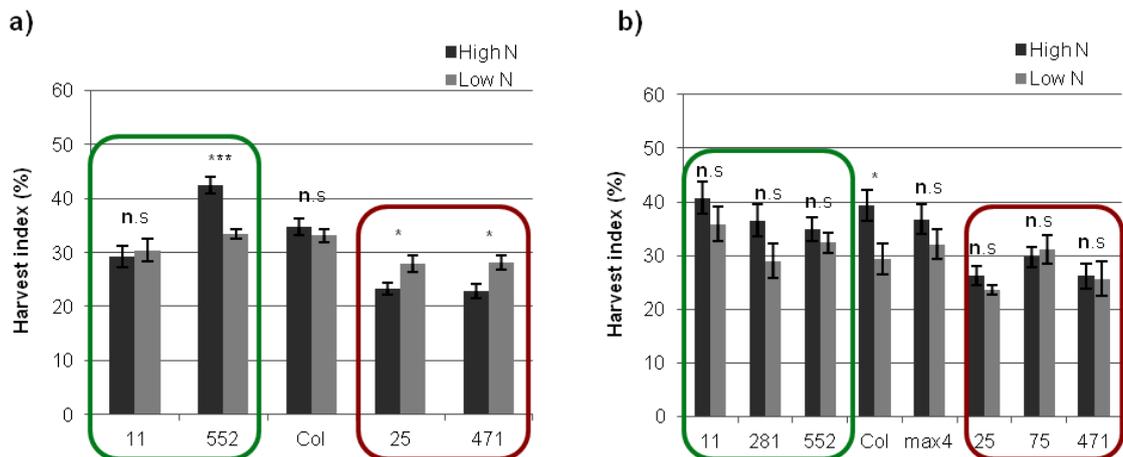


Figure 3.18 Harvest index (%) for different lines grown in a) Summer b) Autumn. Values are means of seed proportion of the total above ground dry matter \pm SE of 8-15 plants. Asterisks indicate significant differences between high and low N treatments (Student's t-test-n.s., not significant ($P>0.05$), * $P<0.05$, ** $P<0.01$, *, $P<0.001$).**

3.7.3.6 Shoot – root partitioning

A wide-spread plant response to N deficiency is to increase the root to shoot ratio. In other words when faced with N limitation plants invest in nutrient foraging by elaborating their root system at the expense of the shoot system. As evident from the RSDM, HI and seed yield analyses, the lines studied differed in their resource allocation, suggesting the need to estimate the root to shoot partitioning. Based on the HI results, it could be hypothesised that the less plastic lines may not shift their root:shoot ratios as much as the more plastic lines.

Effect of Nitrate on the overall growth of roots and shoots in young plants was examined. Fresh weights of rosettes and roots were recorded at bolting to assess the root fraction at this important developmental stage (figure 3.19). Washing off roots at this stage was easier and the entire root system could be recovered. Hence these results are considered more reliable.

Preliminary results for the root proportion of lines under study showed that not all the lines followed the general trend of an increase in root-to- shoot ratios on low N (dry weights, figure 3.20 a). The results supported the hypothesis that highly plastic lines shift their root fraction more on low N. These results need to be confirmed further with better systems to recover all the roots. In the present system of study, although every effort was taken to separate all the roots from the sand and Terragreen mixture, some roots were almost certainly lost.

The lines with high plasticity tended to increase their root fractions (fresh weight) on low N, even at this stage (figure 3.20 b). However, in contrast to the dry weight measurements at maturity, the root proportion (fresh weight) was much higher on both high and low N for the less plastic lines. Root proportions (fresh weight) of Col and *max4* are similar in that both lines increase their root proportion on low N at bolting (figure 3.20 b).

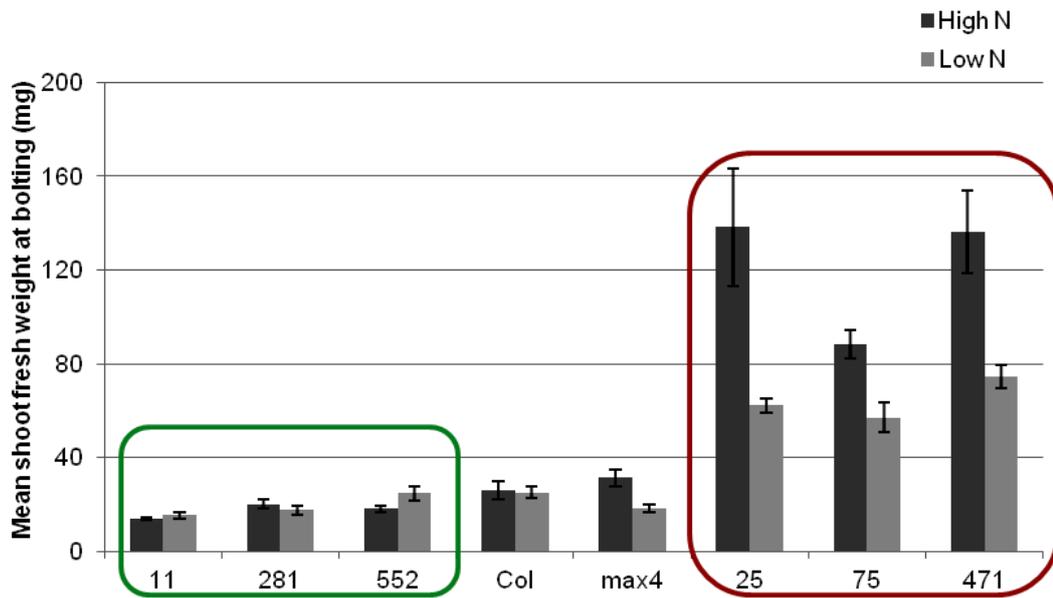


Figure 3.19 Mean shoot fresh weight of different lines at bolting. Values are means \pm SE of 10 plants.

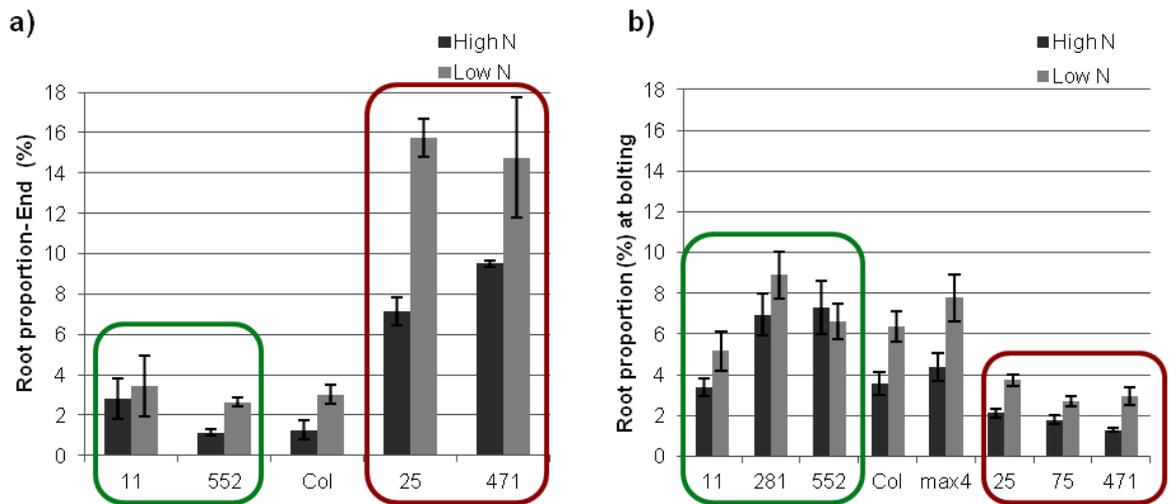


Figure 3.20 a) Root proportion (%) for different lines at the end of the experiment from dry weights. b) Root proportion (%) for different lines at bolting from fresh weights. Values are means \pm SE of 8-15 plants.

3.7.4 Shoot and seed Nitrate concentrations

Nitrate concentrations of the residual shoot dry matter (RSDM) and of the seeds were measured and results showed that the high plasticity lines tended to accumulate similar amounts of Nitrate in the vegetative tissues regardless of N status (figure 3.21 a, b), although this effect was clearer in summer. The low plasticity lines, accumulated more Nitrate in the RSDM if N was sufficient. Nitrate levels in seeds revealed that again, the high plasticity lines accumulated similar amounts of Nitrate on high and low N in both seasons, where as the low plasticity lines accumulated more Nitrate in seeds when N was sufficient (figure 3.22 a, b). However in general, all the lines seem to accumulate less Nitrate in seeds in autumn, regardless of N supply (figure 3.22).

Considering the Nitrate levels in the residual shoot dry matter and seeds together, a very interesting result was that the high plasticity lines consistently had similar Nitrate levels on both high and low N, where as the low plasticity lines consistently had Nitrate levels that varied with N supply.

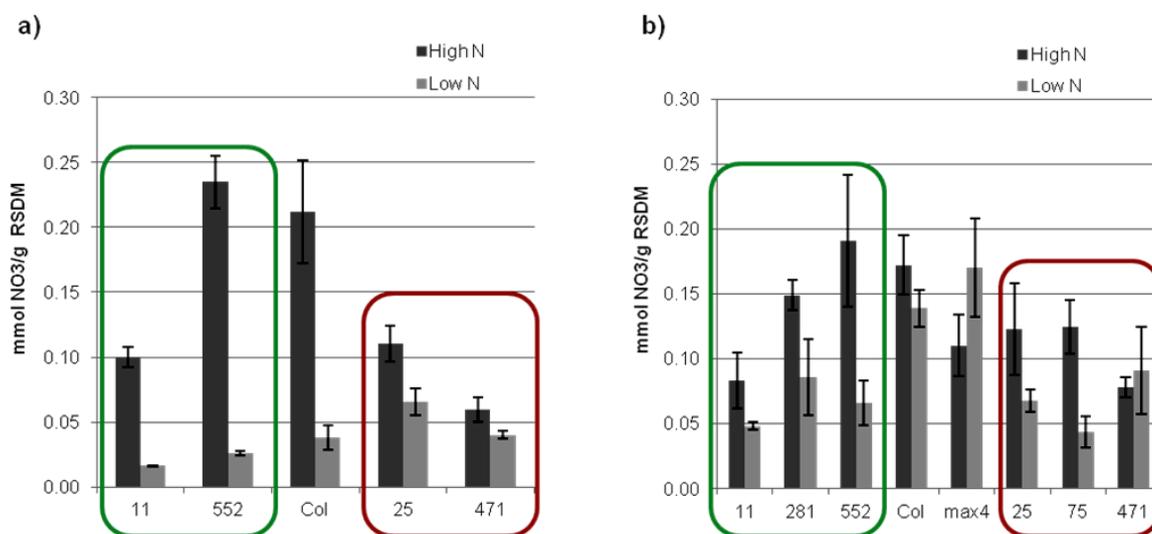


Figure 3.21 Nitrate concentrations expressed as mmol NO₃/g of residual shoot dry matter (RSDM) for different lines grown in a) Summer b) Autumn. Nitrate measurements were performed for three biological replicates with 3 to 5 independent plants/replicate. Three technical replicates were performed for each line. Values plotted are means ± SE of 9-12 plants.

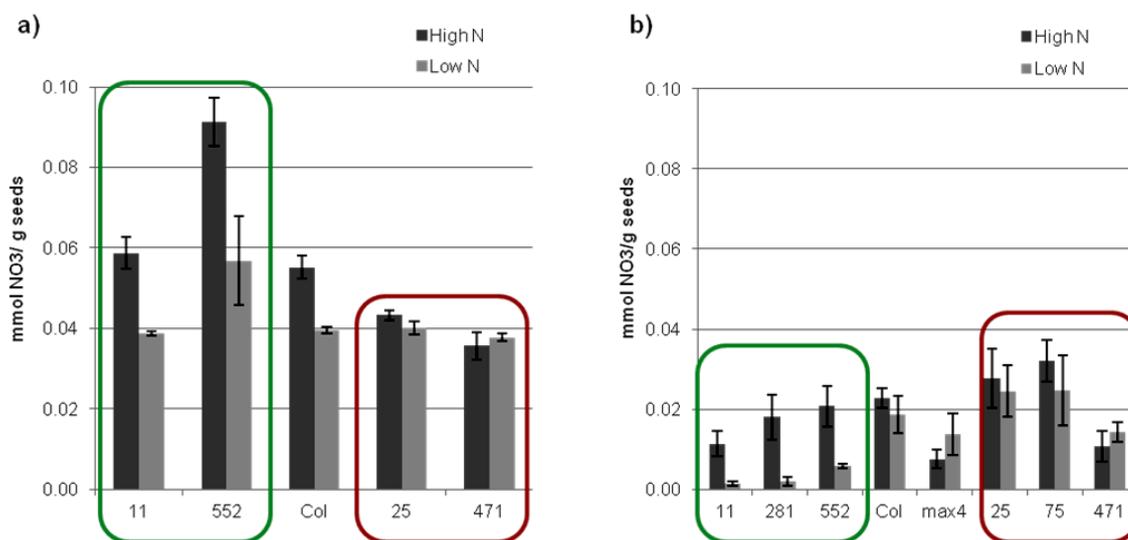


Figure 3.22 Nitrate concentration expressed as mmol NO₃/g of dry seeds for different lines grown in a) Summer b) Autumn. Nitrate measurements were performed for three biological replicates with 3 to 5 independent plants/replicate. Three technical replicates were performed for each line. Values plotted are means ± SE of 9-12 plants.

The Nitrate measurements described previously in this chapter were done at the end of the experiment, when resource allocation was complete. Although this gave an indication of final Nitrate levels, it would be interesting to see what happens early on during development, and might give an indication of the influence or lack of influence of the flowering time component. Nitrate concentrations at different growth stages were hence measured to assess whether the Nitrate level varied among the lines on high and low N and to determine the extent to which flowering time had an influence on the Nitrate levels, if at all. Measurements were made when none of the plants had bolted (12 days after germination) (figure 3.23 a), when each line had just bolted (figure 3.23 b), and, for the later flowering lines, five days before bolting (figure 3.23 c).

Results showed that the variation in Nitrate levels among the lines was apparent early on in the life cycle (12 day old seedlings), however, none of this variation correlated with the plasticity/flowering time phenotype. At bolting stage, there was clearly a major difference between the early and later flowering group (figure 3.23 a, b), with late flowering group having higher Nitrate with low N supply, and the early flowering group having higher Nitrate with high N supply. Interestingly, the later flowering group showed the opposite pattern of Nitrate accumulation 5 days prior to bolting (figure 3.23 c).

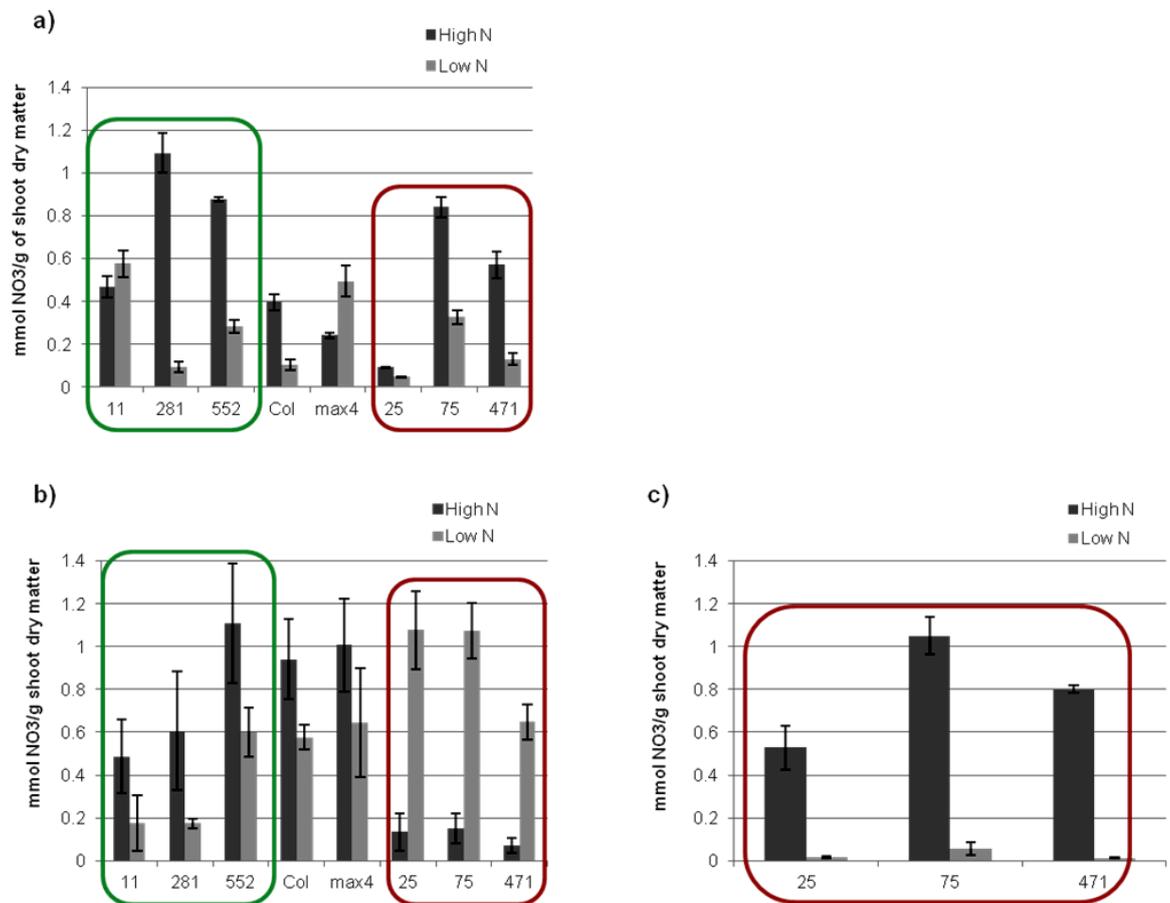


Figure 3.23 Concentration of Nitrate expressed as mmol NO₃/g of shoot dry matter for different lines. Mean Nitrate concentrations expressed as mmolNO₃/g shoot dry matter.

a) 12 day old seedlings

b) Just Bolted

c) Mean Nitrate concentration of later flowering lines (25, 75 and 471) expressed as mmolNO₃/g shoot dry matter - 5 days before bolting.

Nitrate measurements were performed for three biological replicates with 3 to 5 independent plants/replicate. Three technical replicates were performed for each line. Values plotted are means \pm SE of 10-15 plants.

3.7.5 ANOVA

A standard analysis of variance was run (in SPSS) for the full combined data set on high and low N from summer and autumn experiments described in this chapter to determine the contribution of genotype, N supply and the interaction between genotype and N supply (G X E) to the variation in the different traits measured (table 3.3). The traits included in this analysis are total branches, FT, total seed yield, single seed weight, RSDM and HI. Histograms showing the percentage of variation attributable to each factor and their interactions are presented in figure 3.24. Error (residual variation) is the variation that cannot be explained by genotype/N supply/replicate. *max4* was excluded from this analysis because, being a null allele of a major branching regulator gene this line might present totally different data from other lines.

ANOVA revealed the relative contributions of different factors. Genotype was highly significant for all the eight traits analysed (table 3.3). N availability and the genotype by environment interactions were significant for most traits except flowering time and single seed weight. The histogram (figure 3.24) showed that N availability explained a highly significant proportion of variation in traits such as the total number of branches and seed viability. Genotype explained a high proportion of variation in flowering time (60%), and smaller proportions of the variation in single seed weight (~20%) and HI (~ 25%). The effect of interactions (G X E) was highly significant for seed viability in addition to the effects of genotype and environment.

Trait	Source of variation			
	Genotype (Line)	Environment (N)	Genotype by environment (Line*N)	Residual Error
Degrees of freedom (df)	6	1	6	268
Total Branches	157.472 (<0.0001)	367.321 (<0.0001)	259.789 (<0.0001)	278.567
Flowering time	6512.177 (<0.0001)	18.093 n.s	22.908 n.s	4462.4
Total seed yield	0.014 (<0.0001)	0.105 (<0.0001)	0.013 (<0.0001)	0.075
Single seed weight	1.00E-03 0.007	1.21E-08 n.s	4.87E-05 n.s	5.00E-03
Seed viability	4897.574 (<0.0001)	846.81 (<0.0001)	363.921 (<0.0001)	7682.224
RSDM	0.339 (<0.0001)	0.537 (<0.0001)	0.236 (<0.0001)	0.339
HI	4741.287 (<0.0001)	260.302 0.023	1041.121 0.003	13376.74

Table 3.3 Analysis of variance of eight traits for genotypes (Lines) of *Arabidopsis thaliana* grown in different environments (N). Type III sum of squares are reported and significant P-values are given in parenthesis. (Significant at 0.05 level or below, n.s.- non significant).

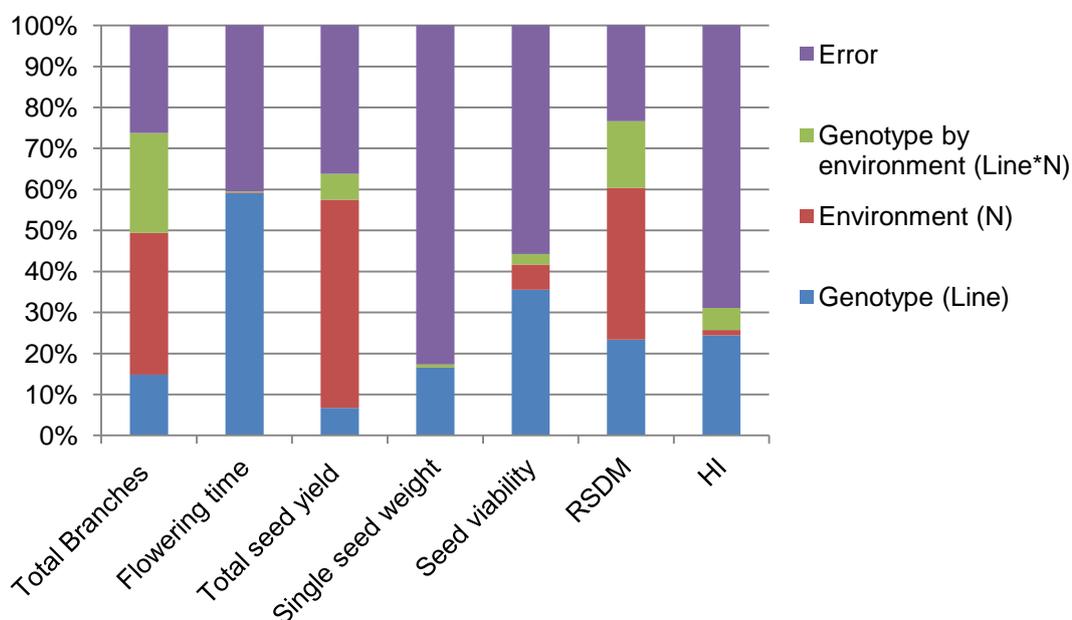


Figure 3.24 Schematic representation of ANOVA with data for eight traits from the combined data set (lines grown on high (9mM) and low N (1.8mM) in summer and autumn). Histogram shows effects due to genotype (line), environment (N) and interactions (G X E) as percentages of the variation explained.

Independent ANOVA analysis was performed on data sets from high and low N (table 3.4) to determine the extent to which season and genotype had an effect on the traits in each N situation. In addition to enabling a comparison of the extent to which each trait was affected by season, the histograms (figure 3.26 a, b) clearly showed the similarities and differences in these traits on high and low N and interactions of genotype and season.

For all traits except single seed weight, the effect of genotype was highly significant (table 3.4) irrespective of the nutrient status. Season did not have a significant effect on the total branch number or seed viability on high N, whereas these two traits were significantly affected by season on low N. Interaction between genotype and season did not affect branch number on high N but affected branch number on low N (table 3.4).

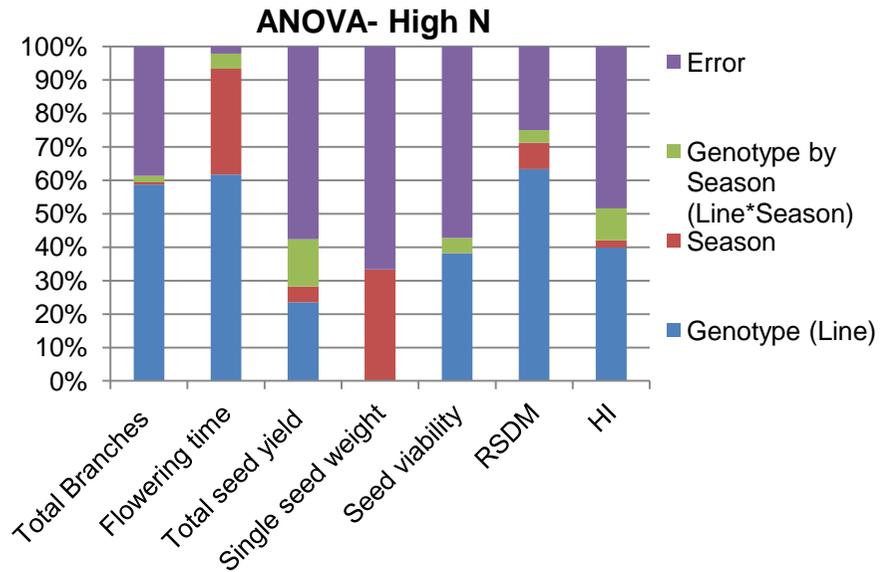
Effect of season was the major significant factor contributing to the variation in single seed weight on both high and low N (table 3.4, figure 3.25 a, b). This trait was least affected by genotype. Season also had a strong effect on flowering time, and a lesser effect on total seed yield and RSDM on both low and high N.

Effect of genotype by season interactions were significant for all traits except total branch number and single seed weight on high N, and total seed yield and HI on low N (table 3.4). HI was not significantly affected by season or interaction between genotype and season on low N.

Trait	Source of variation - High N				Source of variation -Low N			
	Genotype (Line)	Season	Genotype by Season (Line*Season)	Residual Error	Genotype (Line)	Season	Genotype by Season (Line*Season)	Residual Error
Degrees of freedom (df)	6	1	4	129	6	1	4	129
Total Branches	230.44 (<0.0001)	2.88 n.s	7.51 n.s	151.747	154.128 (<0.0001)	4.675 (0.012)	15.931 (<0.0001)	93.964
Flowering time	3588.295 (<0.0001)	1838.331 (<0.0001)	266.957 (<0.0001)	119.35	3257.699 (<0.0001)	1543.54 (<0.0001)	260.552 (<0.0001)	156.265
Total seed yield	0.02 (<0.0001)	0.004 0.002	0.012 (<0.0001)	0.049	0.002 (<0.0001)	0.002 (<0.0001)	3.77E-04 n.s	8.00E-03
Single seed weight	2.285 E-4 n.s	0.001 (<0.0001)	1.128 E-4 n.s	2.00E-03	2.45E-04 n.s	0.001 (<0.0001)	1.09E-04 n.s	2.00E-03
Seed viability	1981.516 (<0.0001)	3.538 n.s	234.728 (0.042)	2969.233	3468.768 (<0.0001)	714.624 (<0.0001)	433.224 (0.003)	3319.933
RSDM	0.504 (<0.0001)	0.063 (<0.0001)	0.03 (0.001)	0.199	0.011 (<0.0001)	0.005 (<0.0001)	0.009 (0.001)	0.033
HI	4576.224 (<0.0001)	258.366 (0.016)	1090.462 (<0.0001)	5561.155	1144.242 (0.001)	44.276 n.s	368.685 n.s	5956.958

Table 3.4 Analysis of variance of eight traits for genotypes (Lines) of *Arabidopsis thaliana* grown on high N (9 mM) and low N (1.8mM) in two seasons (summer and autumn). Type III sum of squares are reported and significant P-values are given in parenthesis (Significant at 0.05 level or below, n.s- non significant).

a)



b)

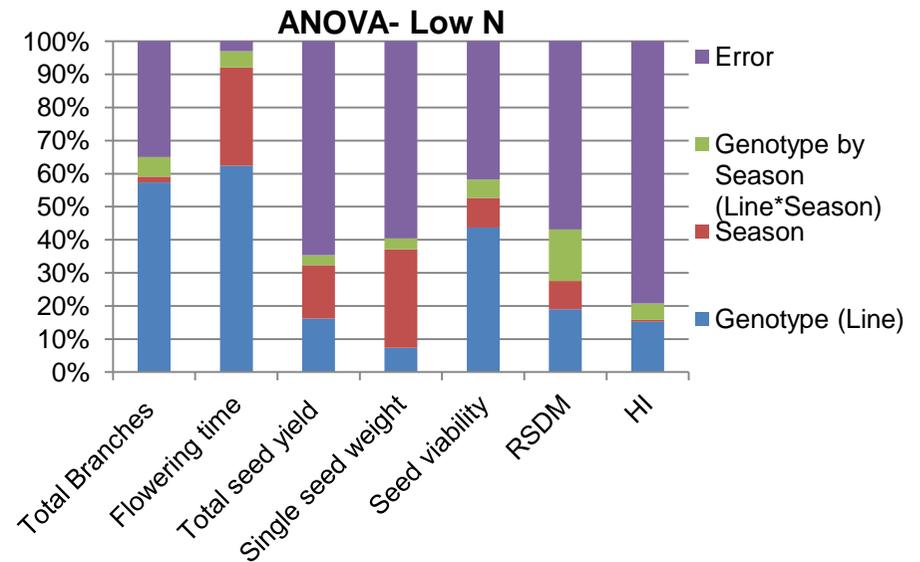


Figure 3.25 Schematic representation of ANOVA with data obtained from different lines in summer and autumn for of eight traits. a) high N (9mM), b) low N (1.8mM). Histogram shows effects due to genotype (line), season and interactions (line X season) as percentages of the variation explained.

3.7.6 Relationship between different traits

In this part of the chapter, data sets from summer and autumn experiments (except *max4*) were combined to investigate the relationship between different traits and to compare how association between traits varied when N was sufficient or limited. As a first step, correlation, positive or negative, between different traits was explored using scatter plots, performed separately on the means of the high and low N data from both seasons. This was based on the rationale that, based on the previous results section, it was reasonable to hypothesise that changes in some traits were correlated with changes in others. While correlation does not suggest causation, it does allow to form the basis for the formulation of hypotheses, especially when correlations between traits differ under different N supply or between the high and low plasticity lines. Individual lines are labelled with colour codes to allow easy visualisation of the trends.

Finally, correlations between traits on high and low N were assessed using Pearson correlation coefficient (table 3.5), which is a measure of the strength of association between the two traits. Mean data for each line on high and low N were used for computing the correlations (in SPSS) to describe and quantify the relationships observed in the scatter plots and to assess which correlations were significant. The table showing Pearson correlation coefficients and significance values (table 3.5) is presented before the section covering relationships between traits for the ease of visualization.

		Totalbranches- High N	Totalbtanches- Low N	Plasticity	Floweringtime- High N	Floweringtime- Low N	Seedyield- High N	Seedyield - Low N	RSDM- High N	RSDM-Low N	Harvest Index-High N	Harvest Index- Low N
Totalbranches- High N	Pearson Correlation Sig. (2-tailed)		-.262 n.s	.832** .001	.232 n.s	.235 n.s	.332 n.s	.118 n.s	.574 n.s	.332 n.s	-.496 n.s	-.266 n.s
Totalbtanches- Low N	Pearson Correlation Sig. (2-tailed)	-.262 n.s		-.752** .005	-.756** .004	-.750** .005	-.173 n.s	.557 n.s	-.631* .028	-.075 n.s	.719** .008	.756** .004
Plasticity	Pearson Correlation Sig. (2-tailed)	.832** .001	-.752** .005		.579* .048	.576* .050	.320 n.s	-.228 n.s	.748** .005	.271 n.s	-.748** .005	-.603* .038
Floweringtime- High N	Pearson Correlation Sig. (2-tailed)	.232 n.s	-.756** .004	.579* .048		.998** .000	.248 n.s	-.607* .036	.472 n.s	-.010 n.s	-.529 n.s	-.734** .007
Floweringtime- Low N	Pearson Correlation Sig. (2-tailed)	.235 n.s	-.750** .005	.576* .050	.998** .000		.274 n.s	-.603* .038	.489 n.s	.003 n.s	-.535 n.s	-.750** .005
Seedyield- High N	Pearson Correlation Sig. (2-tailed)	.332 n.s	-.173 n.s	.320 n.s	.248 n.s	.274 n.s		.162 n.s	.674* .016	.340 n.s	-.266 n.s	-.215 n.s
Seedyield- Low N	Pearson Correlation Sig. (2-tailed)	.118 n.s	.557 n.s	-.228 n.s	-.607* .036	-.603* .038	.162 n.s		.077 n.s	.667* .018	-.039 n.s	.428 n.s
RSDM-High N	Pearson Correlation Sig. (2-tailed)	.574 .051	-.631* .028	.748** .005	.472 n.s	.489 n.s	.674* .016	.077 n.s		.577* .049	-.870** .000	-.604* .037
RSDM-Low N	Pearson Correlation Sig. (2-tailed)	.332 n.s	-.075 n.s	.271 n.s	-.010 n.s	.003 n.s	.340 n.s	.667* .018	.577* .049		-.622* .031	-.379 n.s
Harvest Index- High N	Pearson Correlation Sig. (2-tailed)	-.496 n.s	.719** .008	-.748** .005	-.529 .077	-.535 n.s	-.266 n.s	-.039 n.s	-.870** .000	-.622* .031		.712** .009
Harvest Index- Low N	Pearson Correlation Sig. (2-tailed)	-.266 n.s	.756** .004	-.603* .038	-.734** .007	-.750** .005	-.215 n.s	.428 n.s	-.604* .037	-.379 n.s	.712** .009	

Table 3.5 Summary of correlations between traits on high N (9mM) and low N (1.8mM). This table shows Pearson correlation coefficients computed using SPSS (release 19, SPSS Inc) from mean data of each line. P-values are given below the Pearson correlation coefficients. Significant correlations are shown in red bold letters. (** Correlation is significant at 0.01 level (2-tailed), * Correlation is significant at 0.05 level (2-tailed), n.s- non significant).

3.7.6.2 Relationship between branching and fitness traits

There was a positive correlation between total branch number and seed yield on both high and low N (figure 3.27 a). The correlation was stronger on low N than on high N (table 3.5, $r = 0.332$ on high N and 0.557 on low N), but it was not significant in either case.

As expected, scatter plots clearly showed that seed yield was generally lower on low N. The medium branchy, low plasticity lines (552, 11 and 281) were generally not high yielders on high N when compared to high plasticity lines. The high plasticity lines were very poor yielders when N was limited, but had high yields when N was sufficient (figure 3.27 a). These results might also be useful from a crop breeding point of view, in that although improving the number of branches might be beneficial to improve the yield, the best yielders on nutrient sufficient situations might not be the best yielders when nutrients are limited.

Total seed yield per plant on high N had a positive correlation with plasticity ($r = 0.320$, $P = \text{n.s.}$) as evident from the scatter plot and Pearson correlation coefficients (figure 3.27 b, table 3.5). However, seed yield on low N was negatively correlated with plasticity ($r = -0.228$, $P = \text{n.s.}$). While neither of these correlations was significant, the reversal of the sign of the correlations was interesting. The highly plastic lines made more seed when there was sufficient N, but when N was limited the seed yield was lower than the low plasticity lines. In other words, for these lines, plasticity is associated with reduced yield on low N, probably due to allocation of resources to the root. For the less plastic lines, yield on low N was maintained, associated with a lesser shift in the root fraction. In some of these low plasticity lines, similar seed yields were achieved on both high and low N, but the mean seed yield on high N tended to be lower than that for the highly plastic lines, with high levels of free Nitrate accumulating, suggesting that these lines were unable to take full advantage of all the available N.

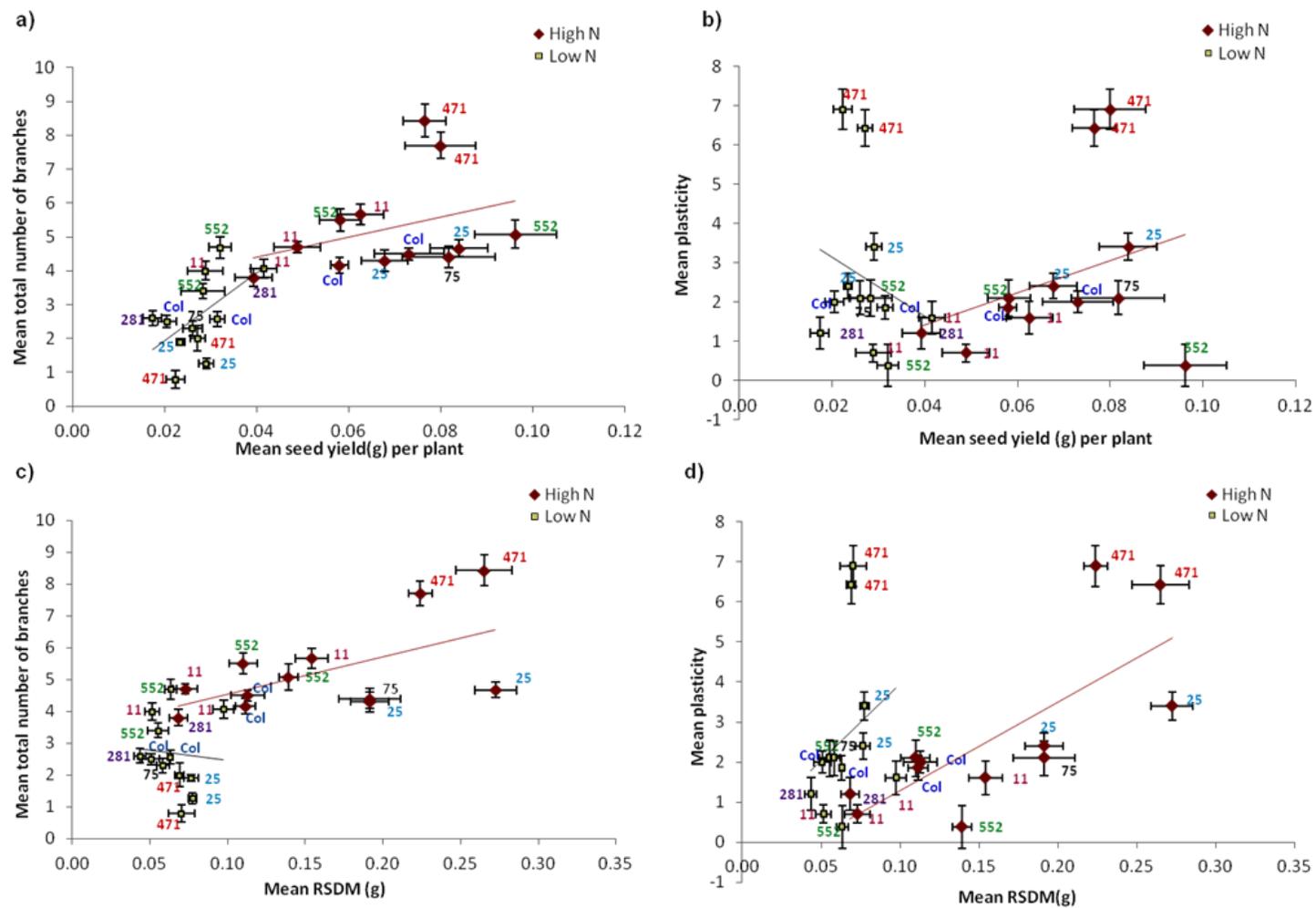


Figure 3.27 Scatter plot showing the relationship between a) Branch numbers and seed yield on high and low N, b) Plasticity and seed yield on high and low N, c) Branch numbers and RSDM on high and low N, d) Plasticity and residual shoot dry matter (RSDM) on high and low N. For each line, the results of two independent experiments are included. Values plotted are means \pm SE of 8-15 plants. The solid lines in the scatter plot are the linear regression lines. (Red line indicates high N and black line, low N).

RSDM and branch number correlated positively but not significantly when N was sufficient (figure 3.8 c, table 3.5, $r = 0.574$, $P = 0.051$), which was not surprising. However, the correlation was very small but negative when N was limited ($r = -0.075$, $P = \text{n.s.}$). This is because highly plastic lines, which make more branches and accumulate more RSDM on high N, make few branches when N is limited, whereas the low plasticity lines maintain a medium number of branches and a similar RSDM on both high and low N.

There was a strong, positive, significant relationship (figure 3.27 d, table 3.5) between plasticity and RSDM on high N ($r = 0.748$, $P = 0.005$). Although the correlation between plasticity and RSDM was positive, it was much weaker and non significant on low N ($r = 0.271$, $P = \text{n.s.}$). This is because highly plastic lines accumulate more above ground biomass than low plasticity lines when there is sufficient N, but when N is limited; all lines have a similar low biomass. The lower RSDM accumulation is likely to reflect their low branch number on low N and resource allocation to the root, but it should also be noted that depending on length and stem thickness different numbers of branches may contribute to the same amount of RSDM, as is evident from the similar RSDMs of the low and high plasticity lines on low N, and the lack of significant correlation between branch number and RSDM on low N.

Harvest index and number of branches (figure 3.28 a, table 3.5) were negatively correlated on high N ($r = -0.496$, $P = \text{n.s.}$). On the contrary there was a strong and significant positive correlation between branch numbers and harvest index on low N ($r = 0.756$, $P = 0.004$). The differences in slope on high and low N could be because on low N the low plasticity lines have higher yields, with high branch numbers and low RSDM, whereas on high N, the high plasticity lines have high yields with high branch numbers and high RSDM.

Plasticity correlated negatively with harvest index on high and low N (figure 3.28 b). Pearson correlation coefficients indicated that the correlation between plasticity and harvest index on high N was strong, negative and significant ($r = -0.748$, $P = 0.005$). Similarly, the correlation between plasticity and harvest index on low N was also negative and significant ($r = -0.603$, $P = 0.038$). This is

3.7.6.3 Relationship between flowering time and other traits

There was a weak positive correlation between the number of branches and flowering time on high N ($r= 0.232$, P - n.s.) and a strong negative correlation between flowering time and branch numbers on low N (figure 3.29 a, table 3.5) ($r=$ etc). Plasticity and flowering time were positively and significantly correlated (figure 3.29 b) on both high ($r= 0.579$, $P= 0.048$) and low N ($r= 0.576$, $P= 0.050$).

There was a strong, significant negative correlation (figure 3.30 b, table 3.5) between flowering time and seed yield on low N ($r= -0.603$, $P= 0.038$) and a weak positive correlation between the two on high N ($r= 0.248$, P - n.s.). Late flowering, high plasticity lines had lower seed yield on low N compared to low plasticity, early flowering lines.

There was no correlation between flowering time and RSDM (figure 3.30 a) on low N ($r= 0.003$, P - n.s.) whereas there was a positive but not significant correlation ($r= 0.472$, P - n.s.) between the two on high N (figure 3.30 a, table 3.5).

There was a negative correlation between flowering time and harvest index on high N ($r= - 0.529$, P - n.s., figure 3.30 c) and a strong, significant negative correlation between flowering time and HI on low N ($r= - 0.750$, P - 0.005, figure 3.30 c).

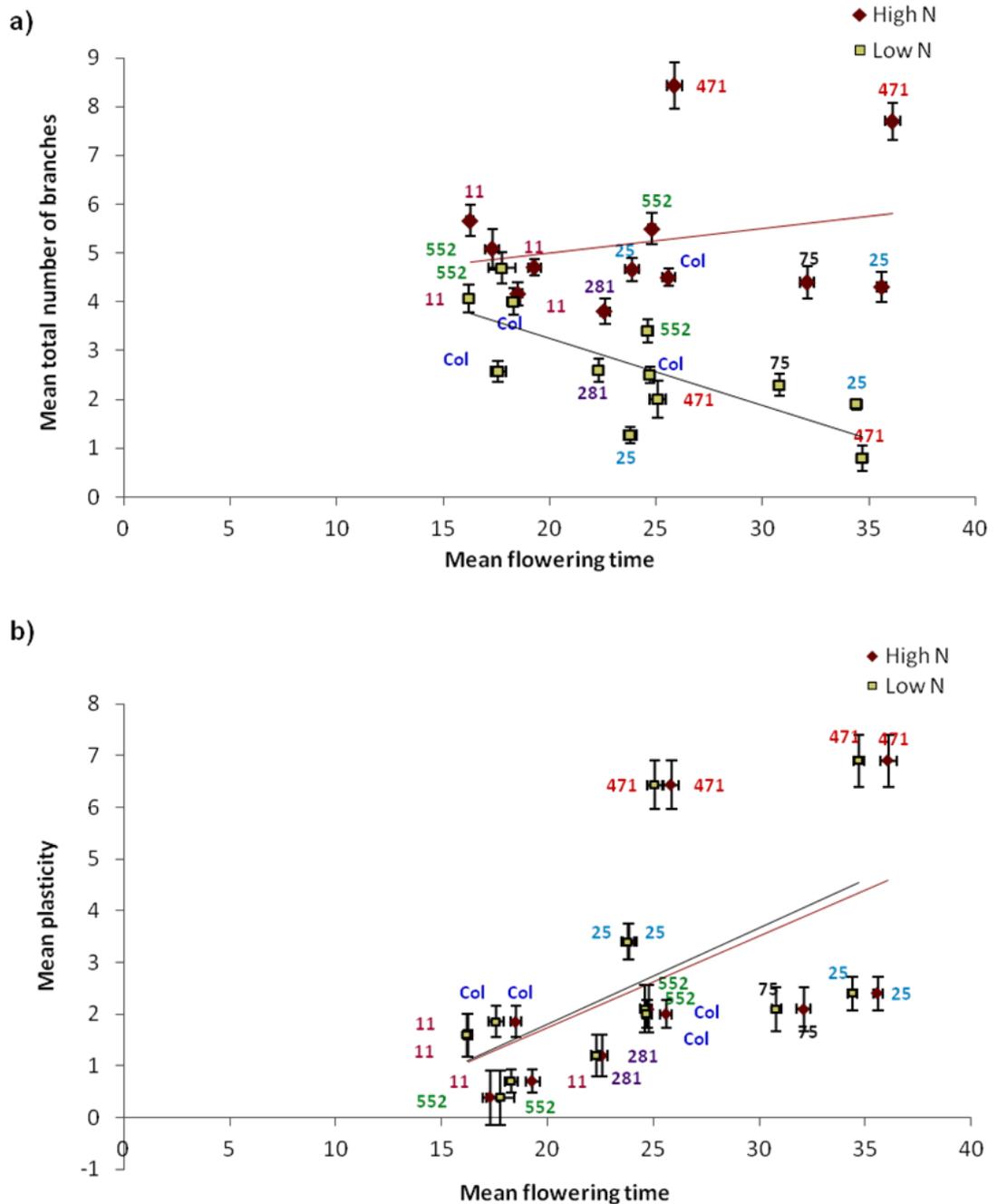


Figure 3.29 Scatter plots showing the relationship between

a) Branch numbers and flowering time on high and low N.

b) Plasticity and flowering time on high and low N.

For each line, the results of two independent experiments are included. Values plotted are means \pm SE of 8-15 plants. The solid lines in the scatter plot are the linear regression lines. (Red line indicates high N and black line, low N).

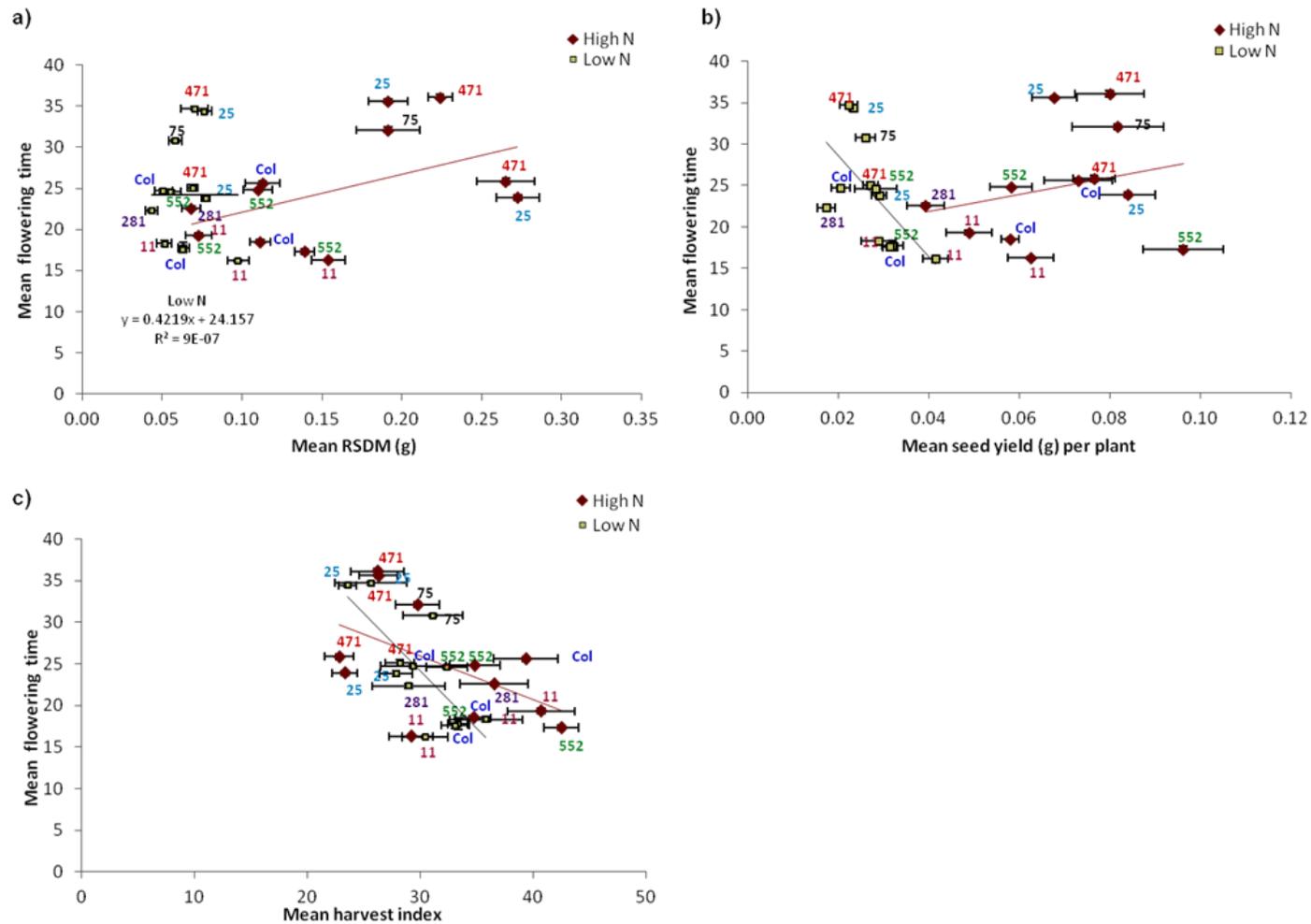


Figure 3.30 Scatter plots showing the relationship between a) FT and seed yield on high and low N, b) FT and RSDM on high and low N c) Flowering time and harvest index on high and low N. For each line, the results of two independent experiments are included. Values plotted are means \pm SE of 8-15 plants. The solid lines in the scatter plot are the linear regression lines. (Red line indicates high N and black line, low N).

3.7.6.4 Relationship between fitness related traits

There was a strong positive correlation between residual shoot biomass and seed yield (figure 3.31) on both high ($r = 0.674$, $P = 0.016$) and low N ($r = 0.667$, $P = 0.018$).

Highly plastic lines that accumulate more shoot biomass when there is sufficient N make less biomass and less seeds when N is limited. Here again, from an agricultural point of view, the low plasticity lines that accumulate medium RSDM on both high and low N may be advantageous if looking for those lines that can perform well on both high and low N.

Harvest index and RSDM had a very strong, significant negative correlation (table 3.5) on high N ($r = -0.870$, $P = 0.0001$). On low N, although the correlation is negative it not strong or significant ($r = -0.379$, $P = \text{n.s.}$).

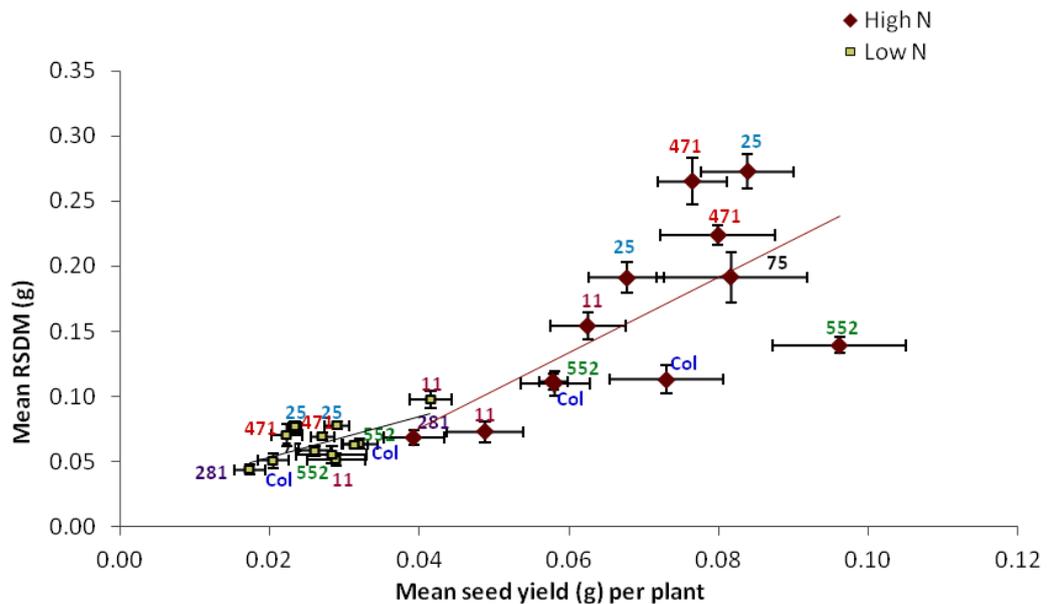


Figure 3.31 Scatter plot showing the relationship between RSDM and seed yield on high and low N. For each line, the results of two independent experiments are included. Values plotted are means \pm SE of 8-15 plants. The solid lines in the scatter plot are the linear regression lines. (Red line indicates high N and black line, low N).

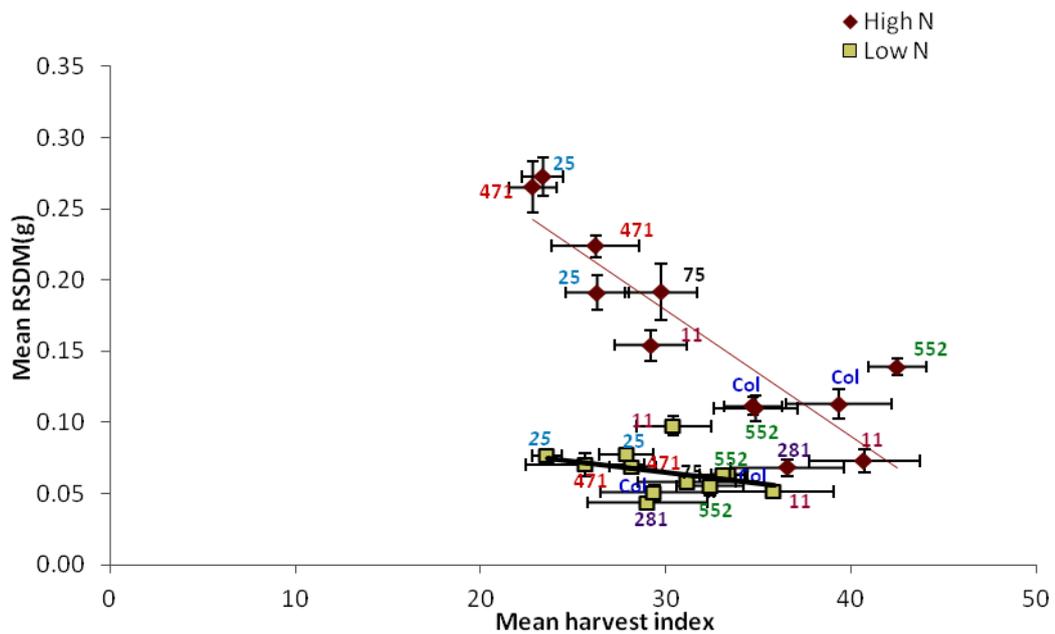


Figure 3.32 Scatter plot showing the relationship between RSDM and harvest index on high and low N. For each line, the results of two independent experiments are included. Values plotted are means \pm SE of 8-15 plants. The solid lines in the scatter plot are the linear regression lines. (Red line indicates high N and black line, low N).

3.7.6.5 Summary of correlations between traits

The summary figure (figure 3.33) of correlations revealed very interesting results about differences on high and low N (table 3.5). Among all the traits analysed, the total number of branches produced when there was sufficient N was highly and significantly correlated with plasticity ($r= 0.832^{**}$) but not to any other trait. However, when the nutrients were limited, total branch number exhibited strong highly significant negative correlations with both plasticity ($r= -0,752^{**}$) and flowering time ($r= -0.750^{**}$) and a strong significant positive correlation with HI ($r= 0.756^{**}$). There was a significant positive correlation between plasticity and flowering time ($r= 0.579^*$ - high N, $r= 0.576^*$ - low N) and highly significant and strong negative correlation between plasticity and harvest index ($r= -0.748^{**}$ - high N, $r= -0.603^*$ - low N) irrespective of the N status. There was a strong, positive and highly significant correlation between RSDM and plasticity ($r= 0.748^{**}$) only

when N was sufficient. But seed yield was positively and significantly correlated with RSDM in both N sufficient ($r= 0.674^*$) and limited situations ($r=0.667^*$).

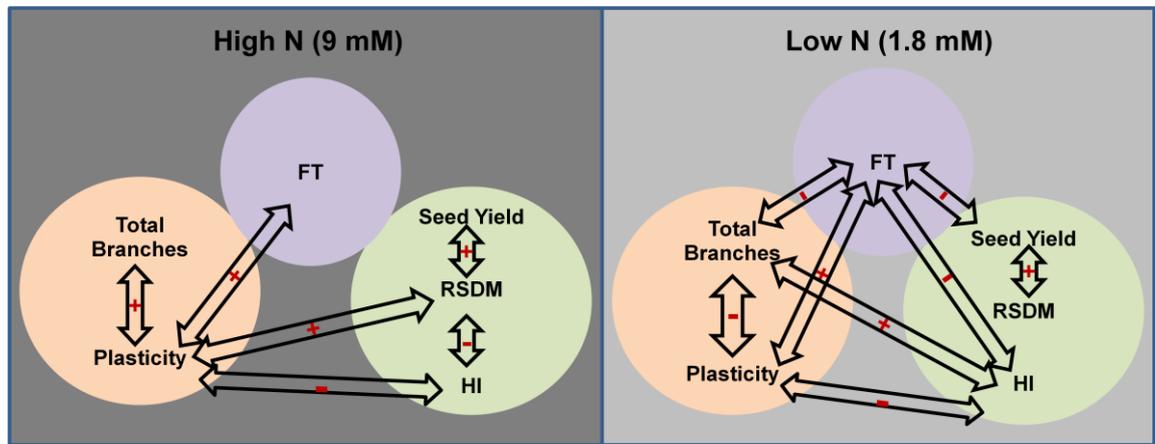


Figure 3.33 Schematic representation of significant trait correlations on high and low N.

3.8 Discussion

This chapter describes a detailed study of branching and branching plasticity in selected MAGIC lines on two different N regimes with the objective of identifying the nature of relationships between traits. In addition, links between plasticity and other traits, particularly fitness-related traits and flowering time were also explored. The main findings are discussed below.

3.8.1 Major variation in branch number and its response to N correlates with flowering time

The study of shoot branching and branching plasticity of selected lines on high and low N revealed that N availability affected branching substantially, and that there was substantial genetic variation in this response to N. The results of this study support and confirm results of previous studies suggesting that environmental signals such as nutrient availability, herbivory and day length, strongly influence quantitative variation in shoot branching (Bonser and Aarssen, 1996).

Analysis of shoot branching and branching plasticity among 15 lines in the pilot study and a detailed subsequent study of selected MAGIC lines showed that the lines studied could be grouped into two distinct categories. The first category had many branches on high N, but very few on low N, characterising them highly plastic and hyper sensitive to N limitation (for example, MAGIC lines 25 and 471). The second category included less plastic lines which had a moderate number of branches on high N, but did not lose many on low N (for example, lines 11 and 552). These lines did not seem to respond to N availability. This relationship between branching and branching plasticity among the subset of selected lines was consistent with the strong correlation observed in the baseline study involving 400 MAGIC lines (figures 3.1 a, b, and 3.26).

Interestingly, this correlated with flowering time (FT). When comparing the flowering time of the lines studied, it was evident that the early flowering lines had only a medium number of branches on high N and they did not respond to N limitation. The strategy of these lines seemingly was to make as many branches as possible, when they could with the available N, within their short life cycle. It is likely that the branch number of these lines on high N was limited by Carbon (C), and C was limiting because of the short time for its accumulation. Alternatively this could be considered as a strategy to produce a reasonable number of seed quickly, for example allowing a second generation within the year. In this context it was interesting that 552 was more plastic in the autumn.

In contrast, the later flowering lines had a lot of branches on high N but these lines made only very few branches on low N. In other words the strategy of these lines which had a longer life cycle was presumably to assess the N availability and branches are made depending on the N availability. It is possible that the late flowering lines, maybe less carbon limited on high N because they have more leaves, and so could make more branches on high N.

As previously mentioned at the beginning of the chapter, there was a lack of strong correlation between flowering time and branch numbers in the 400 line

baseline experiment. The strength of this correlation increased while considering lines which had an FT below 30 days (summer 2008, figure 3.2). All of the lines in my study belonged to this category in summer. And indeed the correlation between FT and branch numbers on low N were negative and significant (figure 3.29 a, table 3.5), further confirming that branch numbers decreased in the later flowering lines when N was limited.

When thinking of causal links of why being late flowering might cause high plasticity in shoot branching in response to N, one could hypothesize that these plants became more N-deficient because they were growing for longer on low N and hence their low branching on low N. However, Nitrate measurements of the late flowering lines provide evidence against this hypothesis. Similar amount of Nitrate was present in the shoots and seeds of these lines on both high and low N (figures 3.21, 3.22). An alternative hypothesis is that the effects of N limitation could be hormonally controlled. Support for this hypothesis comes from the *max4* mutant which is highly branched on both high and low N. This argues against the mechanism mentioned in the previous paragraphs about N and C limitation and suggests that N limitation does not directly cause lack of branching.

3.8.2 Plasticity in shoot branching could be seasonally regulated

In the first part of this chapter, results are presented allowing comparison of experiments carried out in two seasons. These represent independent experiments performed in the same green house in summer 2009 and autumn 2010. The decision to present the results as a comparison of summer and autumn data was based on the observations made, during repetition of branching plasticity analysis on different MAGIC lines during different seasons. There was variability in branch number and branching plasticity among some lines in the repeats conducted during different seasons (table 3.2). Results of repeats performed in autumn were comparable to winter, but were different in summer. This variability in branching response to N exhibited by some lines during different seasons was also observed in the results of the pilot study using 15

MAGIC lines which was conducted in winter (table 3.2, figure 3.7), when compared to the baseline experiment carried out by Williamson et al in summer 2008 (Williamson et al, unpublished). This led to the assumption that branching plasticity in response to N is itself a plastic trait, varying in response to seasons.

A particularly striking result that emerged from comparison of branching plasticity from six separate experiments of the selected lines over seasons (table 3.2) was that branching plasticity switched between seasons for some lines but that some lines exhibit more consistent plasticity than other lines. This shift in the degree of plasticity exhibited by some lines is very interesting. In my study, there were lines such as 552 that could switch their response between seasons (low plasticity in summer and high plasticity during other seasons). Other lines such as 25 had similar, but less extreme variations in plasticity which correlated with seasonal changes. On the other hand, lines such as 11 (early flowering, low plasticity) and 471 (later flowering, high plasticity) retained similar levels of plasticity during all the seasons. This consistency in plasticity exhibited by some lines, despite the differences in flowering time (early or later depending on season), suggests independent regulation of plasticity and flowering time. These results further emphasise the need to unravel the genetic basis of plasticity to assess how these changes occur.

3.8.3 Effect of seasonality on plasticity of other traits

Differences in the growth season in which the experiments were carried out might help to explain the differences observed in various traits among the same lines. Although not planned, experiments were repeated in summer and autumn in consecutive years. There could have been differences in temperature, light intensity and light quality during these seasons, even though plants were grown in controlled green houses with supplemental lighting to give equivalent day lengths. In my study, season seems to have a clear effect on the flowering time, but all lines responded in the same way (figures 3.9 a, b). All the lines shifted their flowering time to a similar extent between summer and autumn, with all of

them flowering nearly 10 days later in autumn. Results of ANOVA performed independently on high and low N also suggested that season influenced flowering time significantly on both high and low N, and to a similar extent (table 3.4, figure 3.25 a, b). This confirmed the observation above that all lines responded to season to a similar extent on high and low N. Several studies have found similar links between plasticity of different traits and season. For instance, Burns et al (2011) reported significant effects of season and interaction of genotype with season on Nitrate accumulation in Lettuce accessions. Similarly, Searle and Coupland (2004) reported synchronisation of developmental changes such as flower initiation to changing seasons. Another example of a seasonal effect on plasticity was the dramatic difference in bolting time between different seasons and sites reported by Weinig et al, in her study of the genetic basis of variation in reproductive timing in Arabidopsis (Weinig et al., 2002). Her study suggested that the photoperiod pathway could be regulated differently in different seasons. Seasonal variation in branching plasticity observed in lines such as 552 and the lack of variation in lines such as 471 was reproducible in the several repeats (table 3.2). However, due to the lack of replicates for other traits during each season it was not possible to separate out the seasonal effect on other traits. Apart from studies of flowering time, the effect of seasonality on plasticity of other key traits has not been studied extensively. More studies about seasonal regulation of plasticity in other traits might be useful in this context.

3.8.4 Nitrate has no strong effect on flowering time

Given that Nitrate is crucial for all plant developmental processes, it could be speculated that N limitation could have an impact on flowering time of Arabidopsis. Several lines of evidence, including the 15 line pilot study and the detailed analysis using a more limited number of lines (figures 3.6 b, 3.9 a, b) strongly suggests that Nitrate has no effect on flowering time. This result was consistent with the results of the 400 line background experiment, and with the recent study of Castro Marin et al (2011) suggesting that Nitrate did not have an effect on flowering time in Arabidopsis.

Two way ANOVA results confirmed that genotype was the major significant contributory factor for the variance in flowering time and that the effect of Nitrate and the interaction between genotype and Nitrate was non-significant (table 3.3, figure 3.24). As previously mentioned season had a similarly big effect on flowering. The variation among different genotypes in response to season was an exception to lack of response of FT to Nitrate. For example, in autumn high N delayed flowering by 2 days for some lines (figure 3.9 b). The slightly earlier flowering time of these lines on low N could be thought of as a general plant response to low nutrient availability allowing the rapid deployment of the limited nutrients to seeds.

3.8.5 Branch loss of high plasticity lines under N limitation is partially overcome by decapitation

Removal of the bolting stem, including the primary and cauline bud shoot apices, and hence the apical auxin sources by decapitation released buds that would have been otherwise dormant, on both high and low N, in all the lines studied (figure 3.12). This result was consistent with current understanding about regulation of apical dominance by apical auxin. There were differences among the lines in their response to decapitation, which could be linked to their degree of branching plasticity (figures 3.12 a, b). Decapitation response of high plasticity lines suggested that these lines were capable of making additional branches, which was consistent with their active branch suppression on low N. In contrast, low plasticity lines did not make many additional branched when decapitated on low N, resulting in fewer branches being made compared to the intact control. This is consistent with the relatively weaker branch suppression by low plasticity lines on low N.

3.8.6 High N is crucial for higher yields but yield retention on low N is different for different lines

Seed yield results of selected lines confirmed the importance of N nutrition in yield (figure 3.13). This is well known and not surprising given that Nitrogen

fertilization is crucial to maintain high yield in agriculture (Sinclair and Vadez, 2002) and that low Nitrogen fertilization leads to significant yield reduction (McCloud, 1998). Increased use of nitrogenous fertilizers in the past 50 years have helped to increase crop yields and one of the main challenges faced by plant breeders is to optimise seed yields when N is limited. This is particularly important because until recently, selection for higher grain yields was often carried out under non-limiting N conditions (Presterl T et al., 2003, Bänziger M et al., 1997). Similar reduction in seed yield in N limited situations has been previously reported in Arabidopsis by Lemaitre et al (2008). A study by Masclaux-Daubresse and Chardon suggested that when N was limited, fewer seeds were produced which is similar to my results (Masclaux-Daubresse and Chardon, 2011). However, their study found that although there was a reduction in seed numbers, seeds were heavier. In my study, greater seed yield on high N was caused by greater overall seed number and not by an increase in seed size, as the single seed weight was unaffected by N supply (figure 3.13, 3.16). Greater seed number could be caused by a greater number of seeds per silique and/or more flowers leading to more siliques. More flowers could result from either more flowers per branch, or more branches. Nevertheless, branches can stay vegetative and in Arabidopsis the inflorescences are indeterminate. So there is no solid basis to assume that more branches would necessarily mean more flowers or more seed. Indeed results clearly demonstrated a lack of significant correlations between total branch numbers and seed yield irrespective of whether N was sufficient or limited (table 3.5, figure 3.27 a). This result is in contrast to the findings of others, such as Doust and Kellogg (2006) who found that in annual grasses, branch numbers might have a direct influence on the seed numbers produced by a plant (Doust and Kellogg, 2006).

Moreover, results from my study become interesting when considering the links between seed yield and branching plasticity. Lines which were high yielders on high N were not high yielders when N was limited. Low plasticity lines had higher seed yields on low N and were better at retaining yield on low N than high plasticity lines, indicating the two different strategies of these two groups. The

strategy of low plasticity lines could be considered as a relatively successful one on low N, with reasonable seed yield. But they were not so successful on high N, with smaller increases in seed yield and often less absolute yield than the high plasticity lines. High plasticity lines invested more resources into roots when N was limiting, increasing the chances of improving N acquisition. These lines had lower yields on low N. In real life, the strategy of high plasticity lines to search for more N when N was limited cannot be considered as a less successful strategy, as it might indeed lead to improved N acquisition. Thus the low plasticity strategy is likely only selectively advantageous in chronically N-poor conditions.

Furthermore, when considering the behaviour of individual lines, it was observed that consistency/ lack of consistency in plasticity of the different lines was reflected in their yield retention (figure 3.14). Lines which had consistently low branching plasticity (figures 3.11 a, b) such as 11, displayed consistently high yield retention irrespective of season (figures 3.14 a, b). 471 behaved similarly (consistently high plasticity and consistently low yield retention regardless of season). Yield retention of 552 was high or low corresponding to the seasonal variation in branching plasticity response of this line. This suggests that there could be some link between branch number and seed yield, despite the above mentioned strategies.

3.8.7 Single seed weight was unaffected by N limitation

Nitrate supply, and interactions between genotype and Nitrate supply did not contribute very much to the overall variation in the single seed weight (table 3.3, figure 3.24). This was consistent with results from studies on different Arabidopsis accessions, in which N limitation did not affect single seed dry weight (Lemaître et al., 2008, Masclaux-Daubresse and Chardon, 2011). My study also showed that with a given Nitrate supply, of the factors assessed, season contributed most to the observed variation, although its effect was still small (table 3.4, figure 3.25 a, b). There were no differences between the seed yields of high or low plasticity

groups (figure 3.16) which together with no differences between single seed weights suggested that single seed weight was a robust trait.

Results concerning seed viability (figures 3.15 a, b) did not reveal any strong patterns. Partly, this could be due to the fact that seed viability could be affected by external conditions during drying on the plant and during storage. Similar variability in germination between seed batches and a lack of clear effect of Nitrate on Arabidopsis seed germination was reported by Hilhorst and Karssen (Hilhorst and Karssen, 1988). In my study, the germination rates of most lines ranged between 90% and 100% (figures 3.15 a, b). However there was some difference in germination between summer and autumn seasons (figure 3.15 a, b). This effect of season was not significant on high N and was more pronounced on low N (table 3.4, figure 3.25 a, b). Despite this, the relative contribution of season was very small. Some studies have found evidence that Arabidopsis seed germination is influenced by seasonal and environmental changes (Footitt et al., 2011) and that the basic life history of plants could be influenced by seasonal dependence of germination timing (Donohue et al., 2005).

3.8.8 Harvest index is affected by N supply

The results of the ANOVA clearly showed that although genotype was the major contributing factor to variation in HI, both Nitrate supply and the interaction between genotype and Nitrate supply also influenced this trait (table 3.3, figure 3.24). This is in contrast with the findings of Masclaux-Daubresse and Chardon, and Barraclough et al in Arabidopsis and wheat, that N nutrition had no impact on harvest index (Masclaux-Daubresse and Chardon, 2011, Barraclough et al., 2010). Season did not significantly affect HI on low N (figure 3.25, table 3.4). When comparing the low plasticity and high plasticity groups, the low plasticity lines had higher HI on both high and low N than the high plasticity lines, irrespective of the season (figure 3. 18 a, b). As HI is the seed proportion of the above ground dry matter this supports the hypothesis that the low plasticity lines

follow the strategy of completing their life cycle quickly, without responding to N supply, setting as many seeds as possible within their short life span.

3.8.9 Root proportion is affected by N

Shifts in resource partitioning between developing shoots and roots is an adaptive response to environmental cues such as N availability. In the current study residual shoot dry matter (RSDM) accumulation was higher on high N for all lines, as expected (figures 3.17 a, b). This result is similar to other studies specifically in *Arabidopsis* where it was shown that low N leads to a reduction in shoot dry matter (Loudet et al., 2003b). Here again, a comparison of high and low plasticity lines showed that these two groups used the same limiting resource in different ways. On high N, the high plasticity lines accumulated more RSDM than low plasticity lines. But low plasticity lines maintained higher RSDM on low N. Lines that had consistently high branching plasticity such as 11 and 471 displayed consistently low RSDM retention on low N (figures 3.17 c, d). The trend of low plasticity lines having higher RSDM retention on low N might contribute to higher seed yield retention (figures 3.14 a, b) on low N by these lines. The fact that RSDM and seed yield are strongly and positively correlated (table 3.5) adds weight to this hypothesis. Shoot to root ratio increases in plants grown on high N, has been previously reported by Scheible et al (2004). This is in agreement with my preliminary studies. It was observed that highly plastic lines shifted their root fractions more on low N (figure 3.20). In contrast, the low plasticity lines do not shift their root fraction as much, but rather maintain the same resource allocation programme on both low and high N, making a similar amount of root, shoot and seed. The later flowering highly plastic group on the other hand, adjusts their growth habit to optimise nutrient capture, by elaborating their root system.

3.8.10 Similar Nitrate levels in shoots and seeds in high plasticity lines regardless of the N supply.

The measurement of Nitrate concentrations in the RSDM and seeds revealed very interesting and differing patterns of Nitrate accumulation among the high and

low plasticity groups. Nitrate concentrations were consistently higher only on high N for the low plasticity group in both RSDM and seeds (figures 3.21, 3, 22). In *Arabidopsis*, most of the Nitrate taken up through roots was transported to shoots, assimilated in shoots and deployed for synthesis of amino acids (Masclaux-Daubresse et al., 2010). One could argue that the short life cycle of these plants contributes to them not making full use of the Nitrate available to them. However, the reasoning against this is that these lines have less time to take it up as well as to use it, so flowering early does not mean that they don't use the N. If it was C limitation, then these lines have no advantage in wasting C by taking up N that cannot be used. Several studies have suggested that N reserves in plants are an indicator of how well they can cope with limiting N conditions (North et al., 2009, Richard-Molard et al., 2008). The analysis of shoot Nitrate levels of the lines studied does not follow the above mentioned hypothesis. None of the lines had particularly high levels of Nitrate when N was limited. There was substantial variation in both shoot and seed Nitrate when grown in two different seasons. Enormous variation in endogenous Nitrate levels among seed batches from one species has been reported by Derkx and Karssen (Derkx and Karssen, 1993) in plants grown under greenhouse conditions. Further repeats are necessary before any conclusions are made about the seasonal influence on Nitrate accumulation. It would have been interesting to have measured total N content in the shoot dry matter and seeds in this study, which might have given better information about N resource allocation. Samples were collected and prepared for estimation of total N; however this work could not be completed due to a machine fault.

3.8.11 Strategies adapted by different lines

Plasticity observed in shoot branching can be considered as an adaptive developmental response of plants to environmental cues, which in this study was N limitation or abundance. It was evident that there was extensive variation in plasticity among lines of same plant species, and associated differences in their strategies to cope with N limitation. It cannot be intuitively assumed that the

strategies of MAGIC lines are adaptive because they are not naturally selected. Nevertheless several lines of evidence generated from this study suggest that the strategies are indeed adaptive. It was evident that even among the relatively early flowering lines (all the lines selected for my study fall within this category) a range of strategies were adopted by different lines faced with different N availability. Lines studied responded to N limitation in two ways:

- 1) They were insensitive to N availability, proceeding quickly through their life cycle, investing existing resources in seed.
- 2) They invested resources in foraging for additional N

Among these, the first strategy, adopted by low plasticity early flowering lines, appears to improve fitness-related traits on low N, in comparison to the second strategy. The second strategy of the late flowering, high plasticity lines, may be favoured in real life if there is a likelihood of more N arriving. The strategy of these lines maximize the chances of acquiring more N as they have increased time available for completing the life cycle, increased carbon accumulation as a consequence of having more leaves, which is used to elaborate their root system. This leads to modifications in root fraction. Modification of root fraction when N is limited has been well documented (Lea and Azevedo, 2006). A recent study using 23 Arabidopsis accessions grown on N sufficient, N limited and N starved situations identified accessions with different responses and different growth strategies providing a generalized response of Arabidopsis to these conditions (Ikram et al., 2012). However, their study focused mainly on the differences between the N limitation and N starvation response.

A plant makes many changes to adapt to N limitation and the situation on low N exemplifies the co-ordination of many changes in different traits that contribute to the phenotype of whole plant on low N (figure 3.28). More trait combinations are correlated significantly on low N than high N. This could be because on low N, plants are driven to make more trade-offs between traits.

3.8.12 Causal links between correlations

Significant positive and negative correlations between traits were summarized to identify the traits that were most correlated (table 3.5, figure 3.33). Analysis of correlations between traits showed that when plants encountered different environments – in this case N sufficiency or N limitation, all traits were not correlated in a similar way. Because different traits respond differently to N, the magnitude and direction of the correlations between traits varied with the N status. A positive correlation between RSDM and flowering time on high N (although not significant) and a lack of correlation between these two on low N (figure 3.27 c) suggests that delayed flowering does not offer any advantage to plants when resources are permanently limited. However, it could be reasonably assumed that if new N sources arrive in the mean time this strategy is advantageous. Late flowering high plasticity lines had not just higher RSDM but also higher seed yields on high N (figure 3.30 b). In this study flowering time is positively correlated with plasticity regardless of N supply. When considering why these two traits are correlated there are two possibilities. One is that late flowering lines are more N starved and hence respond more strongly to N deprivation, in which case there is a direct causal link. Or it could be that plasticity and flowering time are influenced independently by the same underlying mechanism. The second is suggested by the above discussed data that flowering later in autumn does not automatically make them more plastic. Lack of change in plasticity of some low plasticity lines for example 11, in summer vs autumn, despite the delay in flowering support this possibility.

3.9 Summary

This study provides very good examples of two entirely different adaptive strategies of plants to cope with the same environmental cue – N availability. In one, low plasticity is linked to early flowering, medium number of branches on both high and low N, higher seed yields and yield retention on low N, low RSDM on high N, lower root fraction when N is limited and higher shoot and seed Nitrate concentrations on high N. In the other, high plasticity is linked to late flowering,

high branch numbers on high N, higher seed yield on high N, high RSDM on high N, higher root fraction on low N and similar shoot and seed Nitrate levels on both high and low N.

The first strategy protects yield on chronically low N, but prevents effective exploitation of abundant N. The second strategy involves investing in roots when N availability is low, and consequently these lines have lower yields when N is permanently low. However, they do relatively well on high N because they make more effective use of the additional N available by allocating it to extra branches, extra flowers and extra seed. Neither of these strategies could be considered as the 'best strategy' as the different strategies would be successful or less successful depending on the environment in which the plant is growing and its variability.

Since MAGIC lines have a 'random shuffling' of the 19 parental genomes it is very likely that these strategies may not be optimal as they have not undergone natural selection. However the strategies displayed by these lines do represent possible combinations that could exist in nature and will be informative for future studies.

Chapter 4

Hormone response studies on high and Low N

4.1 Introduction

Chapter 3 identified some of the strategies adopted by plants to cope with external environmental conditions, such as limited nutrient availability. This chapter focuses on an endogenous hormone strigolactone (SL) and its role in integrating of nutrient supply with internal processes. As mentioned in the previous chapter, any change or adaptation a plant makes in response to an environmental cue is not an isolated process. Plasticity exhibited by plants is the result of a highly co-ordinated and interactive network of events, which shapes the final architecture of the plant, and hormones play the role of communication officers. Unlike animals, which have a central nervous system, plants rely on versatile hormones for making timely decisions about growth and development in ever fluctuating environments, throughout their life cycle. As expected from good communication co-ordinators, hormones are multifunctional, mobile, highly responsive, affect different aspects of growth and they relay information both locally and globally within the plant and between the plant and environment.

The effects of hormones on shoot branching and on influencing the architecture of a plant has been a topic of study in several plant species for many years. The main hormones regulating shoot branching in plants are auxins, cytokinins and the recent addition, strigolactones (SL). Among these three, less is known about SLs because of the recent discovery of their shoot branching regulatory role. However, the role of SLs as a rhizosphere signal has been known for longer. SLs were identified in root exudates of the host plants of parasitic plants such as striga, (giving them the name strigolactones) and orobanche and they trigger germination of the seed of the parasitic plants (Humphrey and Beale, 2006). Involvement of SLs in symbiotic relationships between plants and arbuscular mycorrhizal fungi, including inducing hyphal branching, was first reported in 2005 (Akiyama et al., 2005). A study from Harro Bouwmeester's group (Matusova et al., 2005) suggested that SLs had a carotenoid origin and that they were distributed widely in the plant kingdom as expected given their role in mycorrhization. Many recent studies emphasized the involvement of SLs

in nutrient responses in the root through their role in mycorrhization. It has been reported that SL production in plant roots increases when nutrients are limited, which is in accordance with symbiotic fungal association (Lopez-Raez et al., 2008, Yoneyama et al., 2007b, Yoneyama et al., 2007a, Lopez-Raez and Bouwmeester, 2008, Yoneyama et al., 2011). These studies provided evidence that SL levels are upregulated by both P and N deprivation. A dual role of SL as a phosphate acquisition signal and in inhibiting tiller outgrowth in rice for optimum phosphate utilization was proposed by Umehara et al (Umehara et al., 2010a) and in Arabidopsis its role in shoot branching response to Phosphate was reported by Kohlen et al (Kohlen et al., 2011).

Despite the recent increase in studies on new roles of SL, the mechanism of action of strigolactones in inhibiting shoot branching remains a matter for debate since its discovery as a branch regulating hormone. Currently there are two main models explaining the shoot branching regulatory activity of SLs. The first one (Brewer et al., 2009) focuses on the action of SLs as a local bud event, based on the ability of direct application of SLs to inhibit bud outgrowth in Pea and Arabidopsis. The second model (Bennett et al., 2006; Prusinkeiwicz et al., 2009) proposes that SLs inhibit bud outgrowth by modulating auxin transport, based on computational modelling and studies of auxin transport phenotypes of SL mutants, showing that SLs can act systemically to dampen auxin transport (Crawford et al 2010).

4.2 Background and aims

The wealth of information accumulating around SL and the already established tools provided an exciting platform to investigate further the role of this hormone in communicating environmental cues, such as nutrient availability, within the plant. This chapter makes use of hormonal assays, and in particular two node assays, developed by Ongaro et al (2008) to investigate the role of SLs in communication between buds. Ongaro et al established these assays using two consecutive nodes excised from Arabidopsis plants, somewhat similar to the

classical two branch assays in pea and bean used extensively to study apical dominance (Snow, 1929). It was evident from her study with two node systems that consecutive branches on an Arabidopsis stem communicated with each other and that branching inhibitory signals acted in both directions as the upper branch could inhibit the lower branch and vice versa. Suggestions of competition between buds across the stem mediated by auxin were evident in the classical pea and bean assays as well as in the two node Arabidopsis experiments. Although V. Ongaro worked with *max* mutants in her study, SLs were only introduced into the scene after the time of her study and this assay provided a good platform to test the effect of SL treatments.

Information from modelling studies over the years also has been very helpful in understanding apical dominance and different aspects of shoot branching, as well as in generating several hypotheses to test in plant experimental systems. Results from the previously mentioned hormonal assays (Ongaro et al., 2008) have been fed into the simulation modelling studies in the Leyser lab in collaboration with Prusinkiewicz (Prusinkiewicz et al., 2009). This model provided mechanistic explanations for the action of auxin in regulation the timing and pattern of bud activation in different Arabidopsis genotypes. Further modelling studies are ongoing, incorporating SLs to understand the mechanisms of interaction between auxin and SLs. In this context, hormonal assays with SLs can provide crucial information to understand the co-ordination between auxin and SLs.

The work described in this chapter aims to understand the mechanisms by which SLs regulate bud activity and nutrient response, by analysing their effect on bud growth using buds on isolated one, two and three node segments. Some of the questions addressed include,

Is SL involved in setting the level of competition between buds?

To what extent is this affected by nutrient status?

How does interaction between SLs and N affect activation and/or elongation of buds?

Is there any correlation between branching response to N and branching response to SLs in the 2 node and 3 node systems?

Does SL have any effect on the pattern or timing of bud activation and is this affected by N availability?

Do the experimental data fit with the results of ongoing modelling studies?

With MAGIC lines - Is there any match between the whole plant phenotypes and bud hormone responses?

4.3 Plant growth and experimental set up

Plants were grown on F2 compost as described in Chapter 2 (page 52) for the first set of one node and two node assays with Columbia, *max2* and *max4*. For further assays on high and low N, plants were grown on 50% sand and 50% Terragreen in 2" pots and fed by high or low N solutions as described in Chapter 2 (page 53). A schematic representation of the two bud assay set up is shown in figure 4.1.

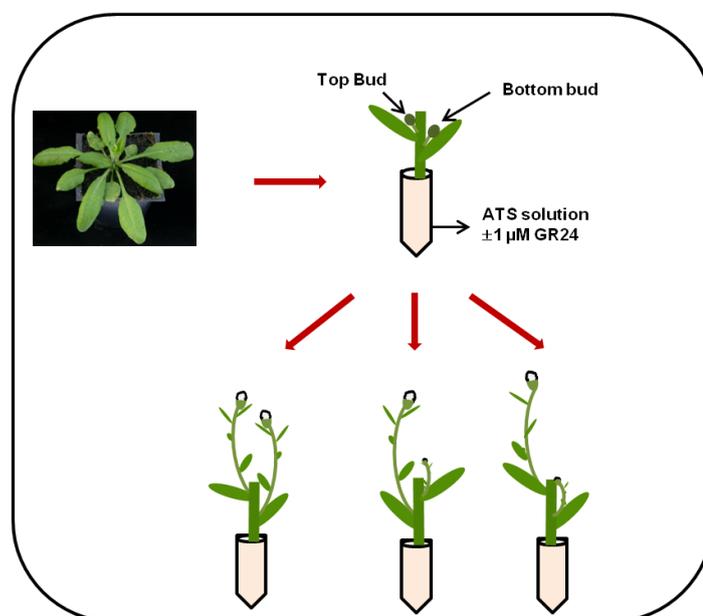


Figure 4.1 Schematic representation of two node assay set up.

4.4 Assays to characterise bud SL responses

4.4.1 Single node assays of Columbia, *max2* and *max4*

To determine the effect of SL on buds on isolated nodal segments, Col, *max2* and *max4* plants were grown on F2 compost. Nodal segments were excised at the developmental stage shown in figure 4.2. Applying synthetic SL analogue, GR24 basally had no effect on bud growth in any of the three genotypes- Columbia, *max2* or *max4* (figure 4.3), particularly at earlier time points. Buds of all three genotypes activated and elongated with no differences between the controls and GR24 treatments at day 4 (figure 4.3). *max2* buds (both control and treated) did not elongate as much as either Columbia or *max4* buds, consistent with the reduced stature of *max2* plants. Given that the focus of this study is bud activation, it was decided to focus on these earlier time points in all further one node assays.

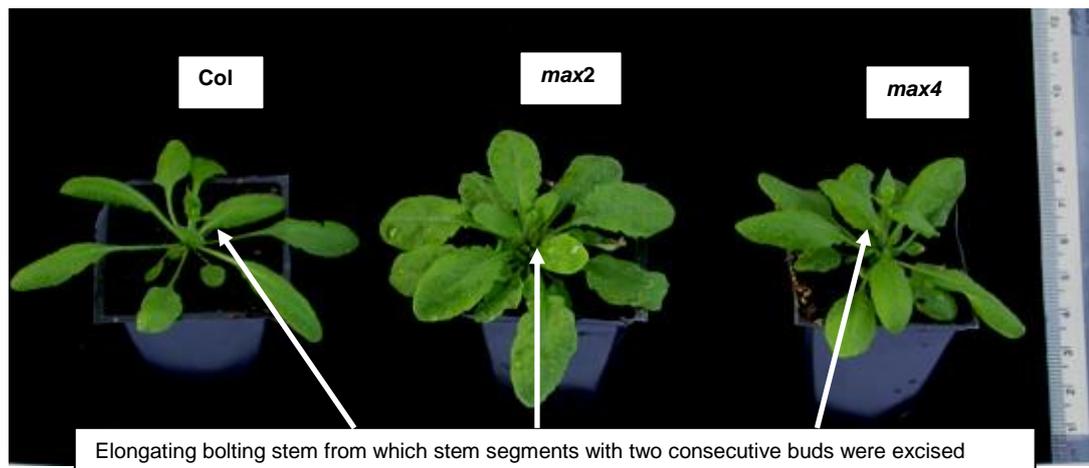
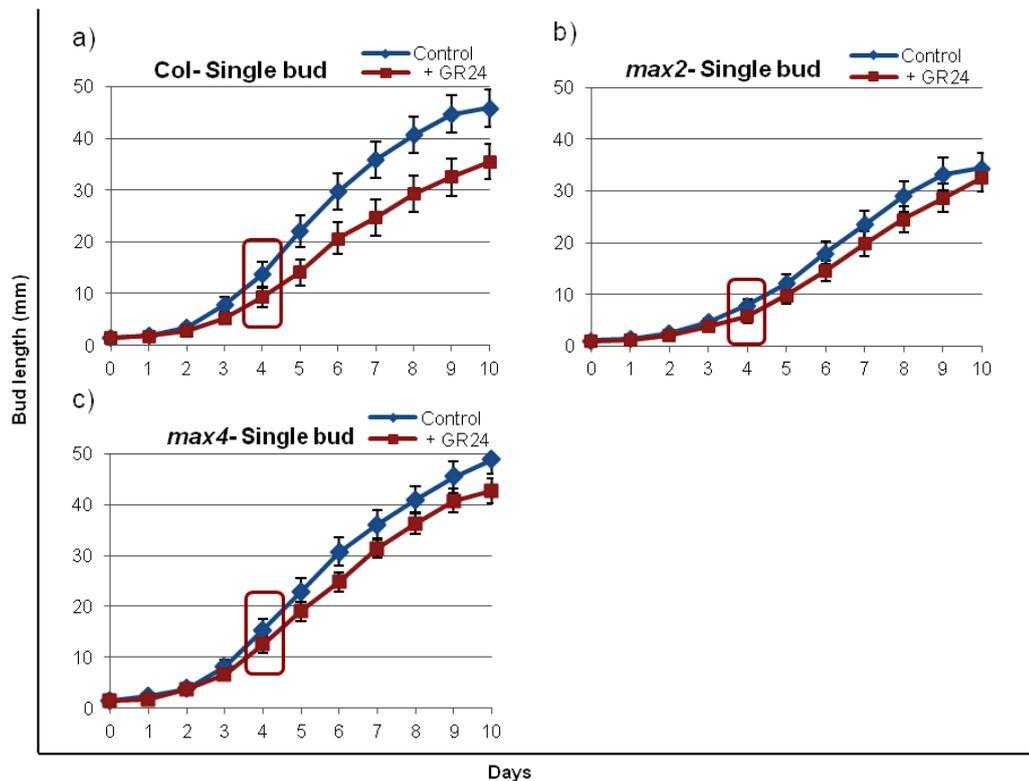


Figure 4.2 Col, *max2* and *max4* plants at the stage chosen for excision of stem segments for bud assays.



4.3 Bud length (mm) of *Col*, *max2* and *max4* buds in one node assays with or without GR24 (1 μ M). Means \pm SE of 16 buds are shown.

4.4.2 Two node assays of Columbia, *max2* and *max4*

In contrast to the one node situation, SLs had a greater effect when two buds were present. The results of two node experiments (figure 4.5) showed that in untreated Columbia explants, while sometimes both buds activated, often one bud grew vigorously, while the other was inhibited. In *max2* and *max4*, both buds activated more frequently. These results are consistent with those of Ongaro et al (2008). With GR24 treatment, *max2* was unaffected, but for Columbia and *max4*, GR24 focused growth into one of the two buds. The mean length of both the top and bottom buds was reduced in Columbia and *max4* (figure 4.5), with the mean bottom bud length being more strongly reduced than that of the top bud. Among the three genotypes, Columbia was the most affected by GR24 treatment, followed by *max4*, with *max2* buds being unaffected. Mean bud lengths are of limited value in this assay, because of the tendency for one bud to dominate the other. The relative growth index (RGI) is a more useful measure, because it can be used to assess the extent to which one

bud dominates (Ongaro et al., 2008). The RGI is defined as the length of the longest bud divided by the sum of the lengths of both buds. It can be between 0.5 (if both branches grow equally) and 1 (if one branch completely dominates the other branch, which does not grow out).

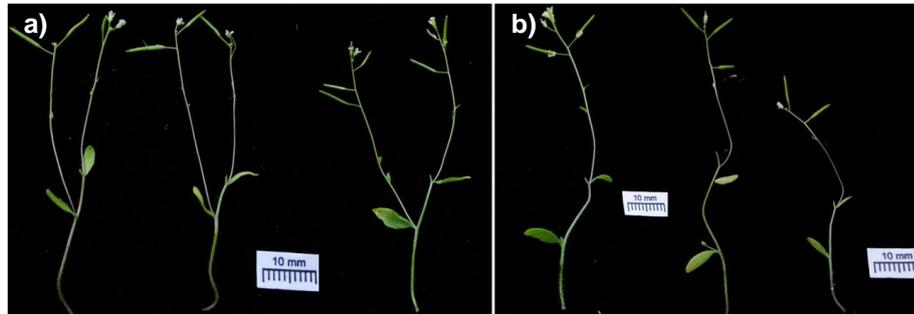


Figure 4.4 a) Explants where both buds grow out, b) explants where one bud dominates.

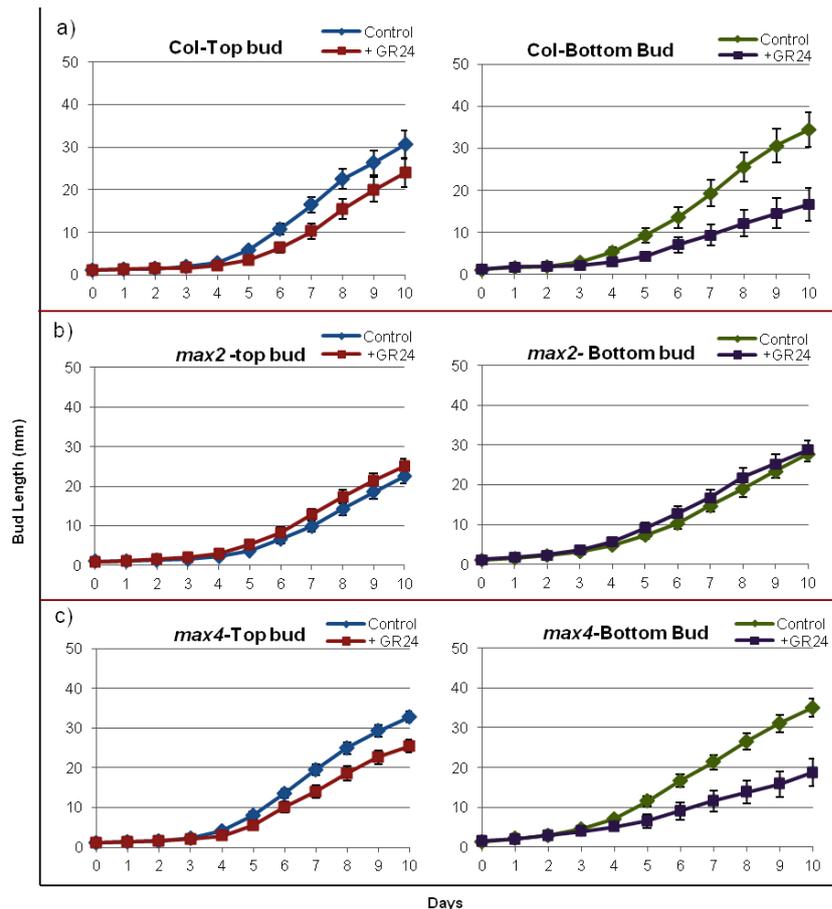


Figure 4.5 - Bud lengths (mm) of a) Col, b) *max2* and c) *max4* in two node assays with or without GR24 (1 μ M). Means \pm SE of 16 plants are shown.

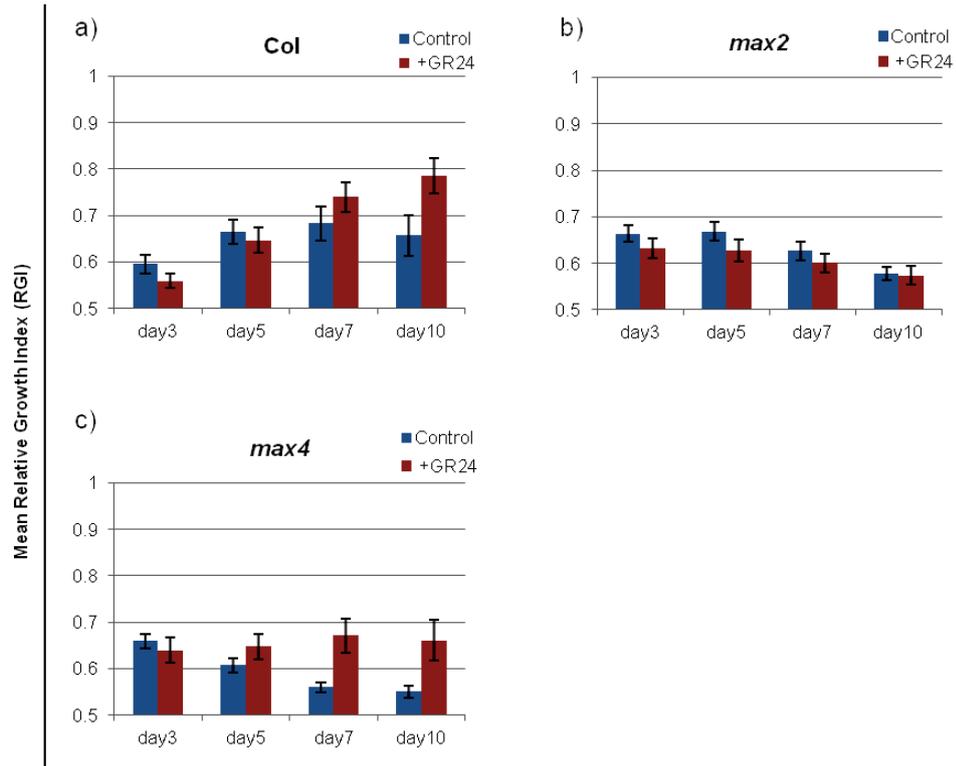


Figure 4.6 - Mean Relative growth index (RGI) of a) Col, b) *max2* and c) *max4* in two node assays with or without GR24 (1µM). Means ± SE of 16 plants are shown.

In the untreated controls, both *max* mutants had lower RGI than Columbia, as previously observed by V. Ongaro (Ongaro et al., 2008). At the beginning of the experiment, all the buds are small and have similar lengths, but when they start growing, the RGI of Columbia increases over time as one bud continues to grow but the other stops growing (figure 4.6 a). In contrast, for the *max* mutants the difference between branch lengths does not increase over time. There is no increase in RGI between day 7 and day 10 for *max4* (figure 4.6 c) and for *max2* the RGI decreases between day 7 and day 10 (figure 4.6 b).

GR24 treatment increased the RGI (figure 4.6) for Columbia (RGI on day 10, Control – 0.65 ± 0.04 and treated – 0.78 ± 0.03), indicative of the dominance of one branch (usually the top branch in this case) over the other. A similar increase in RGI was observed with GR24 treatment of *max4*, although the effect was weaker than Columbia (RGI on day 10, Control – 0.55 ± 0.01 and treated –

0.66 ± 0.04). GR24 treatment did not have any effect on the RGI of *max2* (RGI on day 10, Control – 0.58 ± 0.01 and treated – 0.57 ± 0.01) indicating similar branch lengths for both top and bottom branches.

When comparing bud lengths on single bud and two bud explants, it was also evident that the buds in the two node system activated more slowly than in the case of single nodes (figures 4.3 & 4.5). Bud length on day 6 in the two node system was comparable to the bud length in the single node system on day 4.

4.4.3 Further analysis of Columbia bud behaviour

The different behaviours of buds in the one Vs two node system could be due entirely to the second bud, or to a combination of the second bud and the additional stem and leaf. To determine whether the stem and leaf contributed, assay systems differing from the classic two node assay were set up, in which two node segments were excised, but either the top bud or the bottom bud was removed, leaving the leaves intact (figure 4.7 b, c). The results of these experiments showed that buds activated much faster in the single node system than any of the two node assay system set ups (figure 4.7).

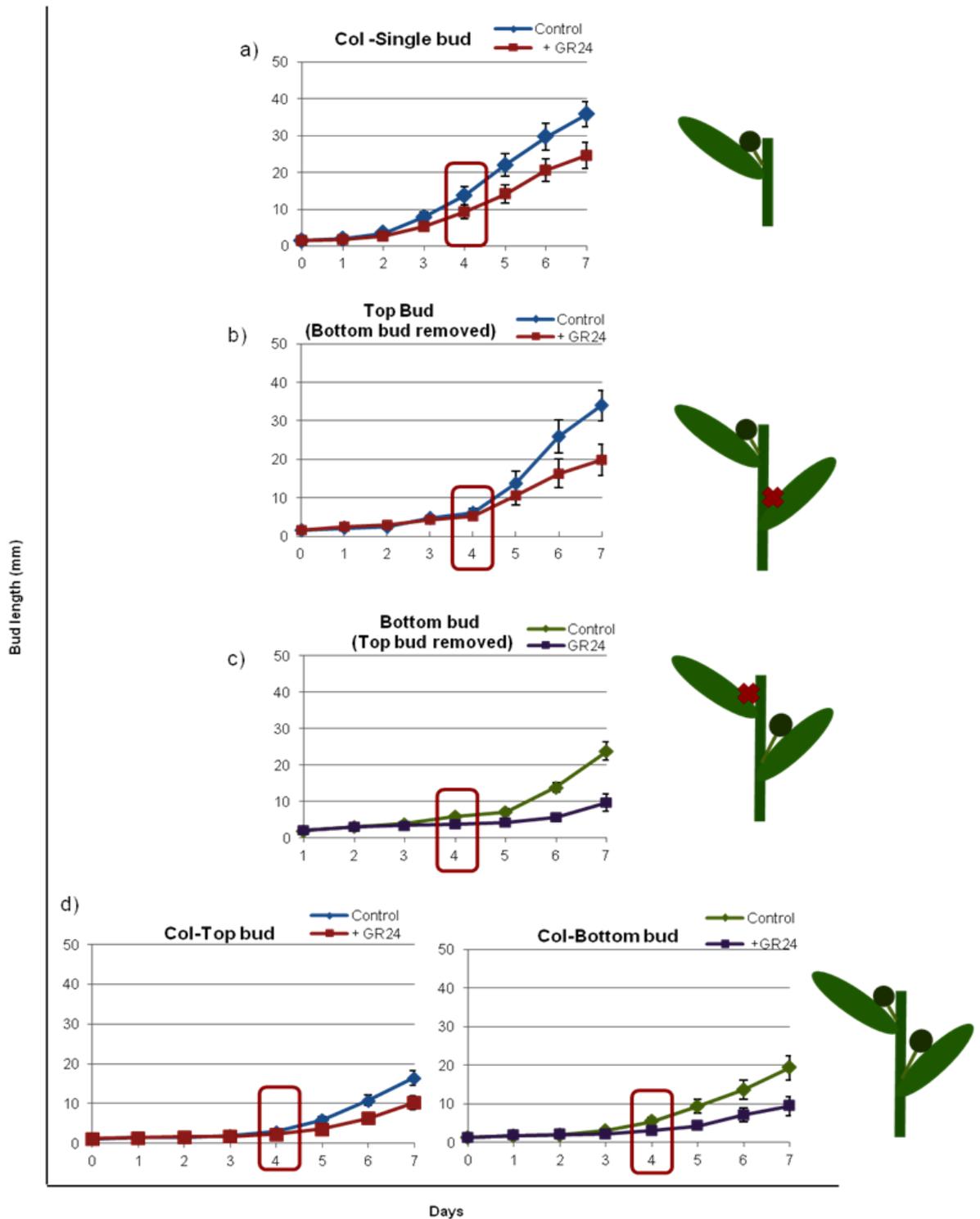


Figure 4.7 Summary of bud lengths (mm) of Col a) single bud b) single top bud (bottom bud alone removed) c) single bottom bud (top bud alone removed) and d) two buds assays with or without GR24 (1 μ M). Means \pm SE of 10-16 plants are shown.

A comparison of the different assay set ups and the differences between them can be viewed in figure 4.8, which shows bud lengths on day 4. These data indicated that GR24 had a greater effect on bottom buds than on top or single buds.

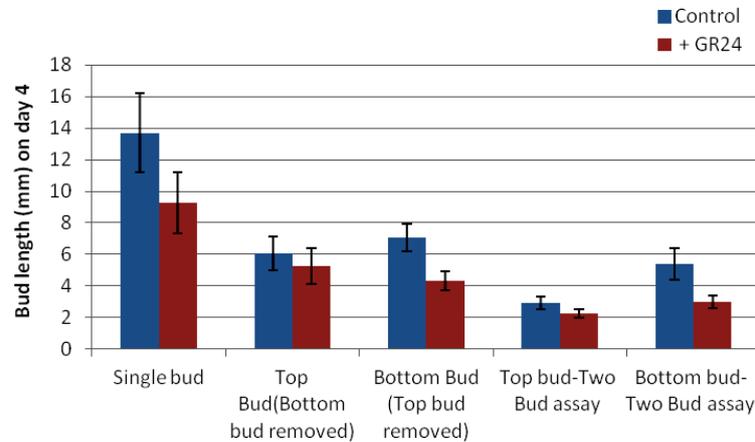


Figure 4.8 Bud lengths of different assay set ups on day 4.

4.5 Hormone response assays on high and low N

To assess the relationship between the shoot branching responses to N and to hormones, response of buds to SL in nitrogen sufficient (high N- 9mM Nitrate) and limited (low N-1.8mM Nitrate) situations were compared. In these assays, an elongating bolting stem segment with one, two or three cauline nodes (figure 4.9) were used.



Figure 4.9 Excised Arabidopsis buds at the stage taken for a) one node, b) two node and c) three node assay set up. Apex left intact in the two node and three node set ups in this figure to indicate the distance from the apex. When performing the assays the main apices were removed.

4.5.1 Single and two node assays of Columbia on high and low N

Columbia buds on single nodes from plants grown on high N elongated more than those on low N, corresponding to the whole plant behaviour on high and low N. There were no differences between the control plants and the GR24 treated plants on either low or high N (figure 4.10).

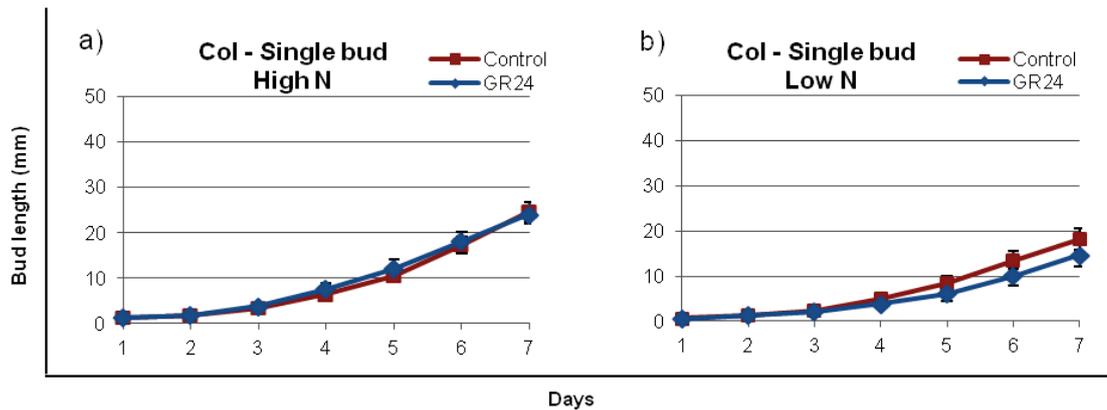


Figure 4.10 Bud lengths (mm) of a) Col in one node assays with or without GR24 (1µM). Means \pm SE of 10-16 plants are shown.

When two node explants were taken from sand-Terragreen grown plants fed with high or low N (figure 4.11), unlike plants grown on F2 compost (figure 4.5), the mean length of the top bud was greater than that of the bottom bud (figures 4.11 a, b). On high N, there was no difference between the control and GR24-treated top branches, whereas GR24 treatment led to complete inhibition of bottom branch (figure 4.11 a). On Low N, both control and GR24 treated buds resembled the equivalent GR24 treated bud on high N. There was no difference between the bud lengths of control and treated top buds, and the bottom buds of both control and treated plants were completely inhibited (figure 4.10 b). These effects of N and SL are clearly depicted in the relative growth index graphs (figures 4.12 a, b). GR24 increased the RGI on high N on day 7 (figure 4.12 a), at which time on low N, the RGI was high even without GR24 treatment (figure 4.12 b). The RGI of both control and GR24 treated plants on low N was comparable to the RGI of GR24 treatment on high N.

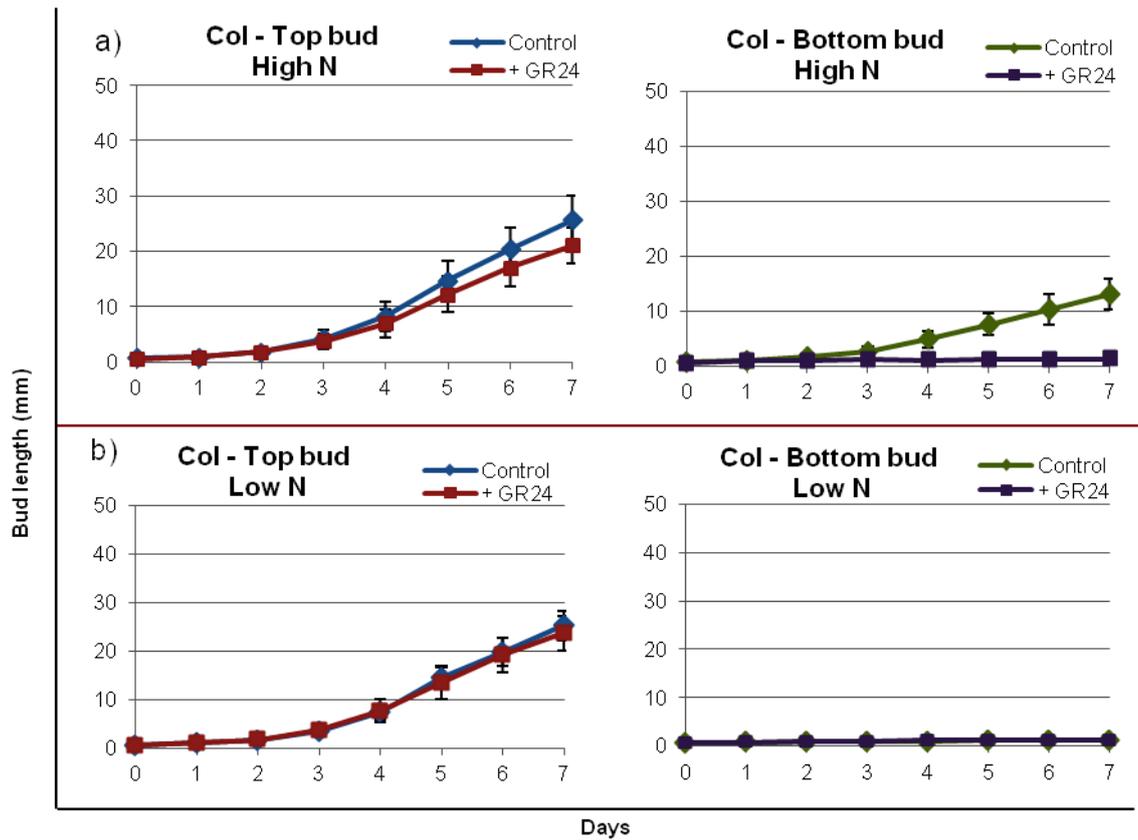


Figure 4.11 Bud lengths (mm) of Col in two node assays on a) high N (9mM Nitrate) and b) low N (1.8mM Nitrate) with or without GR24. Means \pm SE of 10 plants are shown.

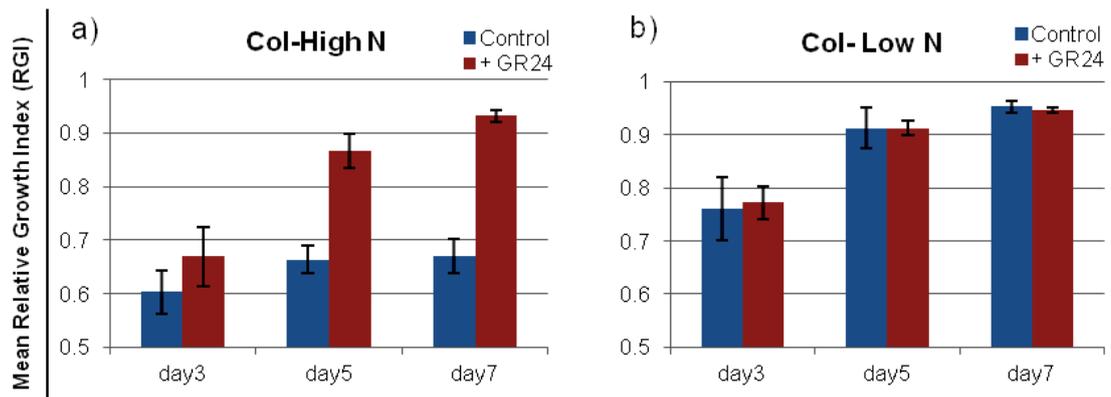


Figure 4.12 Mean Relative growth index (RGI) of Col in two node assays on a) high N (9mM Nitrate) and b) low N (1.8mM Nitrate) with or without GR24 (1 μ M). Means \pm SE of 10 plants are shown.

4.5.2 Comparison of Columbia, *max2* and *max4* two bud assays on high and low N

While investigating the effects of low SL on N response, the same assays were used to compare Col and SL mutants (figure 4.13). Results from Columbia explants on both high and low N (figures 4.14 a, b) confirmed the results of the above experiments (figures 4.11 a, b). For *max2*, bud lengths of neither the top nor the bottom bud were affected by GR24 treatment on high or low N (figures 4.13 c & d). With *max4*, the bottom bud alone was affected by GR24 treatment on high N, similar to Columbia but to a lesser extent (figures 4.14 e, f). However on low N, bottom *max4* buds grew, but were inhibited by GR24, unlike the complete inhibition of the bottom bud observed in Columbia plants (figure 4.13 b, f).

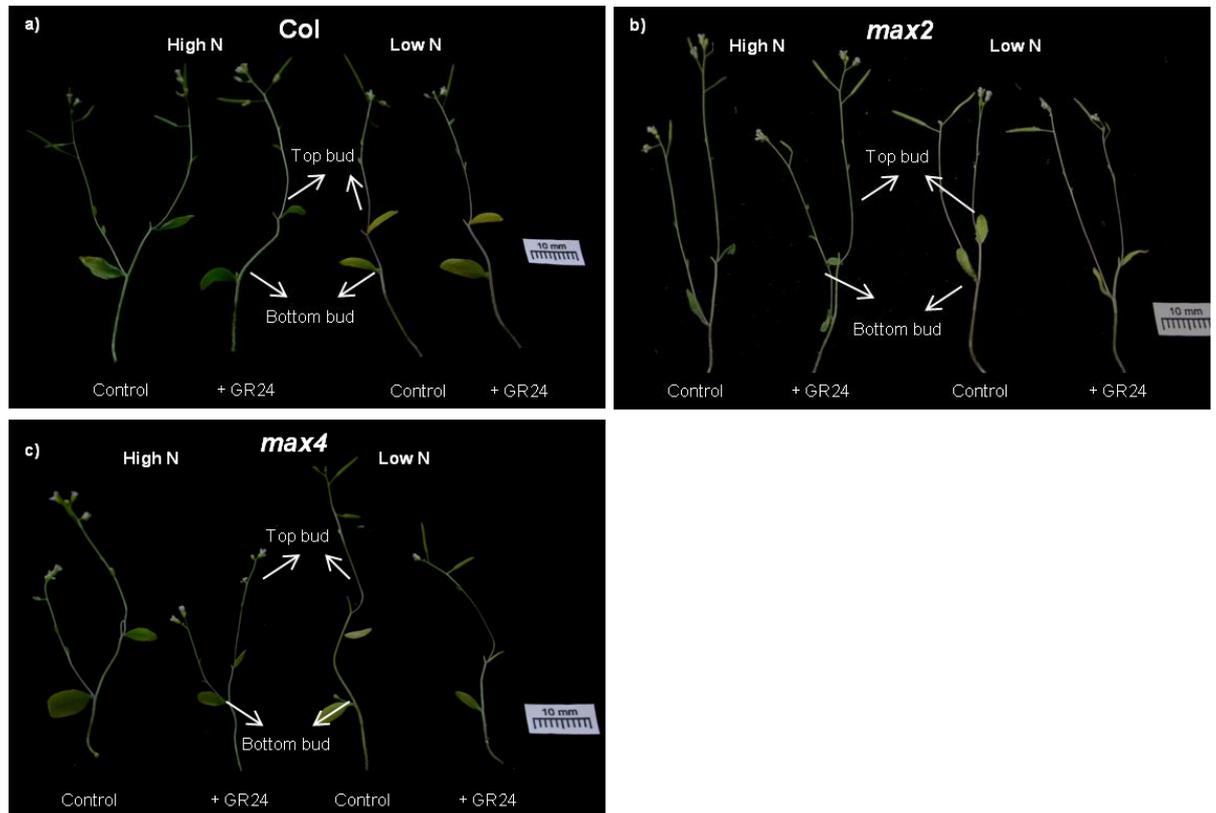


Figure 4.13 Bud elongation of control and treated buds of a) Col, b) *max2* and c) *max4* on day 7 in two node set up.

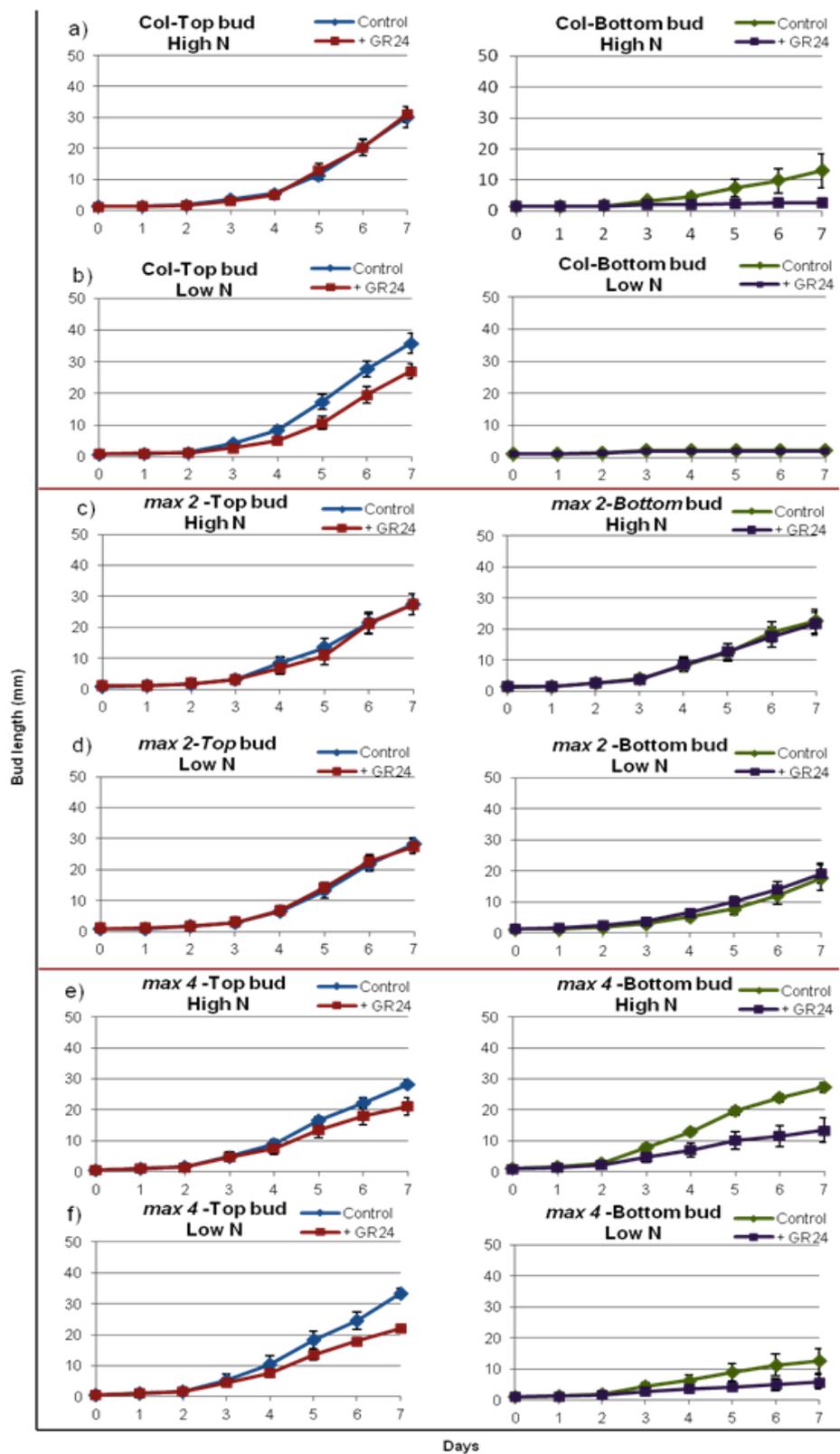


Figure 4.14 Bud lengths (mm) of Col on a) high N b) low N, *max2* on c) high N d) low N and *max4* on e) high N f) low N in two node assays with or without GR24. Means \pm SE of 10- 12 plants are shown.

Mean bud lengths of all the three genotypes on day 3, 5 and 7 were plotted (figure 4.15) to allow direct comparisons of the trends in bud lengths on high and low N at those time points. A comparison of the RGI of the three genotypes on high N (figures 4.16 a, c & e) clearly showed that both *max2* and *max4* had lower RGIs than Columbia in both control and GR24 treated plants on day 5 and 7. RGI increased over time in the GR24 treated explants for both Columbia and, to a lesser extent in *max4*, and the RGI of *max2* was unaffected by GR24 (figures 4.16 a, c & e).

On low N, there was no massive difference between the RGI of control Vs GR24 treated samples for any of the three genotypes. GR24 treatment did not increase RGI on low N, but the low N RGIs were higher than those on high N for both Col and *max4* (figures 4.16 b, d & f). There was no difference in the bud lengths of control compared to GR24 treated plants on day 7 for Col, *max2* or *max4* (figures 4.15 b, d & f). All the three lines had similar RGI for both control and GR24 treated plants on low N. This supports the idea that low N response is mediated by SL. *max2* does not shift its RGI at all with GR24 treatment either on high or low N (figures 4.16 c & d). When comparing the effect of low N on RGI in the different genotypes, while Col shows a significant increase in RGI on low N, *max4* RGI is slightly affected (figures 4.6 e, f) and *max2* RGI is unaffected (figure 4.16 c, d).

4.15

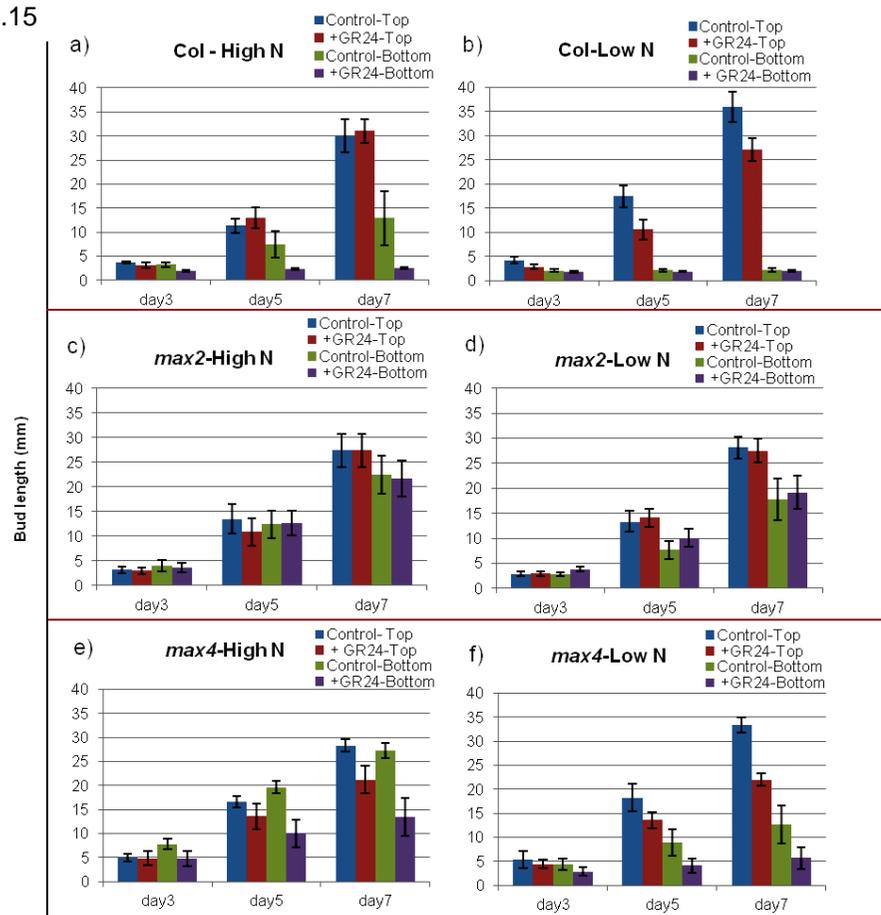


Figure 4.15 - Mean Bud length of a) Col on high N b) Col on low N c) *max2* on high N d) *max2* on low N and e) *max4* on high N f) *max4* on low N in two node assays on days 3, 5 and 7 with or without GR24. Means \pm SE of 10-12 plants are shown.

4.16

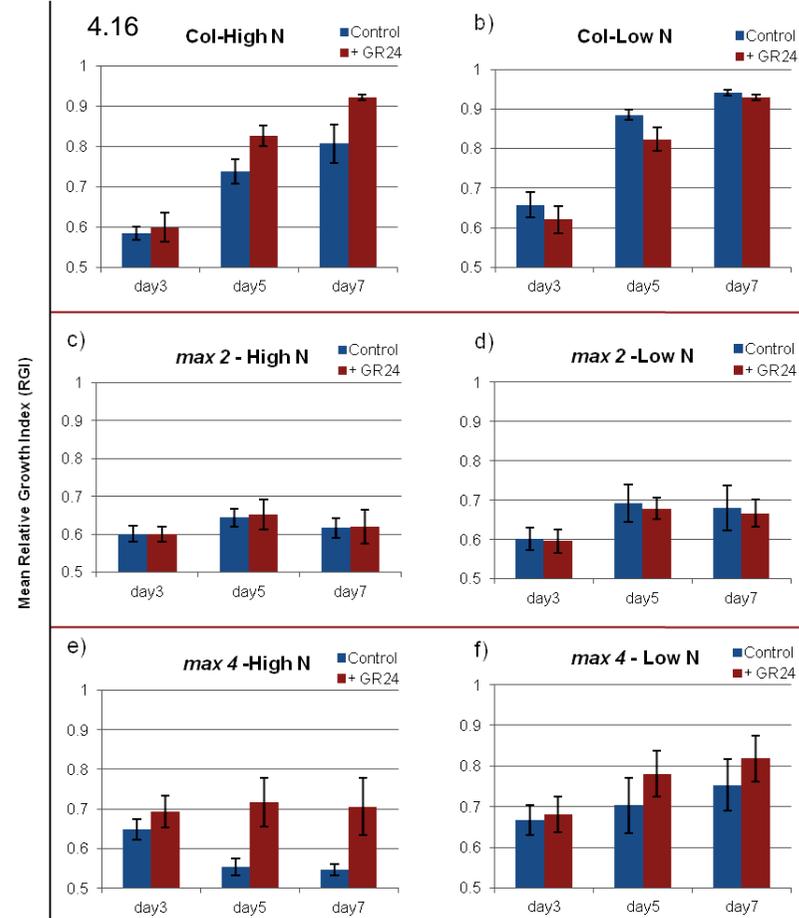


Figure 4.16 - Mean Relative growth index of a) Col on high N b) Col on low N c) *max2* on high N d) *max2* on low N and e) *max4* on high N f) *max4* on low N in two node assays on days 3, 5 and 7 with or without GR24. Means \pm SE of 10-12 plants are shown.

4.5.3 Single node assays of Columbia with shift in N status

While the studies on response of buds from single and two node systems to SL on high and low N were performed, it was noticed that buds from plants grown on low N were smaller at the stage taken for assay than those grown on high N. However, it was not clear whether they were at a similar or different physiological and/or developmental stage from the buds on high N. To try to control this, the starting material was normalised by growing all plants on high N, and then shifting them onto high or low N solutions at the time of assay set up, ensuring uniformity among the buds at the start of the assay. The reciprocal shift, from low N to high N was also investigated.

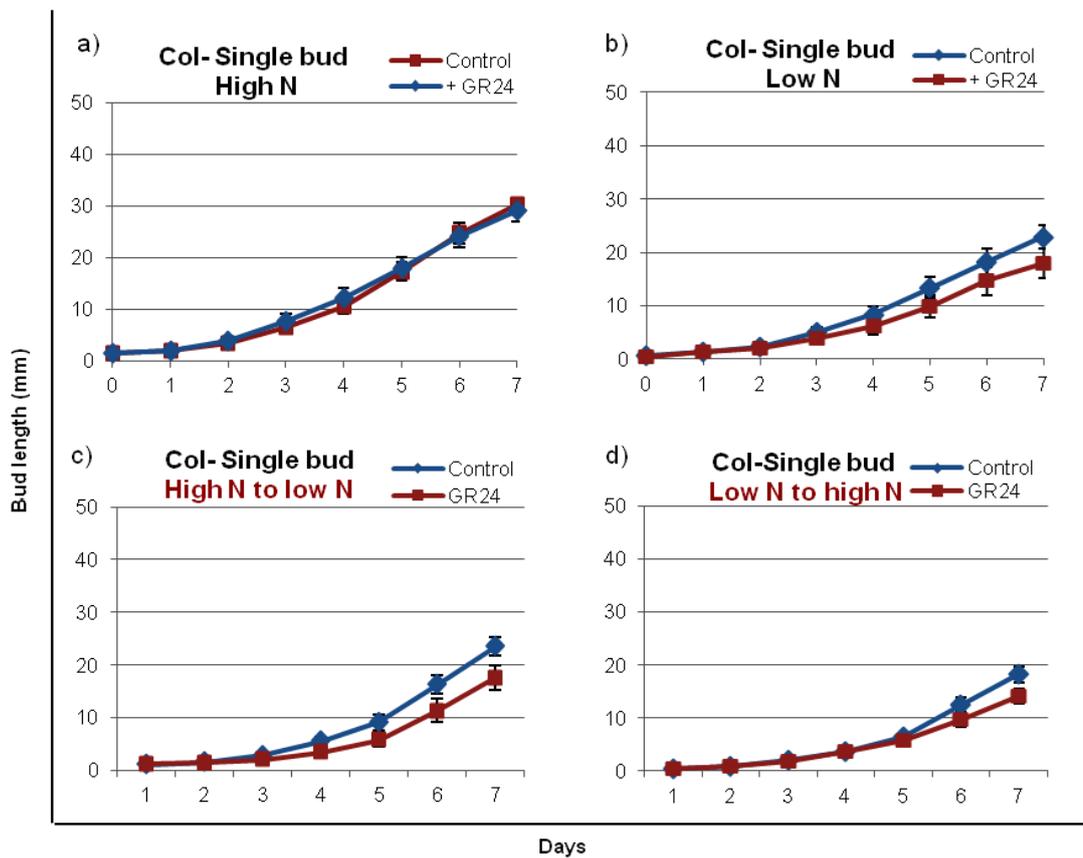


Figure 4.17 Bud lengths (mm) of Col single bud on a) high N b) low N, c) high to low N d) low to high N in one node assays with or without GR24. Means \pm SE of 10 plants are shown.

Figure 4.17 shows a comparison of four different single node assay set ups. Plants were grown on high N and the excised single nodes were transferred to high N nutrient solution with or without GR24. As seen in the previous one node assay set ups, GR24 was unable to inhibit bud outgrowth. Similarly, when plants were grown on low N and single nodes transferred to low N nutrient solutions with or without GR24, the buds did not respond to GR24. Both control and treated buds elongated to a lesser extent than on high N as previously observed (see final length at day 7).

When plants were grown on high N and excised single nodes transferred to low N (figure 4.17 c) they behaved similar to those grown continuously on low N (figure 4.17 b) with respect to bud length and response to GR24, indicating a very rapid response to N status shifts. However, when plants were grown on low N and excised single nodes transferred to high N (figure 4.17 d), bud elongation was not restored to the levels observed for buds grown continuously with high N (figure 4.17 a).

Although the graphs in figure 4.17 show a big effect of N and a small effect of GR24 on bud length it was difficult to assess the timing of bud activation from this analysis. Hence an attempt was made to consider the timing of bud activation and bud elongation separately, although these two processes overlap. In this analysis (developed by M. Domagalska) a threshold bud length of 3mm was chosen and buds were defined as active once they reached this threshold. Figure 4.18 shows the mean number of days it took for buds to activate according to this definition, for different Nitrogen and GR24 treatments. GR24 had no effect on timing of bud activation when buds grown on high N were transferred to high N. But GR24 delayed bud activation when buds grown on high N were deprived of N. GR24 had very little effect on buds grown continuously on low N or those transferred from low to high N.

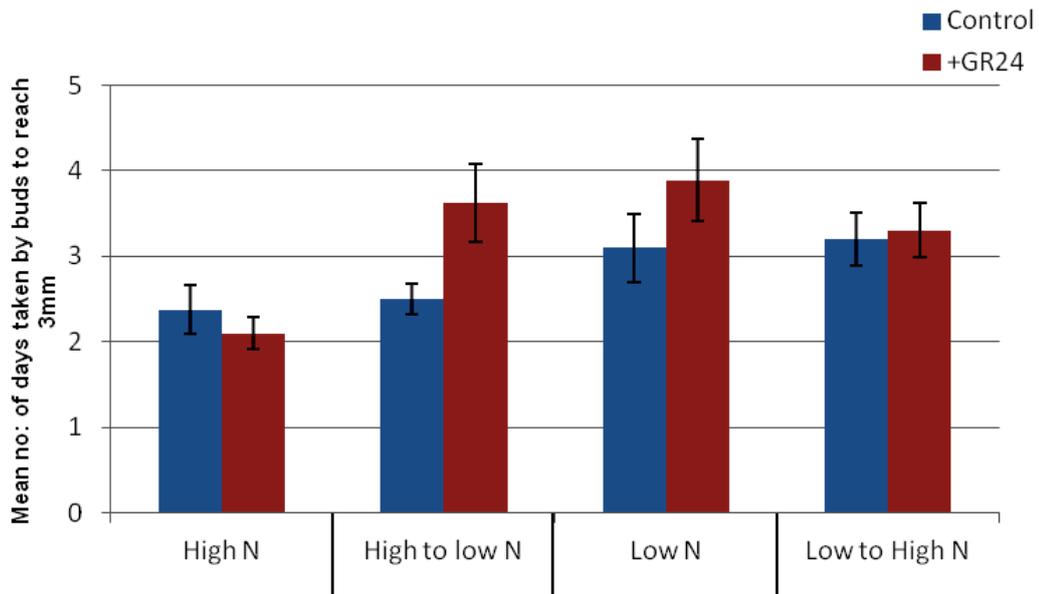


Figure 4.18 Mean number of days taken by Columbia buds to reach 3mm with different N treatments. Means \pm SE of 10 plants are shown.

4.5.4 Two node assays of Columbia with shift in N supply

Two node assays were set up with Columbia under the same N regimes as in the one node assays described above.

The effects of shifts in N supply (figures 4.19 c, d) were compared with results of two node assay set ups on high N alone (figure 4.19 a, previously presented in figure 4.11 a) and low N alone (figure 4.19 b, previously presented in figure 4.11 b). When plants were switched from high to low N (figure 4.19 c), they responded immediately to the switch, and behaved like buds grown continuously on low N. When buds were shifted from low N to high N, they behaved in same way as buds grown continuously on high N. Low to high N transition restored bottom bud activation. Here again, buds responded immediately to N availability.

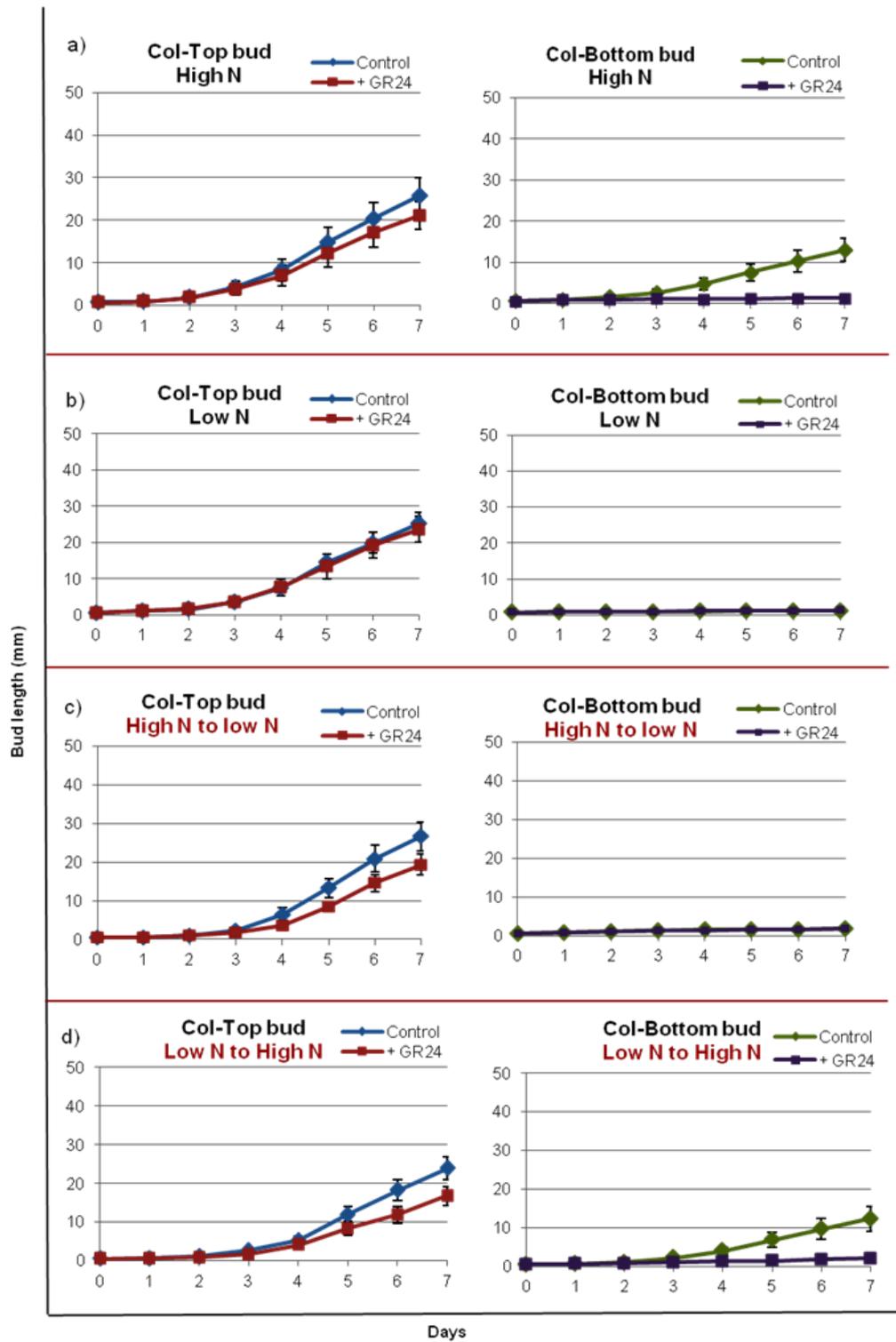


Figure 4.19 Bud lengths (mm) of Col on a) high N b) low N, c) high to low N d) low to high N in two node assays with or without GR24. Means \pm SE of 10 plants are shown.

The response of plants to switch in N supply was also clearly depicted in the RGI plots (figure 4.20). When buds were on constant high N, GR24 treatment increased the RGI and on low N, the RGI was close to one, even without SL. When shifted from high to low N, the RGI was close to 1, as with continuous low N grown plants. Shifting from low to high N restored a lower RGI, and SL-induced increase in RGI.

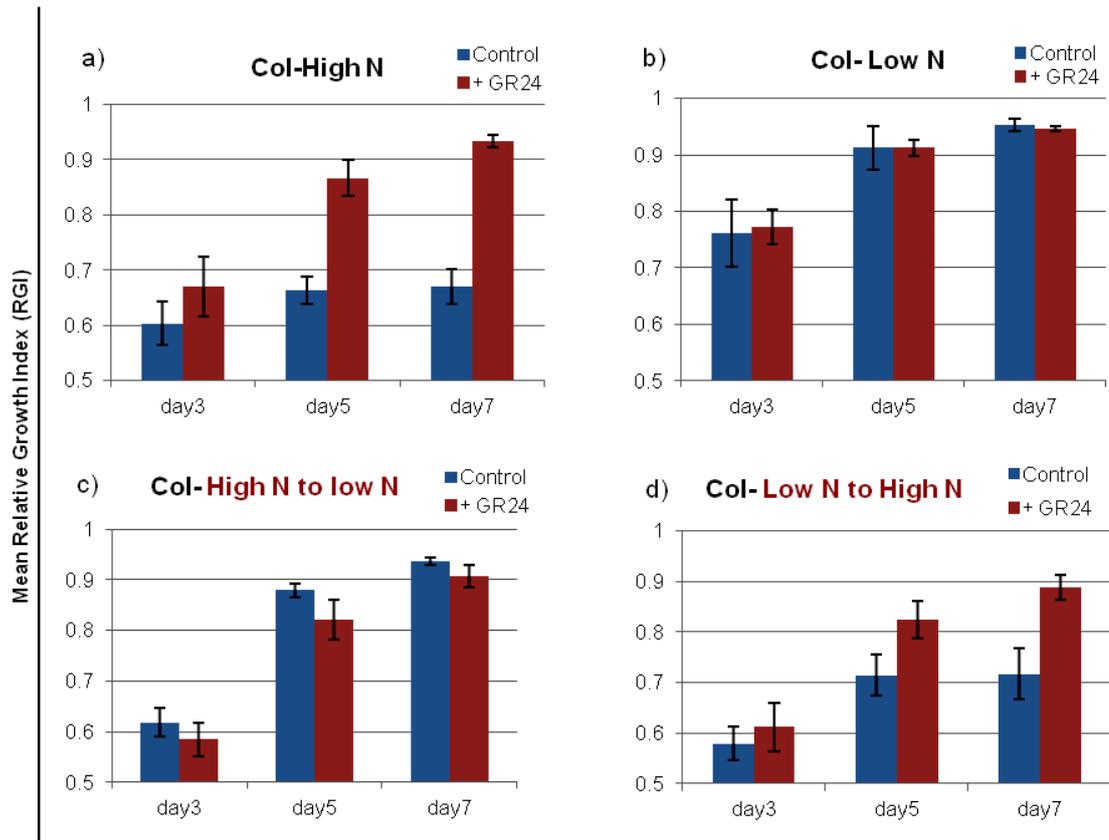


Figure 4.20 Mean relative growth index (RGI) of Col on a) high N b) low N, c) high to low N d) low to high N in two node assays with or without GR24. Means \pm SE of 10 plants are shown.

4.5.5 One node assays with increased stem length

To investigate the effects of having either more or less stem above or below the bud (and hence presumably more or less auxin in the stem), further experiments were performed (figure 4.21 b, c) in which the top or bottom leaf and bud were excised.

On high N, buds did not respond to GR24 in any of the one bud configurations tested (figure 4.21 a-c). A comparison of these to the standard two bud system can be seen in figure 4.21 d, where when treated with GR24, the top bud was unaffected, but the bottom bud was inhibited.

The timing of bud activation in all these configurations is compared in figure 4.22. GR24 was able to delay bud activation only in the case of bottom buds in the classical two bud set up, with two intact buds and leaves (figure 4.22 d). The time taken for bud activation was lowest for the single node alone and for the bottom bud with the top bud and leaf removed (approx. 2 days). Activation of the bottom bud was delayed by the presence of the top bud. Top buds activated with similar timing regardless of the presence of the bottom bud and leaf, after approx 4 days, similar to the bottom bud in the 2 node situation.

On low N (figure 4.23), buds show a stronger response to GR24 than on high N in all four situations. Both elongation and the timing of bud activation were affected, except that in the classical two bud situation, as previously described, GR24 had little effect on the timing of activation of the top bud, while significantly delaying the bottom bud. Interestingly, in contrast to the high N situation, on low N, untreated buds took a similar time to activate in all the four configurations (approx 3 days) except in the case of bottom bud in the two bud configuration where it was completely inhibited (figure 4.24).

4.21

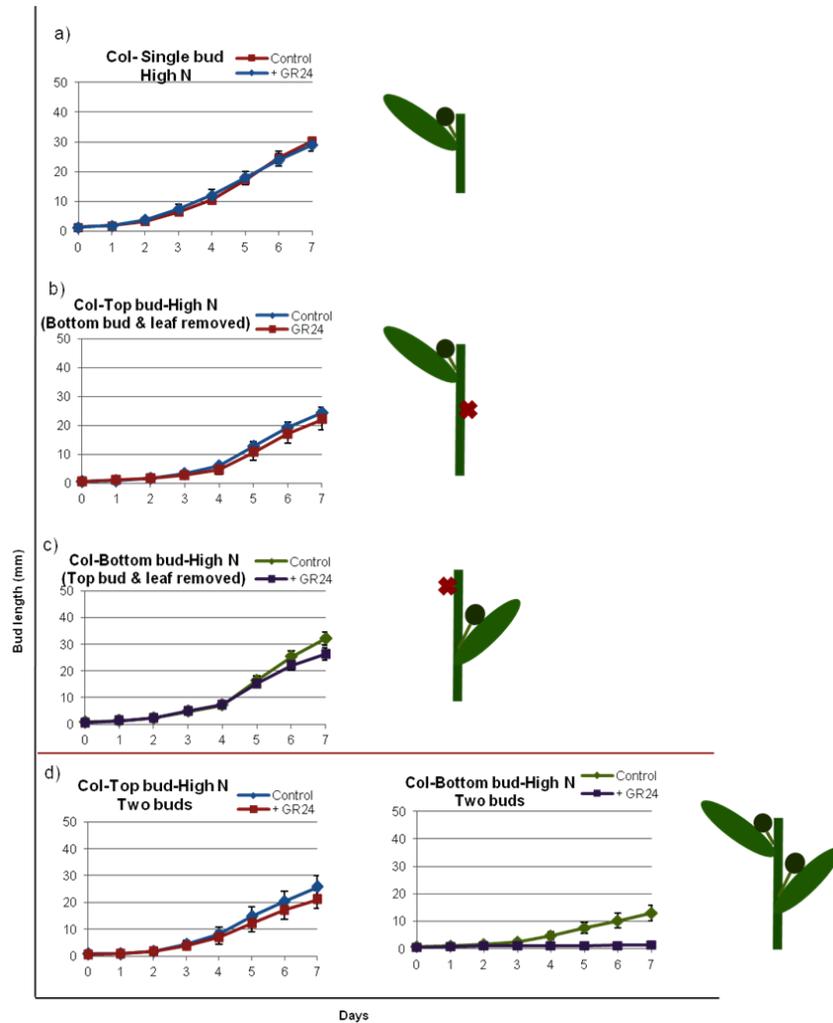


Figure 4.21 Bud lengths (mm) of Col a) single bud, b) top bud in a two node system with bottom bud and leaf removed, c) bottom bud in a two node system with top bud and leaf removed d) top bud and bottom bud – two nodes in assays on high N, with or without GR24. Means \pm SE of 6 - 10 plants are shown

4.22

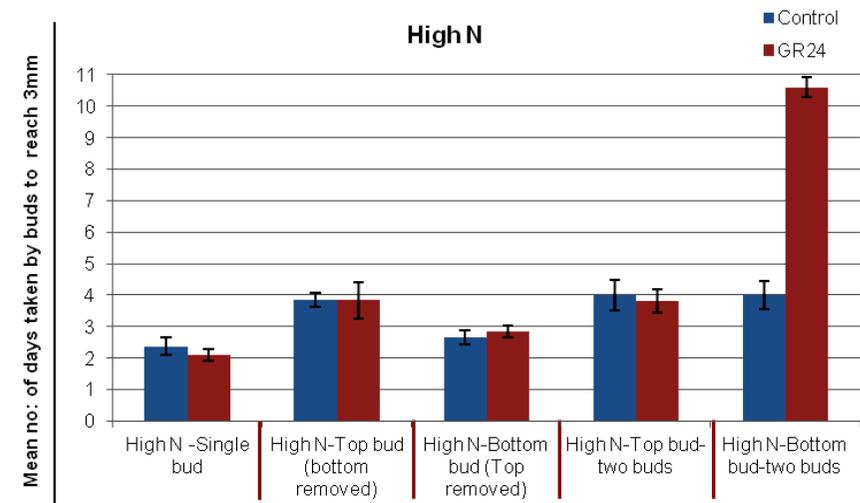


Figure 4.22 - Time taken by buds to reach 3mm on high N. Means \pm SE of 6 - 10 plants are shown.

4.23

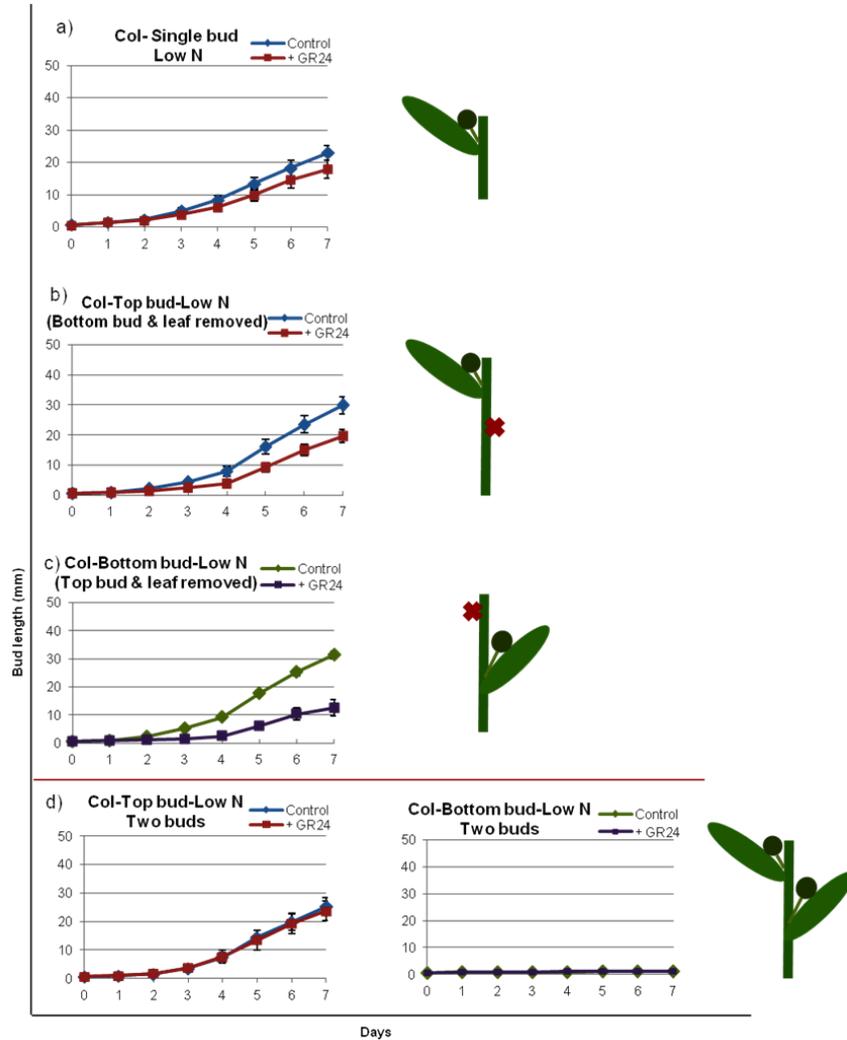


Figure 4.23 Bud lengths (mm) of Col a) single bud b) top bud in a two node system with bottom bud and leaf removed c) bottom bud in a two node system with top bud and leaf removed d) top bud and bottom bud – two nodes in assays on low N, with or without GR24. Means \pm SE of 6 -10 plants are shown.

4.24

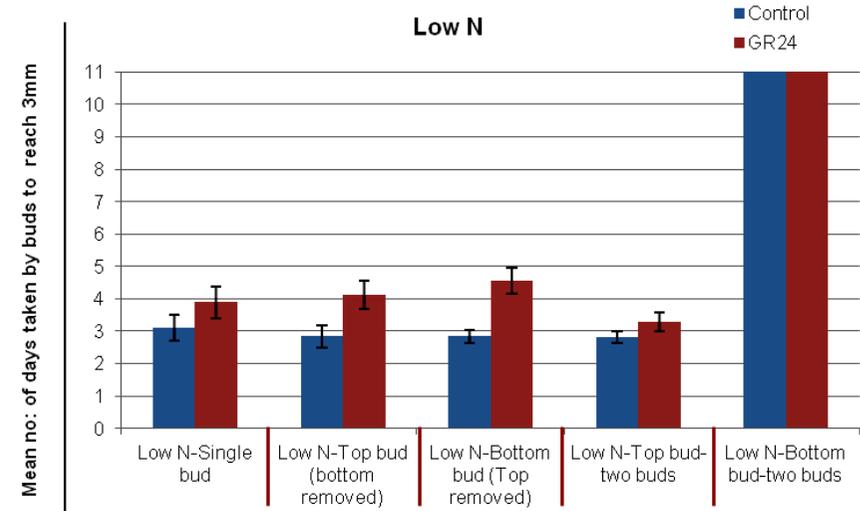


Figure 4.24 Time taken by buds to reach 3mm on low N. Means \pm SE of 6 -10 plants are shown.

4.6 Hormonal assays with MAGIC lines

4.6.1 Single node assays on high and low N

The one and two node assays were used to assess whether variation in the branching behaviour of the representative low and high plasticity MAGIC lines, 552 and 471, is associated with variation in hormone responses.

In the absence of GR24, Columbia buds take more time to activate than either 552 or 471 regardless of N levels (figure 4.26), and for all lines, N availability had little effect on activation time or final bud length, except in the case of 552, where low N apparently promoted elongation (figures 4.25, 4,26).

Single nodes of Columbia do not usually respond to GR24 on either high or low N (see above) although in this particular experiment they do show some inhibition at later time points, both with respect to delays in the timing of bud activation and reduced bud elongation (figures 4.25 a, b, 4.26).

For the MAGIC line 552, GR24 delayed timing of bud activation and bud elongation on high N, but not on low N (figures 4.25 c, d, 4.26). MAGIC line 471 did not respond to GR24 on high N in the timing of bud activation or bud elongation (figures 4.25 e, 4.26). With respect to bud elongation on low N, GR24 reduced 471 bud elongation on low N, but in contrast had little or no effect on 552 bud elongation (figures 4.25 d, f).

4.25

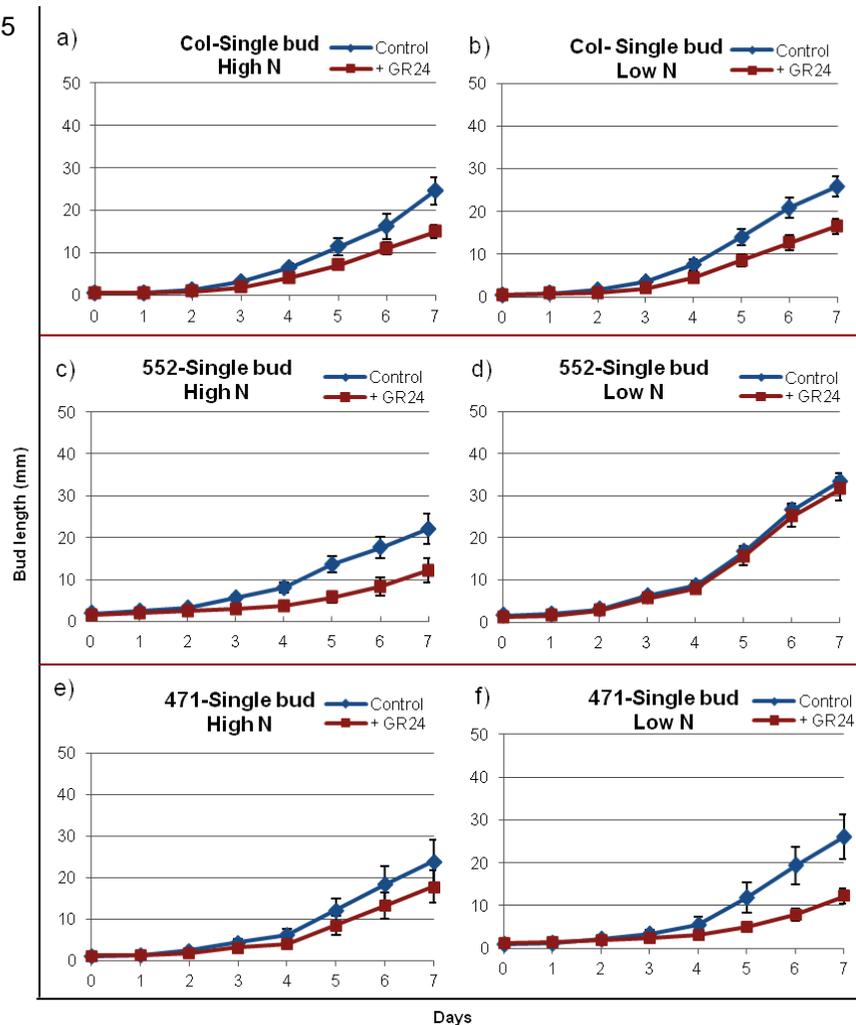


Figure 4.25 Bud lengths (mm) of single buds of a) Col on high N b) Col on low N, c) MAGIC line 552 on high N, d) MAGIC line 552 on low N, e) MAGIC line 471 on high N, f) MAGIC line 471 on low N in one node assays with or without GR24. Means \pm SE of 8-10 plants are shown.

4.26

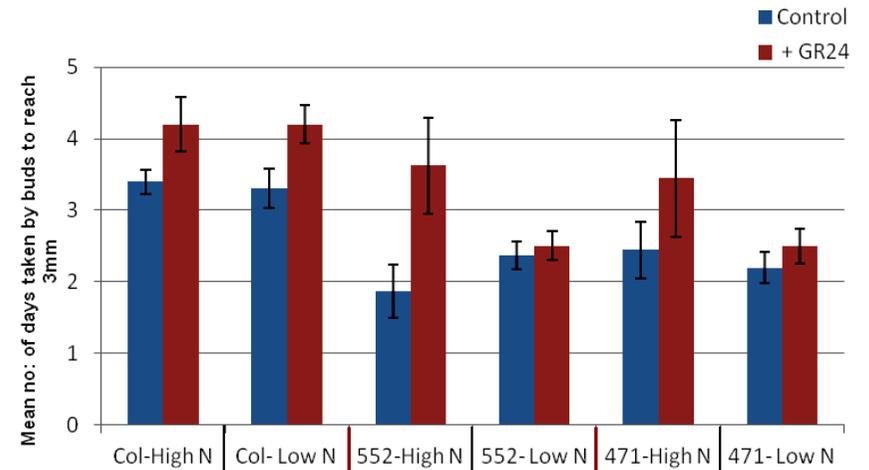


Figure 4.26 Mean number of days taken by Col, 552 and 471 buds to reach 3mm on high N and low N. Means \pm SE of 8-10 plants are shown.

4.6.2 Two node assays on high and low N

On high N, when comparing the top and bottom buds of Columbia, 552 and 471, it was evident that the bottom buds of both 552 and 471 were more vigorous and elongated more than the top buds, unlike Columbia, where usually the top bud dominated (figure 4.27 a-c). However, in general, 552 and 471 buds elongated less than Columbia. It could be that the buds are shorter at the end of the experiments because they started elongating later, rather than growing slower. Both top and bottom buds of control Columbia plants reached 10 mm by day 4, whereas this took 5 days for 552 and 6 days for 471 (figure 4.27 a-c).

On high N, for Columbia and 471, GR24 inhibited only the bottom bud, but both top and bottom buds of 552 were affected (figure 4.27 a-c). However, the weak growth of 552 and 471 buds made the inhibition difficult to interpret.

Weak growth has a major impact on RGI, such that the RGI data presented in figure 4.28 should be interpreted with caution. On Low N, Columbia behaved as previously described with increased RGI caused by greatly reduced growth of the bottom bud (figure 4.27 d, 4.28 e). In this experiment, there was some elongation of the bottom bud on low N, which was abolished by GR24 treatment, reflected in increased RGI. In the 552 and 471, on low N, bud growth was more vigorous than on high N, especially for 552. For both 552 and 471, RGI was unaffected by GR24 treatment on high N, but the 471 bottom bud was completely inhibited by GR24 on low N, while 552 was GR24 resistant (figure 4.27 e-f, 4.28 e-f)

4.27

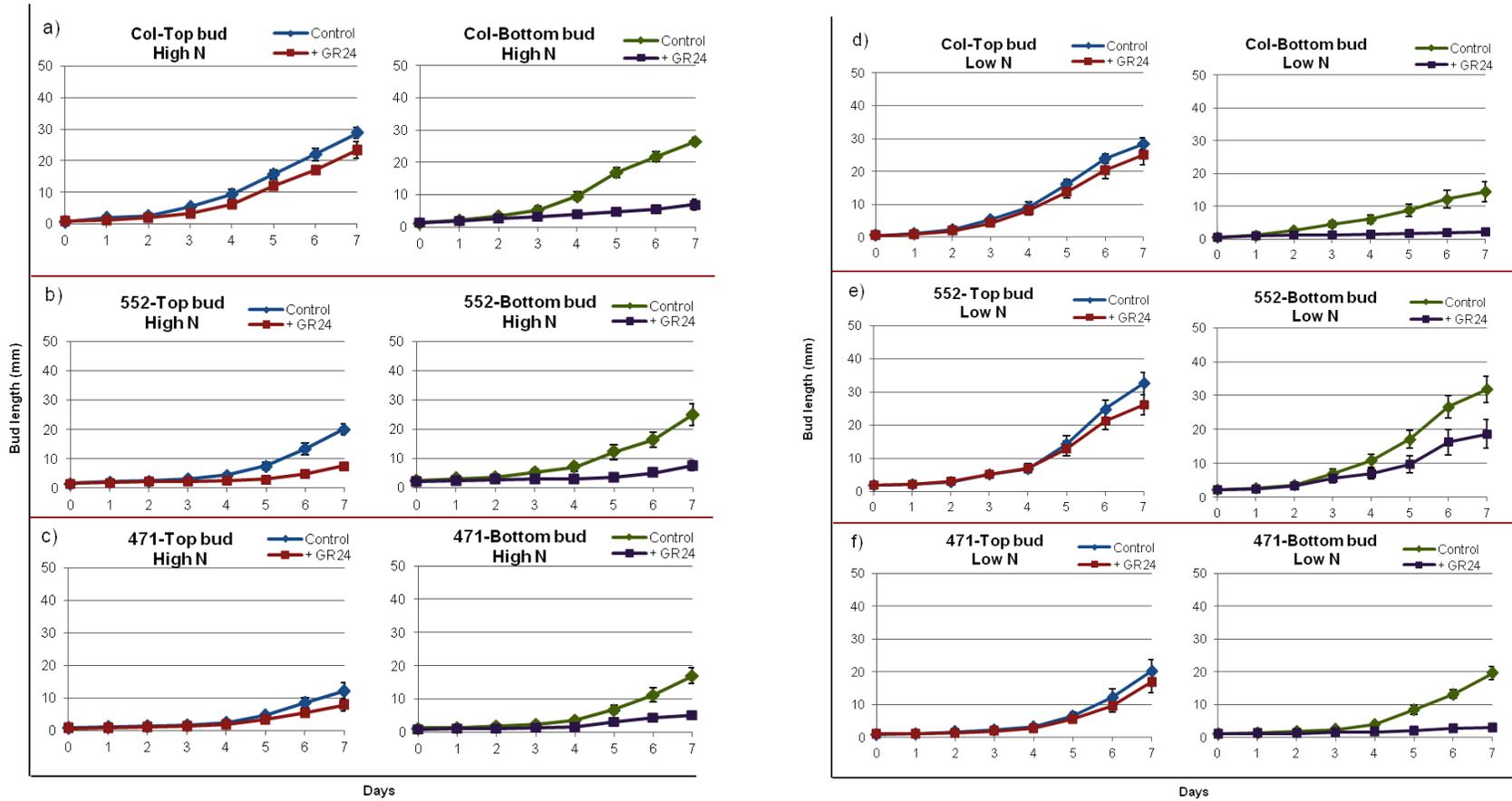


Figure 4.27 Bud length (mm) of Col, MAGIC line 552 and MAGIC line 471 on high (a, b, c) and low N (d, e, f) in two node assays with or without GR24. Means \pm SE of 8-10 plants are shown.

4.27

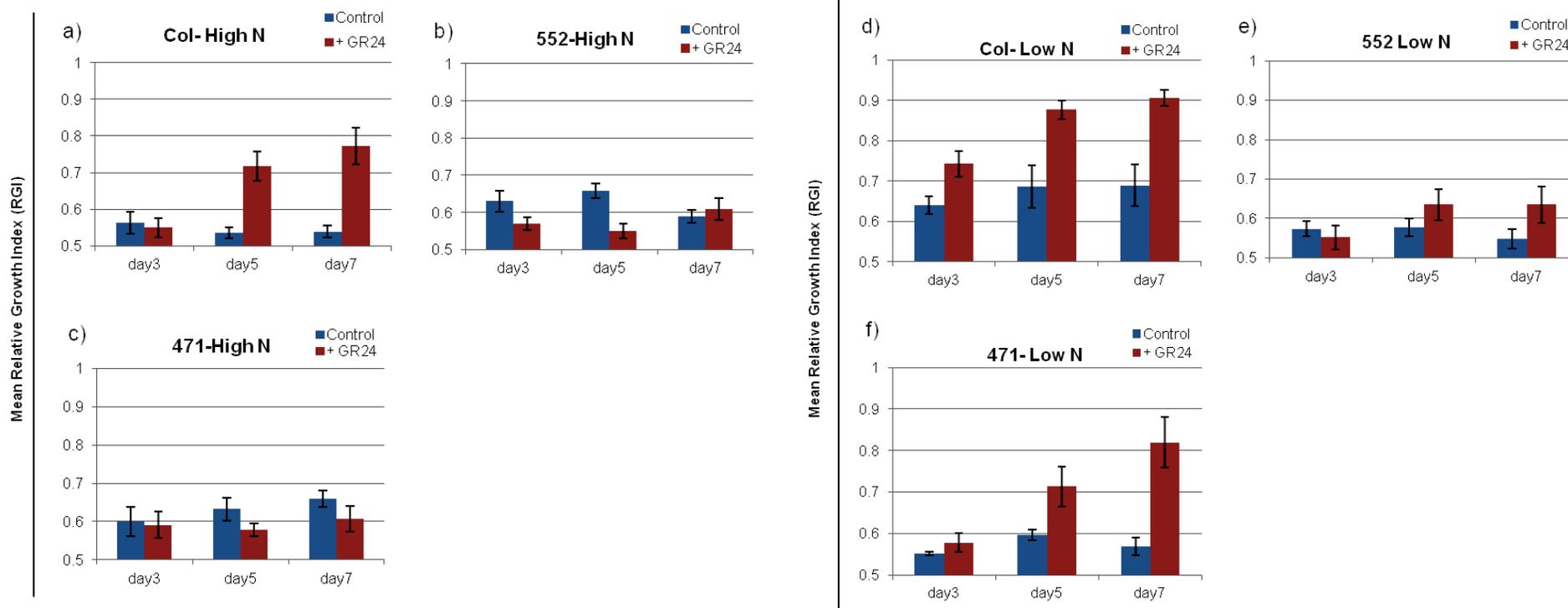


Figure 4.28- Mean Relative growth index of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and low N (d, e, f) in two node assays with or without GR24. Means \pm SE of 8-10 plants are shown.

4.6.3 Two node assays of MAGIC lines with shift in N supply

The experiments described above clearly demonstrated the rapid response of buds to changes in N availability. Similar experiments were performed with 471 and 552 to investigate whether they differed in their responses to shifts in N supply (figure 4.29). On high N (figure 4.29 a-c, 4.30 a-c), Columbia buds behaved similarly to the previous results. On contrast, in this experiment, 552 buds were much more vigorous than in the in the previous experiment. However, they still maintained similar mean growth of the top and bottom buds. The mean length of both top and bottom buds of 552 were reduced by GR24 treatment, and there was a small increase in RGI, indicating less equal growth of the two buds than in the untreated control. With 471, the bottom bud was more vigorous than the top bud, as observed in the previous experiment, and both buds responded to GR24 treatment. In this experiment, although the RGI increased slightly over time between day 3 and day 7, there was no difference in RGI between the control and GR24 treatment.

When shifted from high to low N (figure 4.29 d-f, 4.30 d-f), in this experiment, Columbia did not respond at all, in contrast to the results described above (figure 4.19). Similarly 471 responded very little to the reduced N supply, except that GR24 did not reduce the mean length of the bottom bud. When the MAGIC line 552 was shifted from high to low N, both buds grew less vigorously than in continuous high N treatment, and only the bottom bud responded to GR24 treatment, with an associated increase in RGI by day 7. This behaviour of 552 is similar to the results on low N alone (figure 4.27 e), where just the bottom bud responds, unlike both buds responding to GR24 on high N (figures 4.27 b and 4.29 b).

In general, in this particular experiment the buds of all three genotypes seemed to be more vigorous than the previous experiment, but even so the generally poor growth of 471 makes the results difficult to interpret. Overall, the results of experiments on high N, low N and the shift indicate lower RGIs for both the MAGIC lines than for Columbia. For 552, this was typically caused by vigorous growth of both buds, but for 471 it results from poor growth of both buds.

4.29

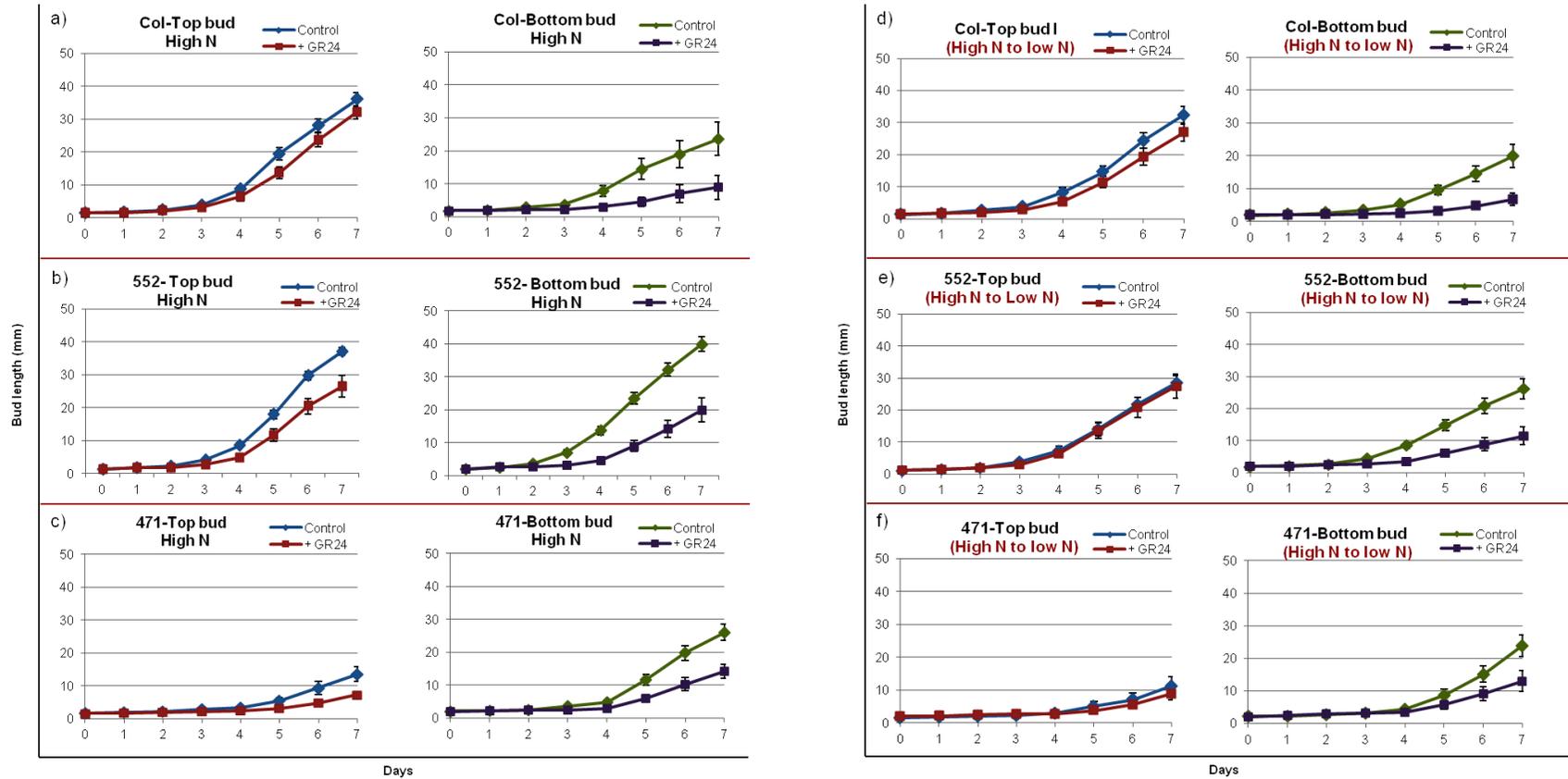


Figure 4.29 - Bud length (mm), of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and shift from high to low N (d, e, f) in two node assays with or without GR24. Means \pm SE of 8-10 plants are shown.

4.30

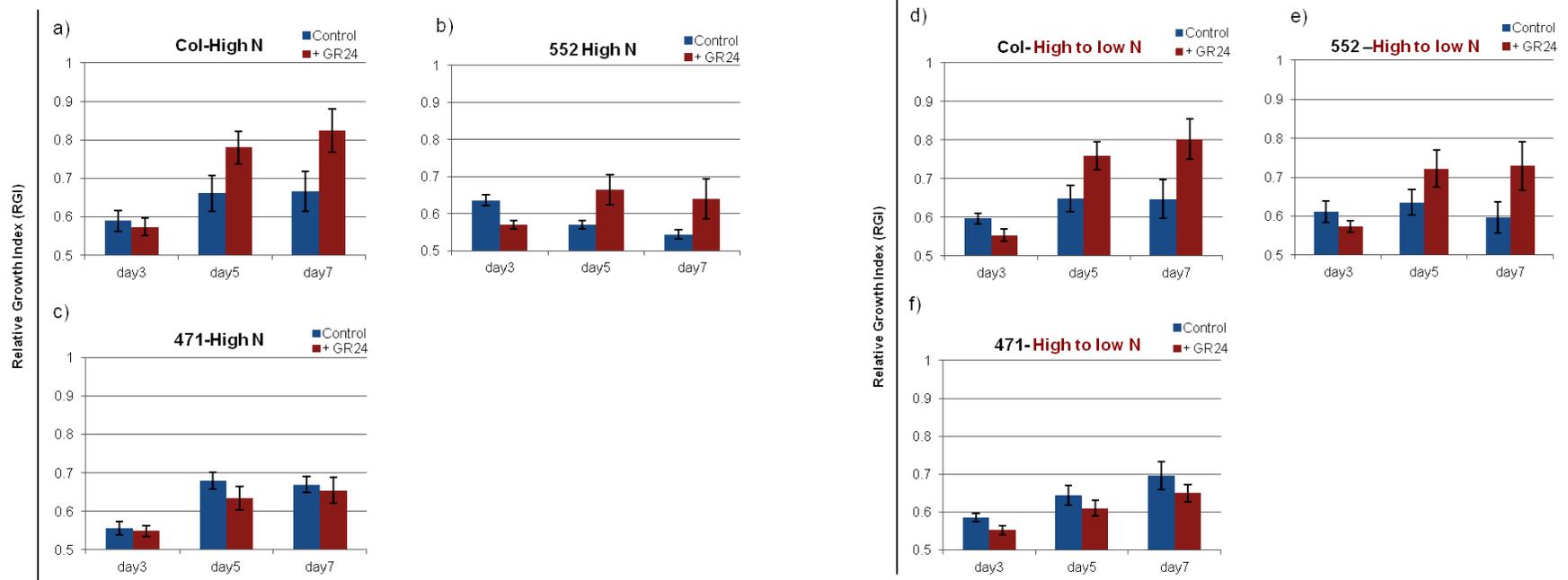


Figure 4.30 Relative Growth Index (RGI) of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and shift from high to low N (d, e, f) in two node assays with or without GR24. Means \pm SE of 8-10 plants are shown.

4.6.4 Three node assays of MAGIC lines

The low RGIs of 552 and 471 suggested lower competition between buds than in Col. Therefore, three node experiments were set up on high and low N to investigate the effects of including more nodes and thereby more competition. In addition, since one bud behaved differently from two buds, it would be interesting to know what happens when there were three buds. The results are shown in figure 4.31.

On high N (figure 4.31 a-c), in Columbia plants the top bud grew vigorously, whereas the middle and the bottom buds did grow very weakly. All the growth seemed to be focused into the top bud. GR24 treatment had very little effect on any of the three buds, although the elongation of top bud was slowed down slightly. All three buds from 552 plants activated near-simultaneously, after which there was a weak acropetal gradient of bud elongation, with the bottom branch being the longest by day 7 (figure 4.31 b). When treated with GR24, the more basal buds were inhibited, with the bottom one being more inhibited than the middle one. 471 buds were less vigorous in elongating when compared to 552 (figure 4.31 c). All the three buds activated at the same time, but by day 7 bottom bud had elongated more than the other two, following a similar acropetal gradient of elongation as observed in 552. Apical buds were unaffected by GR24 treatment and the bottom buds were weakly inhibited.

On low N (figure 4.31 d-e), both control and GR24 treated Columbia buds followed the same pattern as on high N, with only the top bud elongating. Even the weak growth of the middle and bottom buds seen on high N was absent. More buds grew for 552 and 471 on low N than for Columbia, but their growth was less vigorous than on high N. For 552, bud growth on low N resembled GR24-treated bud growth on high N (figure 4.31 b). Of all the genotypes in this experiment, despite being highly sensitive to N in a whole plant context, 471 buds are the most active on low N, with all the buds activating, although only weakly for the bottom bud. Both GR24 treated and control 471 buds on low N follow a basipetal gradient of bud elongation. Here again, the top buds were unaffected by GR24 treatment.

4.31

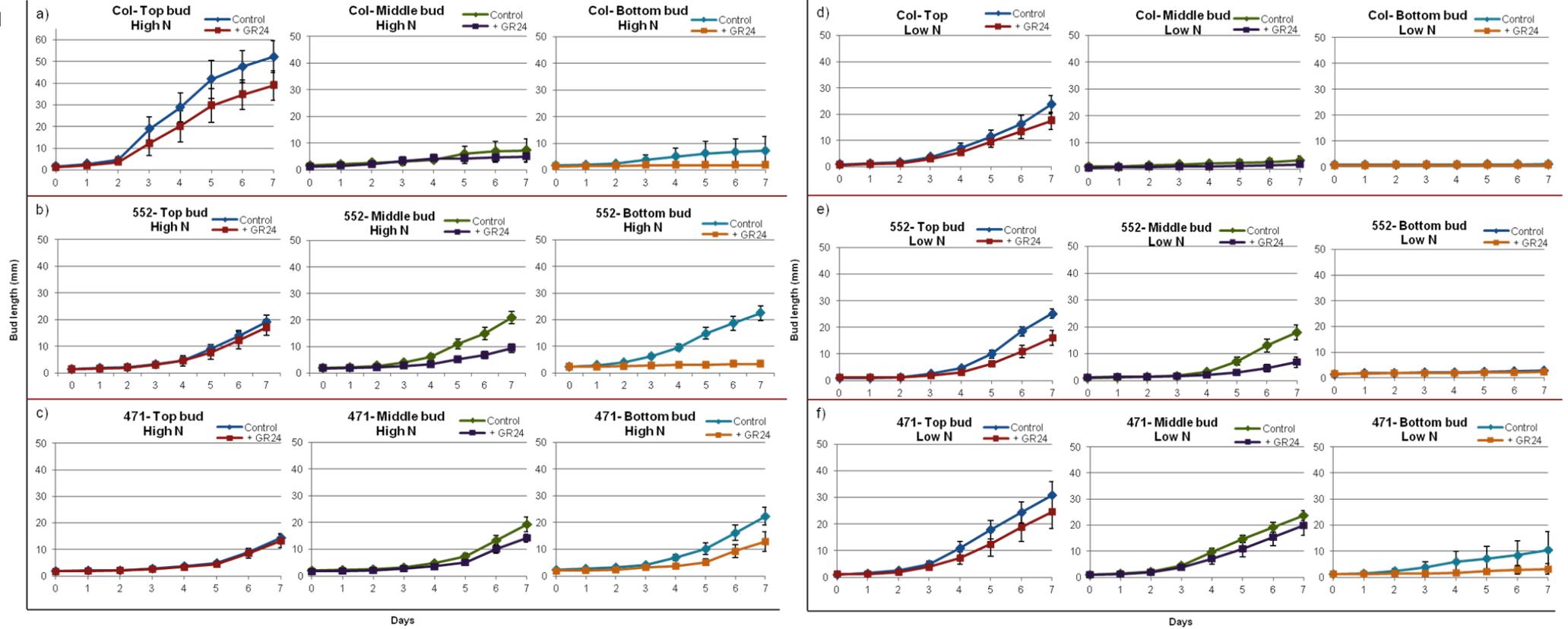


Figure 4.31- Bud length (mm) of Col, MAGIC line 552 and MAGIC line 471 on high N (a, b, c) and on low N (d, e, f) in three node assays with or without GR24. Means \pm SE of 6-8 plants are shown.

4.7 Discussion

The experiments described in this chapter aimed at investigating the role of SL in regulating bud activation and competition between buds, and their responses to N supply, using previously established and modified hormone response assays. The main findings are discussed below.

4.7.1 Mechanism of action of GR24 in regulating bud activation and elongation

The results in this chapter provide evidence that SL is not a straightforward bud inhibitor, but rather its effects could be explained by the auxin transport canalisation model, and the consequent competition between buds. According to the auxin transport canalisation model for the control of bud activation (Bennett et al., 2006b, Prusinkiewicz et al., 2009), buds must be able to export auxin into the main stem to activate. Stem acts as an auxin sink and the young expanding leaves of active or activating shoot apices act as auxin sources. In intact plants, auxin from shoot apex in the PATS prevent establishment of auxin transport out of the bud into the main stem and there by prevent bud activation (Li and Bangerth, 1999, Prusinkiewicz et al., 2009). Consistent with the model, it was recently shown that in pea, following decapitation, activated axillary buds established PAT by increased polarisation of PIN1 which led to increased PIN1 expression levels in the stem (Balla et al., 2011). Prusinkiewicz's model implied that every bud communicates with every other bud on a shoot and they compete for a common auxin transport pathway in the main stem, directed towards the root. Local competition contributes to decisions about which buds to activate; based on factors such as the developmental stage of the bud, light quality etc. Given that SLs reduce the accumulation of PIN proteins on the basal membranes of auxin transporting cell files in the main stem and bud (Crawford et al., 2010), high SL levels make the establishment of auxin transport out of buds more difficult, increasing the level of competition between buds. In all the assays mentioned in this chapter, the apices of plants were removed and consequently the main active auxin source is removed, reducing auxin levels in the main stem, thereby increasing stem sink strength for auxin.

4.7.1.1 SL is not a straightforward bud inhibitor

Results from several experiments with just one bud and the associated stem confirmed that buds on isolated single node stem segments of Columbia (and the *max* mutants) did not respond to basal application of the synthetic SL, GR24. Basal SL had no effect on the timing of activation, or elongation of single buds in any of the genotypes. This is in accordance with the results of the modelling studies in that, when the apex is removed by decapitation and only a single lateral auxin source (bud) is present, canalised auxin flow between the bud and the stem is established, since there is no competing auxin source to prevent this canalisation (Prusinkiewicz et al., 2009). Similar results were obtained using split plate assays in which an isolated nodal segment was held between two agar slabs in a Petri dish (Crawford et al., 2010). In the absence of apical auxin, basal SL supply had no effect on bud growth. In the presence of apically supplied auxin (NAA), basal SL supply was able to enhance the suppression of bud outgrowth.

Apart from some variability observed during different seasons, this result was robustly reproducible. The dose of GR24 used in bud assays (1 μ m) was not high enough to trigger any toxic effects on plants, or to induce stress responses. However, this GR24 concentration was high enough to strengthen the effect of apical auxin confirming that GR24 was active in the assays.

4.7.1.2 SL tends to enhance competition between buds such that only one remains active

Further evidence for the mode of action of SL came from the very informative two node assays. In standard two node assays with Columbia plants, there were typically three scenarios of bud activation; sometimes both buds activated and grew out, and sometimes one of the two buds dominated over time, which could be either the top bud or the bottom bud. These results could be explained based on the auxin transport canalisation model for bud regulation. Both the buds are competing for the PAT pathway in the main stem, and if both buds are activated simultaneously they both establish auxin export and grow out. Alternatively, if either the top or bottom bud activates first, it will export auxin to the PAT first, and inhibit the outgrowth of the other bud. So essentially whichever bud activates first and establishes robust auxin transport into the stem becomes dominant over time.

Competition between buds in a two node system with either the basal or apical bud dominating, as previously shown with Columbia plants in assays by V. Ongaro (Ongaro et al., 2008), was reproduced in my assays with Columbia control plants. However, when treated with basal GR24, there was enhanced competition between Columbia buds, with one bud, almost always the basal one, often being inhibited completely. Inhibition of just one of the two buds by GR24 in this system strongly suggested that SL does not simply inhibit the growth of all buds that it reaches. Besides, when treated with GR24, while Columbia showed a significant increase in RGI over time, the SL signalling mutant *max2* did not exhibit any increase (figures 4.6 a, b). This demonstrated the MAX2 dependence of this effect. In general, the RGIs of both *max* mutants were lower than those of Columbia indicative of reduced competition between *max* mutant buds (figure 4.6).

All these provide evidence that SLs increase competition among buds and these results, together with experimental evidence from other studies, led to the conclusion that SLs set the context for competition for auxin export from buds into the stem (Crawford et al., 2010).

4.7.1.3 SL does not play a role in deciding which bud activates

Although SL enhances competition between buds, this enhanced competition does not say anything about which bud activates. In situations where one bud dominated, in my assays mostly it was the top bud that grew out, and the bottom bud was inhibited. In the experiments of Ongaro et al (2008) it was usually the bottom bud that dominated. This could be because the bottom bud was older and usually bigger. In my experiments, for example, on all the repeats of two bud assays performed on high N, the tendency of the top bud to elongate more was very clear, and SL treatment had no effect on either the activation or the elongation of top buds (for example, figures 4.21 d, 4.22). These results could be explained based on the results of Crawford et al (2010) according to which SLs reduced the rate of PIN accumulation. On removal of the apical auxin source, the top bud was released from inhibition and starts growing first, replenishing auxin in the main stem. Whereas, the bottom bud experiences the decreased auxin slightly later, putting it at a competitive disadvantage with respect to the upper bud. It is interesting that the bottom bud activated faster than the top bud in

experiments where the other bud had been removed in both control and GR24 treated buds (figure 4.22). Besides, the situation on low N could not be explained by the bottom bud getting GR24 fractionally earlier. Here, this would not explain why the top bud activated in situations where one bud was inhibited by low N availability in control plants where no exogenous SL was applied.

One might argue that there was a possibility of basal SL solution being delivered unequally to the buds. This could be ruled out based on the results from a dye accumulation experiment performed on the two bud system. In this experiment the excised stem segment was grown on nutrient medium containing a red food dye and uptake of dye was measured. Results showed that basally supplied solutions reached and accumulated in both buds equally (K. Abley personal communication). Furthermore, the inhibition of the bottom bud in favour of the top bud could not simply be explained based on the nature of SL transport or the proximity of the bottom bud to the SL source alone, because SL is predicted to move in the transpiration stream, which is predicted to deliver solutes rapidly throughout the shoot. On low N, there could be high levels of endogenous SL (Yoneyama et al., 2011) and this could be cited as a reason for one bud (the bottom bud) being completely inhibited in control plants. Even in this situation there is no reason for this endogenous SL to be unevenly distributed between buds.

4.7.1.4 SL has no role in bud elongation

Results in this chapter suggested that the length of the buds at the end of the experiments was a combination of when they activated and how fast they subsequently grew. When comparing the behaviour of plants grown on F2 compost and those grown on sand-Terragreen mixture and fed with high N, single buds behaved in a similar way. However, it was noticed that in two bud assays, there was a tendency for the top bud to be longer, whereas the bottom bud eventually became longer in assays where the plants were grown on F2 compost. Perhaps this was because the older bottom buds grew faster in assays with plants from F2 compost than from those grown on sand-Terragreen. If buds were activating slightly more slowly on sand-Terragreen, then the advantage afforded by being older would be less.

4.7.1.5 SL delays bud activation in the two node system consistent with enhanced competition

On comparing the growth of one node and two node assays it was clear that buds in the two node system activated more slowly than buds in one node assays (figure 4.22, High N-Single bud Vs High N-Top bud and high N-Bottom bud- two buds). This could be due to competition between the buds, slowing the early stages of auxin export establishment for both buds. Results from the experiments where one of the two buds was excised support the idea that the bottom bud was delayed by the top bud, but not so much the other way round. This could be because buds in a single node assay are bottom buds (see figure 4.9) which are comparable to the bottom buds in a two bud assay set up. They have the similar amount of stem under them and they tend to be bigger and have similar sizes. So when the top bud was excised, the timing of activation was similar to the single buds (figure 4.22, High N-Single bud Vs High N-Bottom bud, top removed). And when the additional top bud was present as in the two bud situation, there was delay in bud activation consistent with the enhanced competition as described above. The delay in activation compared to the one node system was independent of GR24.

4.7.2 Mechanism of action of N deprivation in inhibiting buds

Evidence from experiments in this chapter clearly indicated that inhibition of buds on low N was not a direct nutritional effect. In one node assays on high and low N, single buds of Columbia grew out irrespective of whether they had sufficient N or not (figure 4.10). Similar results of other lines (*max* mutants and MAGIC lines) also provide conclusive evidence of the inability of low N alone to bring about bud inhibition. In two node assays with Columbia on high and low N the top bud grew out on both high and low N, whereas the bottom bud on low N was completely inhibited (figure 4.11). The outgrowth of the top bud on low N again provided evidence that N limitation cannot inhibit buds.

4.7.2.1 SL synthesis is upregulated by low N

Several studies had already demonstrated the upregulation of SL synthesis by low N (Yoneyama et al., 2007b, Umehara et al., 2008). Besides SL levels are likely to be up-regulated by auxin. It has been shown that expression of SL

biosynthetic genes is up regulated by auxin (Hayward et al., 2009, Foo et al., 2005, Johnson et al., 2006). On low N there is more auxin (K. Ljung, V.Ongaro unpublished results). There is possibly more SL in the stem as SL levels are upregulated in the root in N deficiency (Yoneyama et al., 2007b, Yoneyama et al., 2011) and as there is a correlation between root and xylem sap levels under P deficiency (Kohlen et al., 2011). More auxin and more SL could explain why on low N the bottom bud is completely inhibited. Consistent with this idea, on low N, but not on high N, SLs could inhibit the growth of the remaining bud from two node segments from which one node had been removed (figures 4.22, 4.24). Increased levels of endogenous SLs coupled with increased levels of auxin in the stem under low N could account for the complete inhibition of the bottom bud on low N.

4.7.2.2 The behaviour of Col buds on low N and with GR24 treatment is similar

Experiments with GR24 and low N treatments undoubtedly showed that low N and GR24 had similar effects on bud activation and elongation (figures 4.14, 4.15, 4.16, 4.23 and 4.24). Furthermore, the lack of response of *max2* buds to low N demonstrated that response to low N requires SL (figure 4.14 d). Thus GR24 response and low N response are both MAX2 dependent. Nonetheless, *max2* and *max4* differed in their responses and the response of *max4* was intermediate between Columbia and *max2* on low N (figures 4.14 f, 4.15 f, and 4.16 f). For example, *max4* RGI was affected by low N whereas *max2* RGI was unaffected.

Combining these ideas with the known effects of low N on SL and auxin levels described above suggests a model for the reduced shoot branching observed on low N. Low N results in increased auxin in the stem. Since auxin upregulates PIN gene expression (Sauer et al., 2006, Petrášek and Friml, 2009) low N is likely to increase stem auxin transport. However, low N also likely increases SL levels and GR24 reduces the PIN accumulation in xylem parenchyma. Therefore, these two effects could balance out, resulting in a stem with high auxin levels, but limited PIN accumulation. This, especially in a high SL environment, would reduce shoot branching, while increasing auxin delivery to roots, promoting root growth and enabling the observed shift in root : shoot ratio on low N.

4.7.3 Response to shift in N supply

The main comparison here is between continuous high N and high N followed by low N. When the starting plant material for two bud assays was normalised by growing them on high N initially and then transferring to low or high N, the interesting result was that plants transferred from high to low N, at least in some experiments, behaved like those grown continuously on low N (figures 4.19 c, 4.20 c). This implied that these plants were able to respond to the N-limitation soon after the transfer was made. This could be true as there is evidence from microarray experiments in *Arabidopsis* roots that of thousands of genes were induced or repressed within a very short time (within 20 minutes) of exposure to Nitrate levels as low as 250 μ M Nitrate (Wang et al., 2003). Another study found that a similar number of genes responded in *Arabidopsis* shoots when exposed to 5mM Nitrate for 2 hours (Wang et al., 2004). These suggest that the rapid response to shift in N supply may not be surprising. Similarly, growth on low N followed by transfer to high N restored bottom bud activation (figures 4.19 d, 4.20 d). It is known that when plants deprived of N were resupplied with N, cytokinin synthesis was increased (Scheible et al., 2004) which could account for the activation of the bottom bud. They showed that several genes involved in CK synthesis were rapidly induced in the shoots upon Nitrate resupply. Furthermore, rapid induction (within 10 minutes) of Nitrate assimilation genes upon Nitrate resupply to Nitrate starved *Arabidopsis* plants was also reported by Scheible et al (2004). All these together implicate that N at the time of assay was more important than earlier growth conditions as observed with the result of shift in N supply and that the response to shift in N was also very rapid, as buds presumably started growth rapidly after transfer.

4.7.4 The MAGIC lines 552 and 471 differ in their N and GR24 responses

4.7.4.1 MAGIC line 552

Behaviour of MAGIC line 552 differed from Columbia in the two bud configuration in that on high N, unlike Columbia both buds of 552 responded to GR24 (figure 4.27 a, b). And on low N, there was quite robust and rapid elongation of both top and bottom control buds of line 552. With regard to GR24 treatment, line 552 exhibited some SL resistance as the bottom bud did not respond strongly to

GR24, unlike the other genotypes (figure 4.27 e). This behaviour on low N corresponded to the whole plant behaviour in that 552 was branchy on both high and low N (Chapter 3, figure 3.10 a). However, it was difficult to interpret the high N response of both top and bottom buds of 552 to GR24 on the basis of the whole plant behaviour. On three bud assay situation with Columbia, there seemed to be even more enhanced competition among the buds than the two bud assay situation. In Columbia, both the middle and bottom bud were inhibited with the bottom-most bud being most affected, which could be explained based on the model. If the buds were competing to export auxin, it could be deduced that fewer buds were activating because there were more buds contributing auxin to the stem. This was indeed observed with Columbia. When the behaviour of line 552 buds on high N were compared to Columbia, all the three buds (top, middle and bottom) activated and elongated with the bottom most one eventually becoming the longest (figure 4.31 b). One possible explanation for this could be that there is less competition between the control 552 buds on high N, when compared to Columbia. However, GR24 treatment enhanced competition among the buds of 552 as evident from the complete inhibition of the bottom bud and the response of the middle bud. On low N again the middle bud exhibited some SL resistance unlike Columbia as observed with the two bud assays (figure 4.31 e). Although behaviour of 552 in two bud assays on low N matched to a certain extent with the whole plant behaviour, when there were three buds the results were opposite to what one would expect. 552, which was branchy on both high and low N, was affected by both low N and GR24. Nevertheless, since the shoot apex was removed in the bud assays, the results were compared to the decapitation response of the whole plants. 552, a low plasticity line did not make that many branches when decapitated, especially in summer (Chapter 3, figure 3.12). Hence it could be postulated that the behaviour of this line in the three bud assays corresponded to the whole plant behaviour when decapitated.

4.7.4.2 MAGIC line 471

In the bud assays, the slow growth of 471 in general made it difficult to interpret the results. In contrast to Col, the control bottom buds of line 471 elongated more than the top bud in two bud assays on high N (figure 4.27 c). Response of bottom bud to GR24 was not that strong. Bud elongation was maintained on low N but

there was good SL response as the bottom bud was completely inhibited like Columbia but different to SL resistance of 552 (figure 4.27 f). In the three bud assays on high N, 471 differed from line 552 and Columbia in that the bottom most bud was resistant to SL (4.31 b). One similarity among all the three genotypes (Col, 552 and 471) was that, on low N, it was the bottom-most bud that was most responsive to GR24 treatment (figure 4.31 d-f). This corresponds to the decapitation response of the whole plants (Chapter 3, figure 3.12). 471, a high plasticity line, was capable of making additional branches when decapitated and on low N, decapitation could overcome the branch loss of this line.

Nonetheless, it followed that although the behaviour of MAGIC lines differed from Columbia in both the N and GR24 responses, it did not relate in a straightforward way to their whole plant phenotypes.

4.7.5 Summary

All the results in this chapter strongly suggest that response of buds to N limitation is mediated by hormones, especially SL, which acts systemically and that N does not affect branching independently of hormones. If SL is the N starvation signal, as proposed by different reviewers (Koltai and Kapulnik, 2011, Xie and Yoneyama, 2010) presumably it could be postulated that many N limitation responses are caused by SL. However, SLs are also upregulated in the absence of not just N, but other nutrients such as Phosphorous (Umehara et al., 2010a, Yoneyama et al., 2011, Lopez-Raez and Bouwmeester, 2008), and is certainly not exclusive for N starvation. Similarly, other hormones regulating shoot branching, such as cytokinin adding to the complexity of the branching regulatory network, for example it is known that removal of apical auxin source by decapitation led to increased CK levels (Bangerth, 1994, Shimizu-Sato et al., 2009). CK is certainly involved in the nutrient–hormone network, especially because Nitrate increases CK biosynthesis (Takei et al., 2004, Miyawaki et al., 2004) making the nutrient – hormone network even more complex.

Chapter 5

N and hormone response studies in *Brassica rapa*

5.1 Introduction

Chapters 3 and 4 focused on studies of shoot branching and hormonal response in N sufficient and N limited situations in the model plant *Arabidopsis*. This chapter explores the possibility of comparing data from *Arabidopsis* and Brassica species, by assessing the shoot branching and hormonal response to N limitation in *Brassica rapa*. Much of our understanding about shoot branching control and the effects of hormones and nutrients come from *Arabidopsis*, providing opportunities to translate the knowledge to Brassica crops.

Among the Brassicaceae crops, oilseed rape (*Brassica napus*) is an important crop in UK agriculture, grown mainly for its oil. *Brassica napus* is an amphidiploid species, and has a complex genetic background as it originates from a cross between two diploid Brassica species, *B. rapa* and *B. oleracea* (figure 5.1). Among the different Brassica species comprising the 'Triangle of U' *Brassica rapa* ($2n=20$) is believed to have undergone domestication first (Prakash S, 1980).

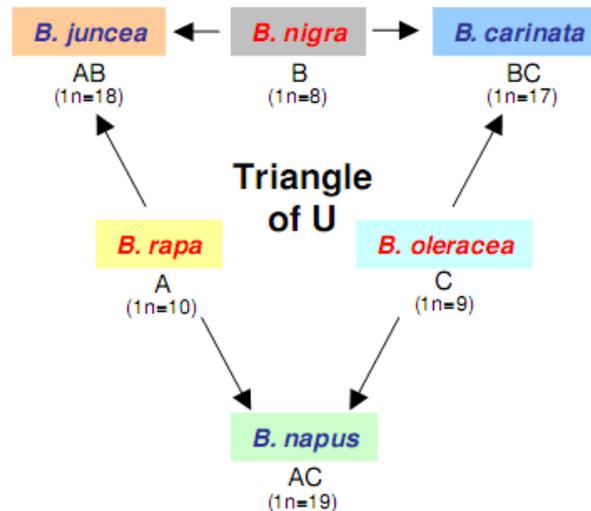


Figure 5.1: Relationship between different Brassica species depicted as the "Triangle of U". Diploid species are indicated by red font, allotetraploid (amphidiploid) species by blue font taken from (Ostergaard and King, 2008).

5.2 Background and aims

Using information from *Arabidopsis* to investigate the physiological and genetic basis of variation in shoot system architecture in *Brassica napus* has been ongoing in the Leyser lab, in collaboration with the Bancroft lab at the John Innes

Centre (JIC). The ultimate aim is to identify alleles and biological knowledge to feed into marker assisted breeding. Mapping data and a dihaploid population generated by Ian Bancroft's group have been used for this work. Both QTL data from the Bancroft lab, and physiological characterisation of a selection of dihaploid lines with diverse degrees of shoot branching in the Leyser lab, suggested that genetic variation affecting the known hormonal network regulating branching in *Arabidopsis* is likely to contribute to at least some of the branching diversity observed in *Brassica napus*.

At the start of this study a new resource for reverse genetics, based on a diploid species, *Brassica rapa* was being developed by L. Ostergaard's group at JIC. R-o-18 is a *Brassica rapa* inbred line which is diploid, self fertilising and has a good seed set and rapid cycling characteristics (Stephenson et al., 2010). Plant architecture of *B. rapa* R-o-18 is similar to the cultivated oil seed crop *B. napus* and studies in *B. rapa* should be easy to translate to the polyploid *B. napus*. Moreover the sequencing of *B. rapa* genome (Wang et al., 2011) was ongoing at that time. This led to the preferential use of R-o-18 in exploring architectural traits such as shoot branching in this study. In addition to this reference line, M3 seeds of three branchy lines (391-8, 349-10 and 340-5), referred to hereafter in this chapter as line 8, line 10 and line 5 from an EMS mutagenised population of R-o-18 were obtained from JIC. These lines were visually selected from among the collection at JIC on the basis of their increased branching phenotypes.

5.3 R-o-18 morphology

To assess the basic branching morphology of *B. rapa*, line R-o-18 plants (figure 5.2) were grown on F2 compost in pots of 7" diameter. At the time of floral transition the plants had an average of eight to nine fully expanded leaves. R-o-18 plants have long internodes and buds big enough for growth measurements, as well as for hormone studies. At maturity R-o-18 plants had between 6 and 7 lateral branches on average on the primary stem. Branches originated in leaf axils (figure 5.2d) and similar to *Arabidopsis*, after making a few leaves, their shoot apical meristems underwent floral transition. (figure 5.2 e). Growth of R-o-18 was indeterminate. Normally, only the buds in the axils of topmost leaves grew out and each of the branches produced an indeterminate inflorescence as in

Arabidopsis. None of the leaf axils had any visible buds during the vegetative phase. Upon floral transition buds were visible in the axils of most apical fully expanded leaves and internode elongation on the main stem continued.

Lateral branching followed a basipetal gradient. Branches from the most apical leaf axils were longest and the length decreased progressively in a basipetal order. There were dormant axillary buds at lower nodes in intact plants, which had the potential to be released, for example in decapitation experiments. Inflorescence of R-o-18 is a raceme and flowers open from the base of the raceme acropetally. R-o-18 flowers are yellow with four diagonally opposite (cruciform) bilaterally symmetrical petals (figure 5.2 b). Just as in other Brassicaceae family members, R-o-18 fruits are dehiscent (figure 5.2 c), encapsulate the seeds and are referred to as pods or siliques, which split longitudinally when mature. It takes five to six months to complete the life cycle from seed to seed.

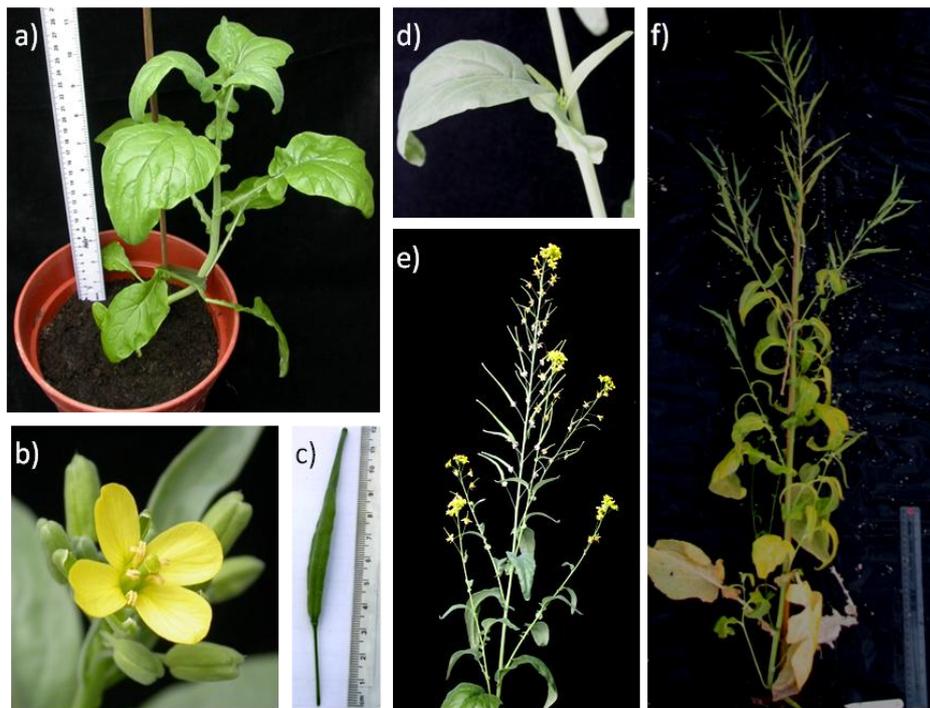


Figure 5.2 a) Four week old R-o-18 seedlings, b) single flower, c) a single pod, d) branch arising from leaf axil e) terminal inflorescence at 7 weeks, f) mature plant 14 weeks after sowing.

5.4 N response studies

5.4.1 Characterisation of the basic effects of Nitrate limitation in *Brassica rapa* line R-o-18

A system similar to Arabidopsis experiments on a 1:1 mixture of sand and Terragreen, fed with controlled amounts of Nitrate (in ATS solution) was used for initial Nitrate response studies of R-o-18. At floral transition, R-o-18 plants had an average of nine fully expanded leaves on high N and eight on low N (figure 5.3). Stem elongation was very rapid after floral transition, such that the height of the plant also increased rapidly. The mean plant height at floral transition was 44 cm and 35 cm on high and low N respectively.

Measurements of total plant height, total number of lateral branches on the primary stem, total number of leaf bearing nodes and total number of pods/silques were made at maturity, when all pods on the primary shoot had filled and started drying. At maturity, mean plant height was 107cm and 83cm on high and low N respectively. As previously observed in Arabidopsis, R-o-18 plants responded to N limitation by a reduction in plant height (figure 5.4 a), and a massive reduction in the number of branches (figure 5.4 b). The total number of nodes did not differ extensively (figure 5.4 c). There was a six fold reduction in the total number of pods produced on low N compared to high N (figure 5.4 d).



Figure 5.3 Six week old R-o-18 seedlings on high and low N. Arrows indicate branches arising from leaf axils on high N.

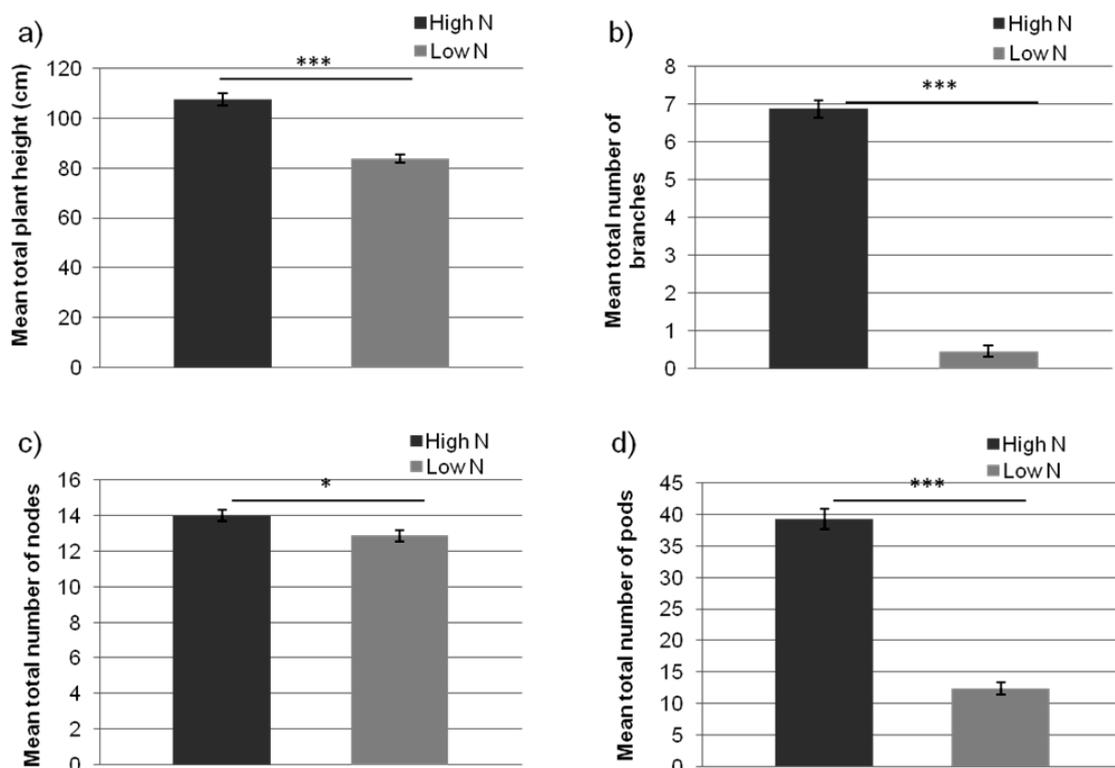


Figure 5.4 a) Mean total plant height (cm), b) mean total number of branches, c) mean total number of leaf-bearing nodes on the primary stem and d) mean total number of pods of R-o-18 at maturity on high (9mM) and low N (1.8mM). Values are means \pm SE of 10 plants. P- values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

5.4.2 N response studies in different lines of *Brassica rapa*

Nitrogen response studies were set up with four *B. rapa* lines, R-o-18 (isogenic background control line) and three lines (5, 8 and 10) identified as highly branched in an EMS mutant screen conducted at JIC. Plant height differed between the four lines grown on high N, with line 8 being the shortest and line 5 the tallest (figure 5.5). However, on low N the height of all four lines was comparable.

While comparing the branching response of the different lines, it was evident that they had very different sensitivities to N. On high N, line 8 had the most branches closely followed by line 10 (figure 5.6), with the wild-type R-o-18 being least branchy, reproducing the results of the original screen. On low N, two among the four lines- line 8 and wild-type R-o-18 did not produce any lateral branches. Line 10 lost half of its branches, whereas line 5 was the least plastic line with little difference between the number of branches on high and low N.

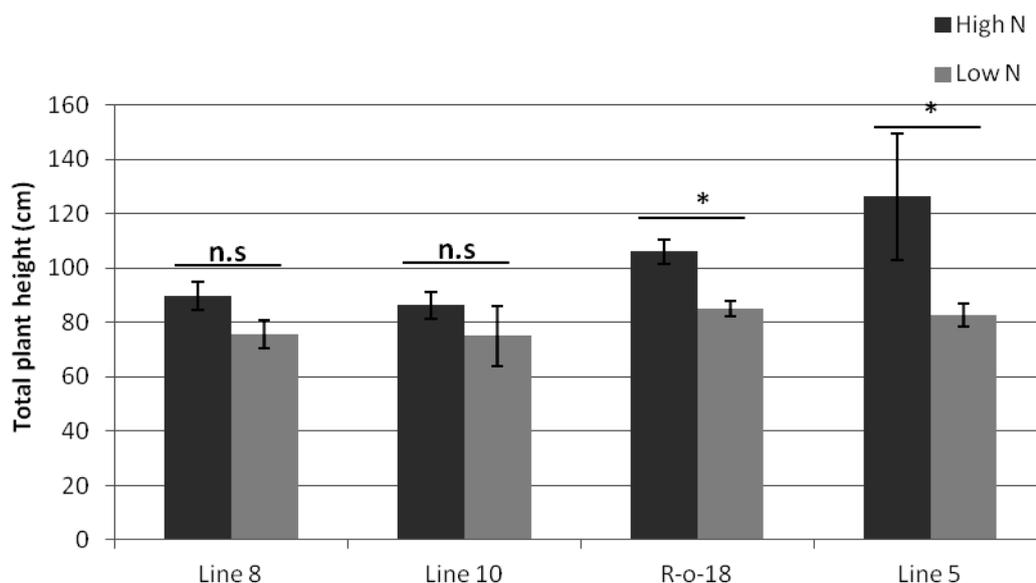


Figure 5.5 Mean total plant height (cm) of mature plants of different *Brassica rapa* lines grown in green house on 50% sand and 50% Terragreen mixture under high (9mM) and low (1.8mM) Nitrate supply. Values are means \pm SE of 10 plants. P-values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

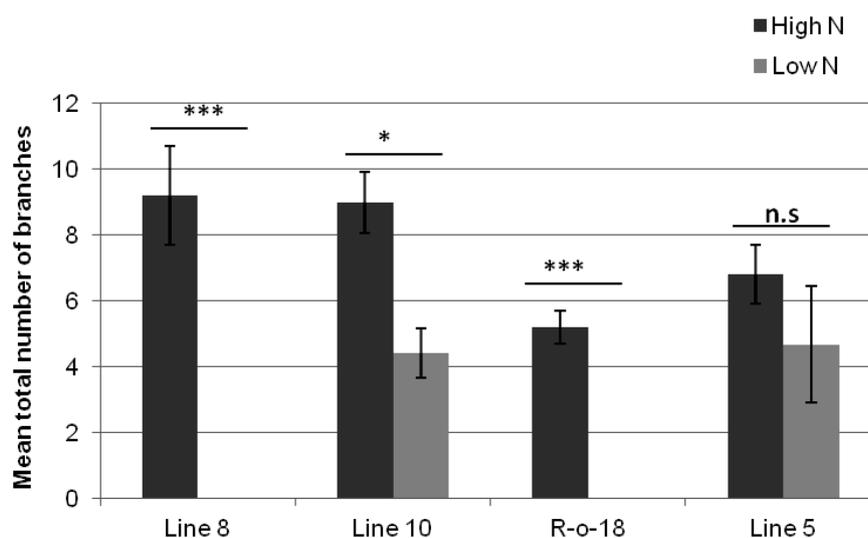


Figure 5.6 Mean total number of first order branches of mature plants of different *Brassica rapa* lines grown on high (9mM) and low (1.8mM) Nitrate supply. Values are means \pm SE of 10 plants. P-values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

5.4.3 Decapitation response of different lines on N sufficient and N limited conditions

There was no information on decapitation studies in *Brassica rapa*, whereas this was well characterised in *Arabidopsis*. With the rationale that buds at different nodes of the plant may have different activation potentials and different responses to decapitation, attempts were made to characterize the decapitation response of different nodes along the primary stem of R-o-18. The shoot tip was excised above the fourth node of young plants grown on both high and low N. Lanolin or NAA (1mM) in lanolin paste was applied to the stump. At this stage all the plants had four fully expanded leaves. Cotyledonary nodes were designated as node 0. Buds at all these nodes would normally have remained inhibited in intact plants. There were normally no visible buds in the cotyledonary nodes and by this stage, the cotyledons had abscised on both low and high N. No buds visible to the naked eye were present in the leaf axils at any of the nodes at the time of decapitation.

Following decapitation on high N, it was always buds at the cotyledonary nodes that grew out instead of buds in the most apical nodes (figure 5.7 a). In 46.9% of plants a single cotyledonary bud grew out compared to buds in both the cotyledonary nodes in 31.25% plants (table 5.1). When auxin was applied, a smaller proportion of plants activated buds compared to the high N control (78.13% in control vs Vs 47.06% in auxin treated plants). Also, in 1 out of 17 plants, the most apical bud grew out. When comparing decapitation on high and low N, the most striking difference was that while none of the most apical buds grew out on high N, a small proportion (12.5%) of apical buds grew out on low N (figure 5.7 b).

	High N	High N + NAA 1mM	Low N
Number of plants from which buds grew	25/32 (78.13%)	8/17 (47.06%)	16/32 (50%)
Both cotyledonary buds growing out	10/32 (31.25%)	3/17 (17.65%)	1/32 (3.13%)
Single cotyledonary bud growing out	15/32 (46.9%)	4/17 (23.53%)	12/32 (37.5%)
Bud at most apical node growing out	0/32	1/17 (5.89%)	4/32 (12.5%)

Table 5.1 Bud outgrowth from R-o-18 plants decapitated at node four on high and low N

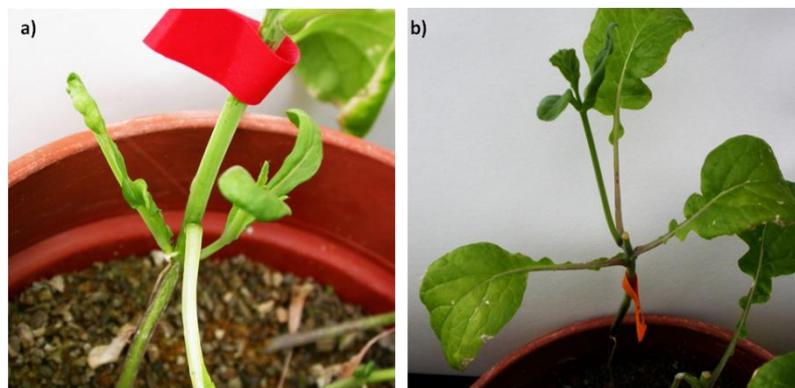


Figure 5.7 R-o-18 plants showing bud outgrowth at ten days following decapitation at node four on a) high N, b) low N.

For further experiments it was decided to decapitate plants at the stage when they just bolted (visible floral cluster at the primary apex), just below the inflorescence cluster so that the developmental stage was comparable to the standard stage for decapitation experiments in Arabidopsis. R-o-18 plants were decapitated above node ten (counting from base) and lanolin paste was applied to the cut stump. Although lateral branches grew out in intact plants, these branches were shorter and at a larger angle from the primary stem. When decapitated, it was observed that the most apical branches grew longer (figure 5.8 a, b) and at a more vertical angle than the branches in the same position in the intact plants. The lengths of most apical buds followed a similar pattern on both high (figure 5.8 a) and low N (figure 5.7 b) although very few intact plants produced branches on low N.

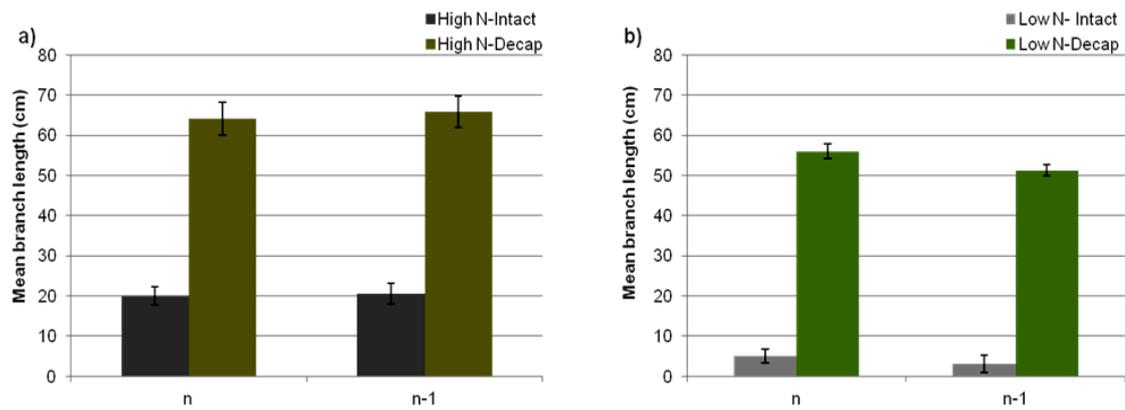


Figure 5.8 Mean branch lengths of two most apical first order branches in intact and decapitated plants of R-o-18. a) on high N b) on low N, measured 28 days after decapitation. Values plotted are means \pm SE of 13-15 plants.

To compare their decapitation response with the response of R-o-18, decapitation experiments were performed also with the three other lines. Plants selected for decapitation from all lines had at least nine fully expanded leaves. At this stage different lines had different numbers of nodes and fully expanded leaves. For example, lines 10 and 8 had twelve to thirteen, and lines R-o-18 and 5 had nine to ten fully expanded leaves below the inflorescence cluster. The phenotype of intact and decapitated plants two weeks after decapitation on high and low N is shown in figures 5.9 and 5.11 respectively.

Removal of the apex by decapitation on high N resulted in release of branches that would not have normally activated (figure 5.10). All the lines responded to decapitation on high N in a similar way, with normally dormant buds activating. Following decapitation, line 8 made marginally more branches compared to the other lines.

Response to decapitation on low N is shown in figure 5.12. Lines 8 and the wild-type R-o-18, which did not normally make any branches on low N, activated more than one additional bud, over-compensating for the loss of the apex. Lines 10 and 5 also responded to decapitation by activating buds resulting in branch numbers not significantly different from the intact controls (figure 5.12).

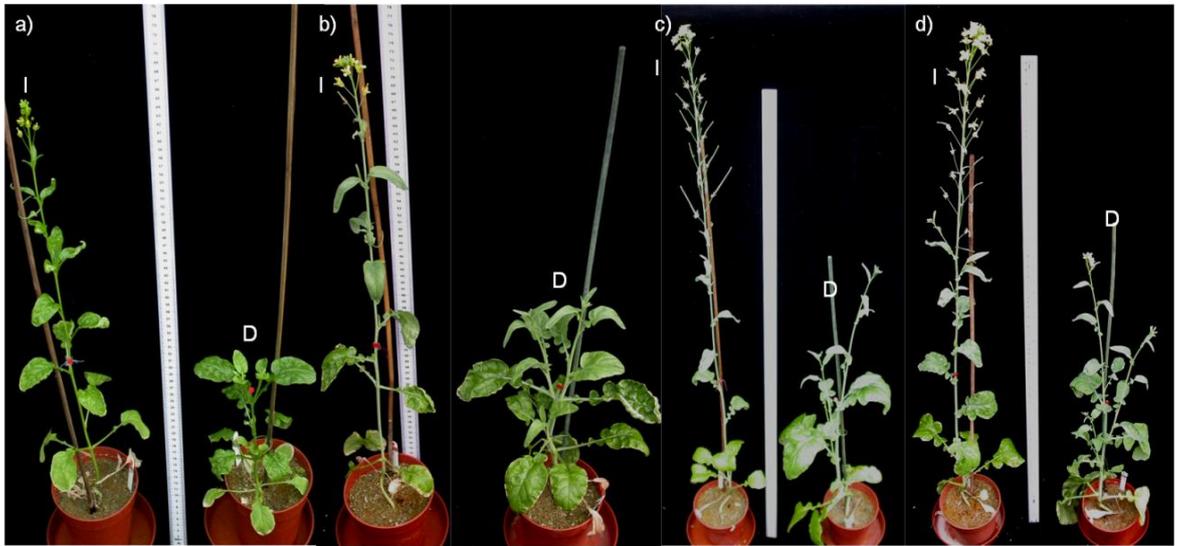


Figure 5.9 Phenotypes of a) line 8, b) line 10, c) wild-type R-o-18 and d) line 5 two weeks after decapitation on high N. I - denotes intact plants and D - denotes decapitated plants. The site of decapitation on decapitated plants and the corresponding node on intact plants are indicated by red dots in the figure.

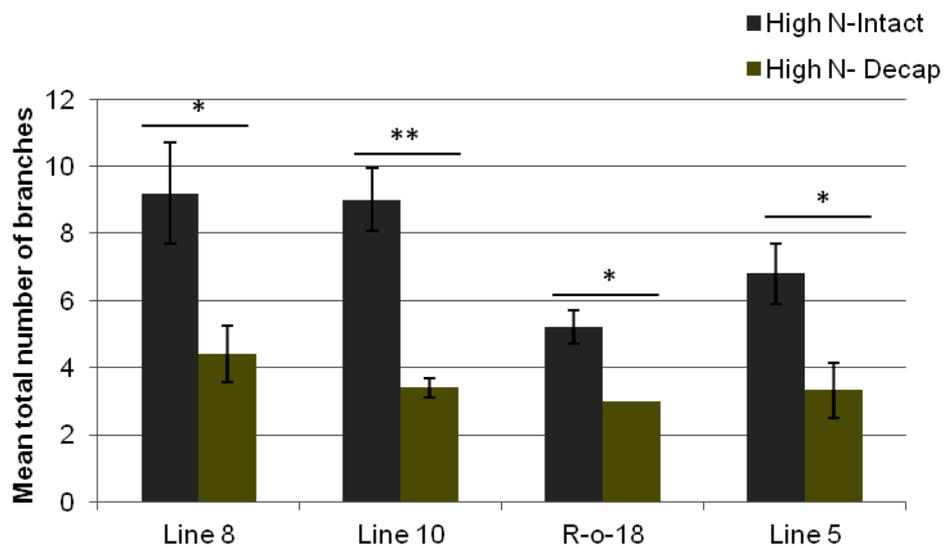


Figure 5.10 Response of different lines to decapitation on high N. Plants were grown in greenhouse in continuous long day conditions on 50% sand and 50% Terragreen mixture under high (9mM) Nitrate supply. Branches were counted 14 days after decapitation. Total branches of intact plants grown under the same condition were compared to branches of decapitated plants. Values plotted are means \pm SE of 6-8 plants. P- values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.



Figure 5.11 Phenotype of a) line 8, b) line 10, c) Wild-type R-o-18 and d) line 5 two weeks after decapitation on low N. I - denotes intact plants and D - denotes decapitated plants. The site of decapitation on decapitated plants and the corresponding node on intact plants are indicated by yellow dots in the figure.

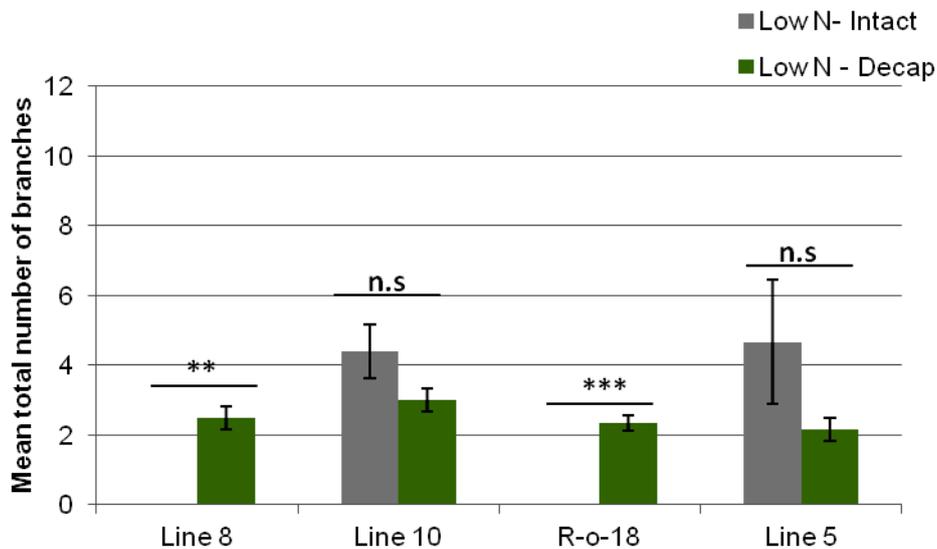


Figure 5.12 Response of different lines to decapitation on low N. Plants were grown in greenhouse in continuous long day conditions on 50% sand and 50% Terragreen mixture under low (1.8mM) Nitrate supply. Branches were counted 14 days after decapitation. Total branches of intact plants grown under the same condition were compared to branches of decapitated plants. Values plotted are means \pm SE of 6-8 plants. P- values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

5.5 Preliminary characterisation of line 8

Among the three branchy lines obtained from the EMS mutagenised population from JIC one mutant; line 8, exhibited several phenotypic aberrations compared to the wild-type line, R-o-18, at all stages of development. It was hypothesised that this mutant might have a hormonal basis, hinted by its pleiotropic phenotype. Some preliminary experiments were performed comparing line 8 to R-o-18 to test this hypothesis.

5.5.1 Morphological abnormalities in line 8

Line 8 exhibited several morphological abnormalities including defects in the leaves, flowers, inflorescences, pods and seeds. These defects are illustrated in figure 5.11. Line 8 plants had a shorter stature than the control line R-o-18. At maturity the mean total plant height of R-o-18 was 106cm compared to 89.75cm in line 8 (figure 5.13 a). This was associated with shorter internodes (figure 5.13 d). As described above, the total number of lateral branches of line 8 was twice that of R-o-18 (figure 5.13 b, c). In addition to the increased first order branching, line 8 also appeared to show increased higher order branching (figure 5.13 d). These features together made line 8 appear much bushier in appearance than R-o-18. Leaves of line 8 were rounder and tended to curl inwards (hyponastic leaves) compared to R-o-18, and they had sunken patches clearly visible on their abaxial surfaces (figure 5.13 f). Furthermore, plants of line 8 senesced later than R-o-18 and had darker leaves. Individual flowers of line 8 were also defective in appearance (figure 5.14 a). On the inflorescences, many flowers were malformed and the petals were irregular in shape. A low proportion of individual plants of line 8 showed fasciation of the primary inflorescences as seen in figure 5.13 e. Corresponding to the floral defects, several pods were also malformed and even the healthier pods were shorter and lacked the long beak of R-o-18 (figure 5.14 b). Line 8 had a high proportion of aborted or malformed seeds compared to R-o-18, which could be seen in figure 5.12 e. The average number of seeds per pod was much lower in line 8 compared to R-o-18. Weight of 50 seeds also differed between the lines, with a slight reduction in seed weight in line 8 (figure 5.14 f).

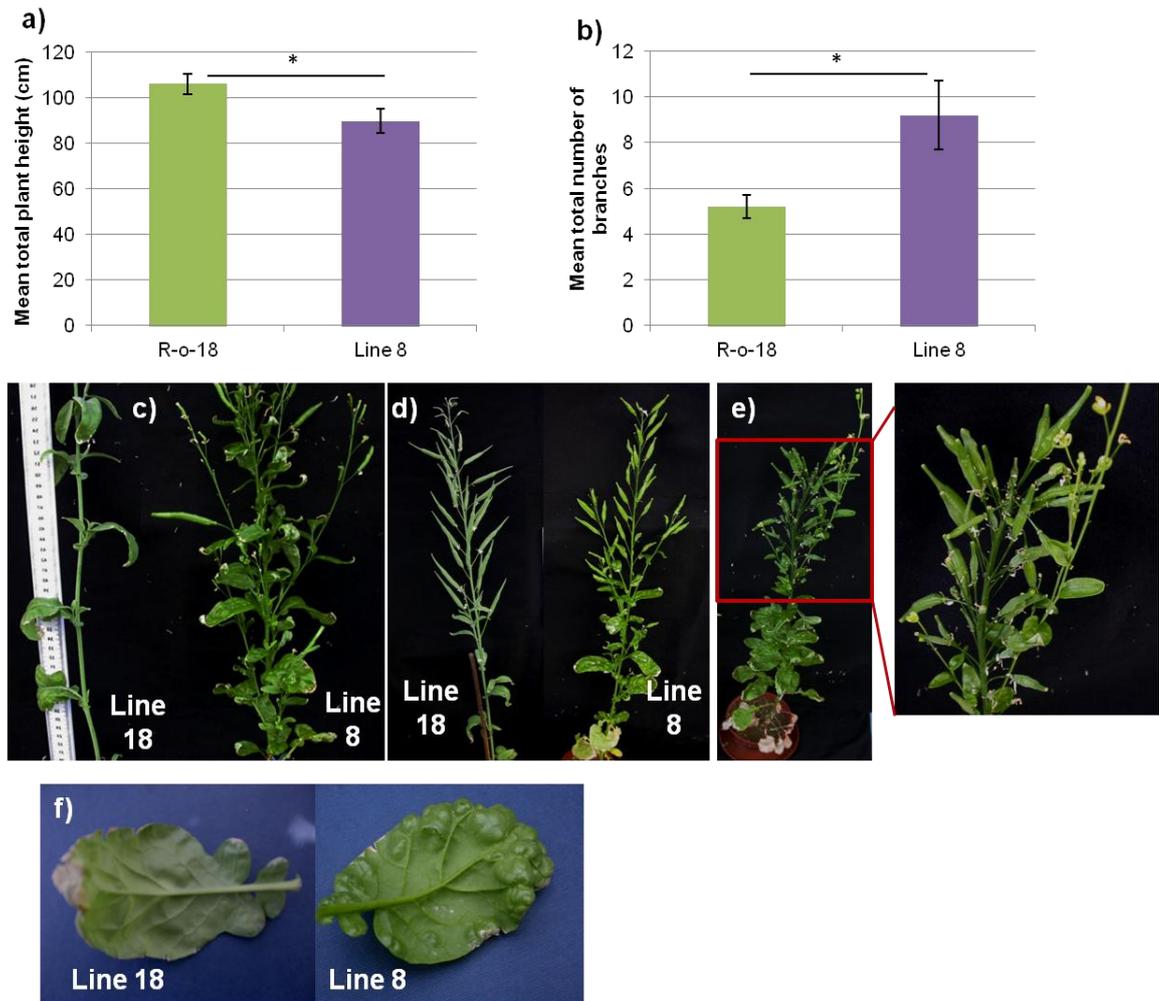


Figure 5.13 Comparison of line R-o-18 and line 8. a) Mean total plant height, b) mean total number of branches, c) branching in line R-o-18 and 8, d) higher order branching in line R-o-18 and 8 e) fasciated primary inflorescence of line 8 and f) abaxial view of single leaves of line 18 and line 8. P- values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; *, $P<0.0001$.**

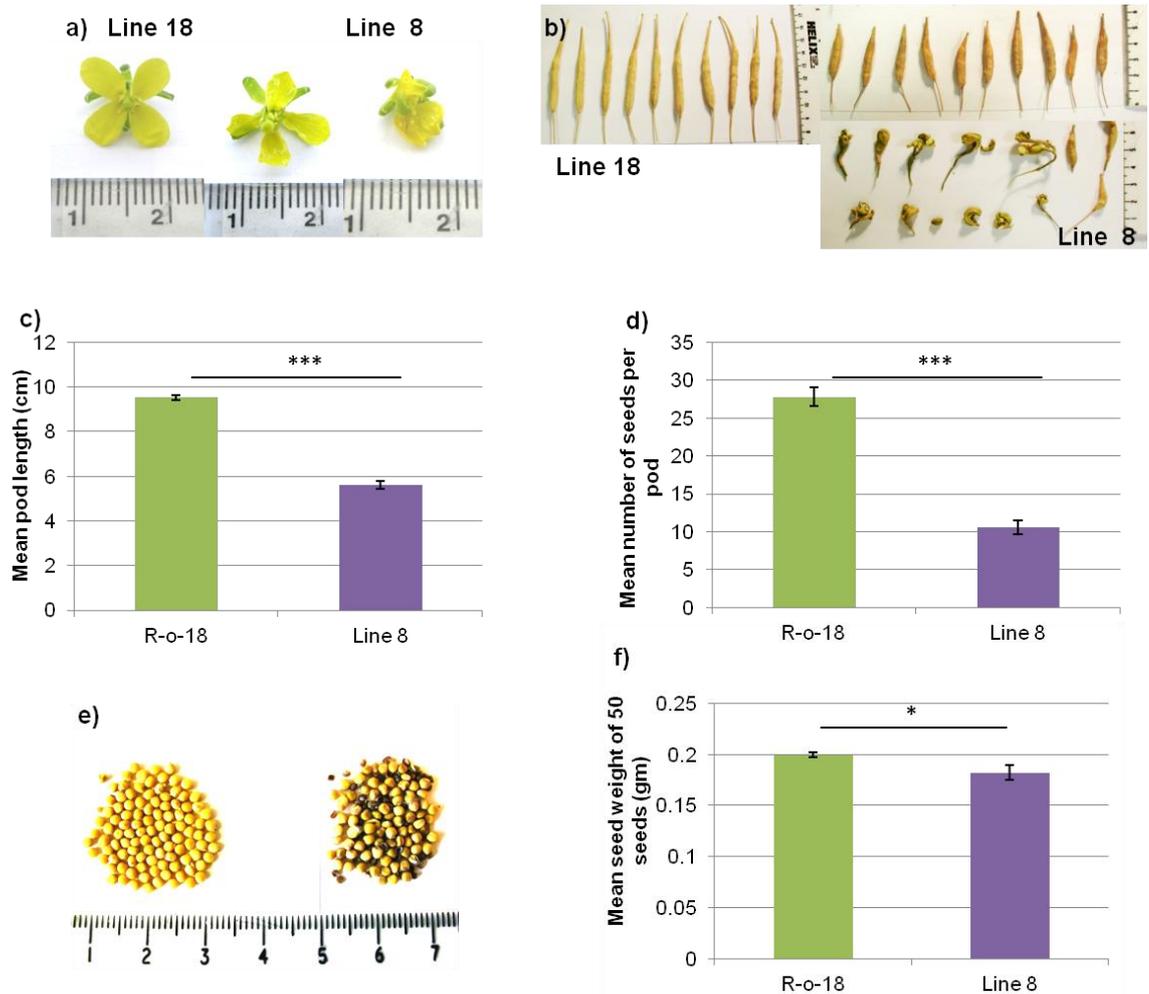


Figure 5.14 Comparison of flowers and seeds of R-o-18 and line 8. a) single flowers b) single pods, c) mean pod length (cm), d) mean number of seeds per pod, e) seeds of line 18 and line 8 and f) mean seed weight of 50 seeds. P- values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; *, $P<0.0001$.**

5.5.2 Preliminary experiments in an attempt to understand the cause of the phenotypes

Hypocotyl elongation is a useful assay for studying hormone and light responses. So this assay was performed with the *B. rapa* mutant. Many of the hormone biosynthetic and signalling mutants in *Arabidopsis* affect hypocotyl elongation. Light is an important environmental signal affecting plant growth, and plant hormones interact with light to bring about different growth responses. Light has a strong influence on hormone levels, transport and responsiveness. For example, it is known that photomorphogenesis in *Arabidopsis* seedlings is regulated by auxin (Halliday et al., 2009). Not much is known about the

response of *B. rapa* seedlings to light and so preliminary experiments were done to assess this.

When seedlings were grown on agar plates supplemented with *Arabidopsis thaliana* nutrient medium (Wilson et al., 1990) in white light, line 8 had shorter hypocotyls (figure 5.15) and longer roots (figure 5.16) compared to R-o-18. In addition to this, it was observed that the cotyledons of line 8 were considerably bigger than those of R-o-18 from the time they expanded. As *B. rapa* seedlings grew too big for accurate assessment on plates, seedlings were grown in pots for the dark and red light experiments. It is known that when seedlings of the *Arabidopsis* auxin resistant mutant, *axr1*, are grown in the dark, elongation of hypocotyls was reduced and elongation of root increased compared to wild-type (Lincoln et al., 1990). When seedlings of R-o-18 and line 8 were grown in the dark, there was no significant difference between the lines in their hypocotyl length (figure 5.17). When grown in red light, line 8 had shorter hypocotyls and more expanded cotyledons than R-o-18 (figure 5.18). This response was similar to that observed in white light.

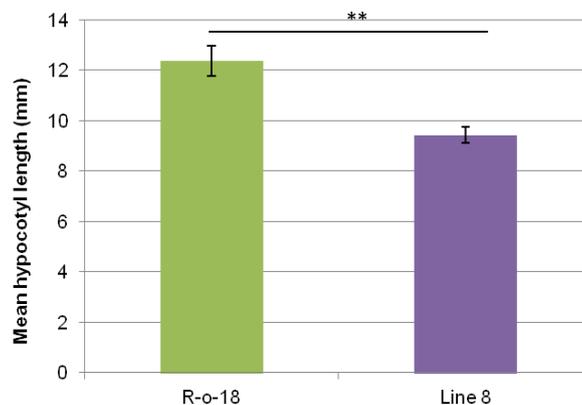


Figure 5.15 Hypocotyl lengths of line R-o-18 and 8 seedlings grown in white light ($\sim 60\text{-}100\mu\text{mol m}^{-2}\text{ s}^{-1}$). Values are means \pm SE of 20 – 25 seedlings. P- values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; *, $P<0.0001$.**

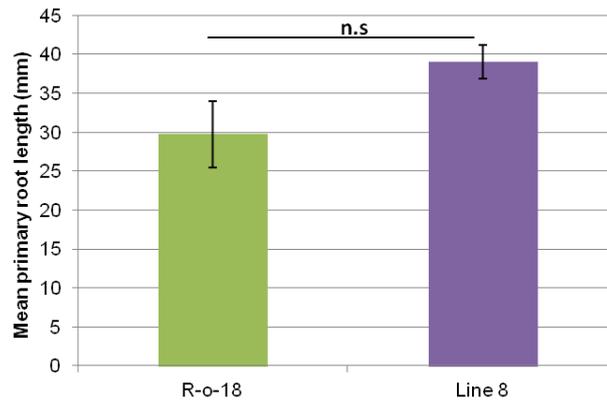


Figure 5.16 Primary root lengths of R-o-18 and line 8 seedlings (day 3) grown in white light ($\sim 60\text{-}100\mu\text{mol m}^{-2} \text{s}^{-1}$). Values are means \pm SE of 20 – 25 seedlings. P-values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

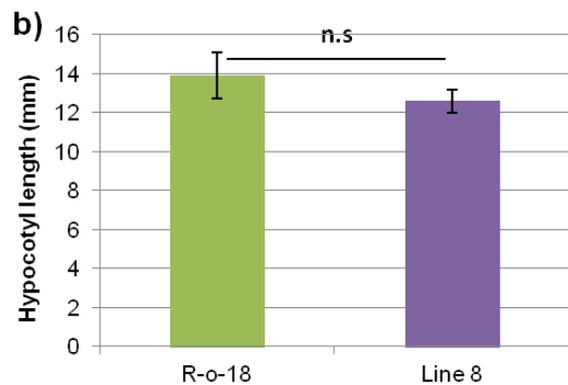
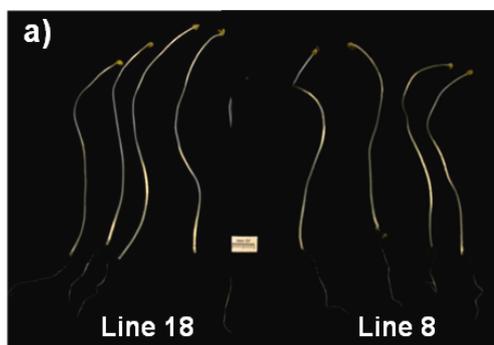


Figure 5.17 a) Phenotype of dark grown seedlings of R-o-18 and line 8 (day 5), **b)** Mean hypocotyl length of R-o-18 and line 8 seedlings grown in dark. Values are means \pm SE of 10 seedlings. P-values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

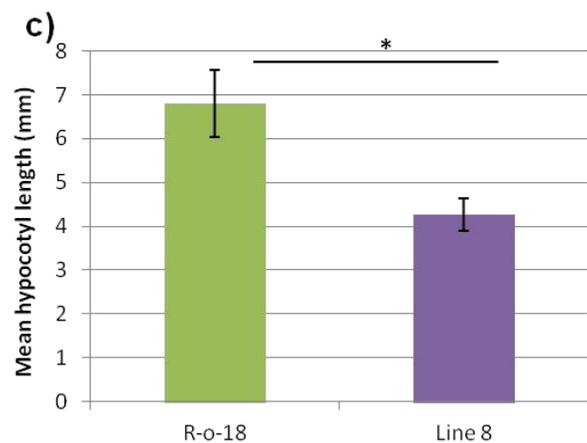
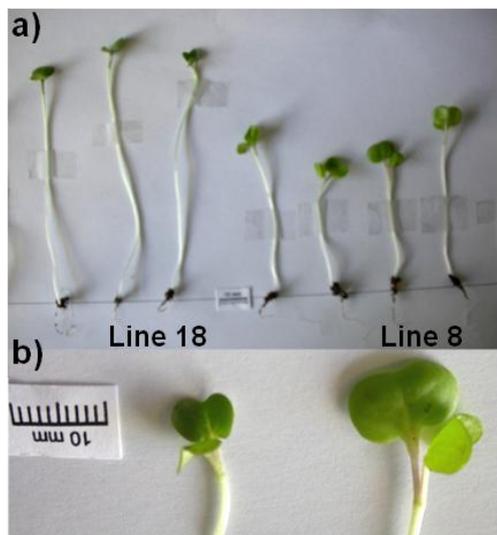


Figure 5.18 a) Phenotype of seedlings of R-o-18 and line 8 grown in red light (day 5), **b)** Enlarged cotyledons of line 8 compared to R-o-18, **c)** Mean hypocotyl length of R-o-18 and line 8 seedlings grown in dark. Values are means \pm SE of 10 seedlings. P-values calculated using Student's *t*-test- n.s., not significant ($P>0.05$); * <0.05 ; ** $P<0.001$; ***, $P<0.0001$.

5.6 Grafting attempts in *Brassica rapa*

Grafting studies have been informative in several plant species in characterising the nature of mutants impaired in hormonal pathways. For example, grafting experiments conducted in *Arabidopsis* with *max* mutants played a crucial role in understanding the nature of SL synthesis and transport. Grafting approaches in *B. rapa* were attempted, which might be useful to provide evidence for any hormonal basis for mutants such as line 8.

Previous attempts at grafting by other people had proven unsuccessful (D. Newman, personal communication) and several reasons; including variability in stem size, difficulty in hardening of the grafted plants etc were pointed out. Initial attempts at grafting in this study using greenhouse-grown plants at different ages and different grafting techniques were not successful.

Since *Arabidopsis* grafts were successfully performed in sterile conditions from plants grown in Petri dishes, attempts were made to grow *B. rapa* seedlings in large Weck jars (1L) and a wedge grafting technique, similar to a technique successful in *Arabidopsis* (Turnbull et al., 2002) was used to join the scion and root stock of these plants in sterile conditions. Tight fit of the graft region was ensured by wrapping micropore tape around the graft union. After two weeks, these plants were transferred to soil and maintained under high humidity conditions by covering with a plastic bag. Self-grafts of the two lines R-o-18 and 8, and a few other grafts were successfully hardened (figure 5.19), grown to maturity and seeds were collected. However, several grafts including self-grafts of line 10, failed due to the difficulty in precisely aligning the graft union and different stem sizes of the seedlings used. Quantitative data of the grafting trials could not be presented as only very few plants were successfully hardened.

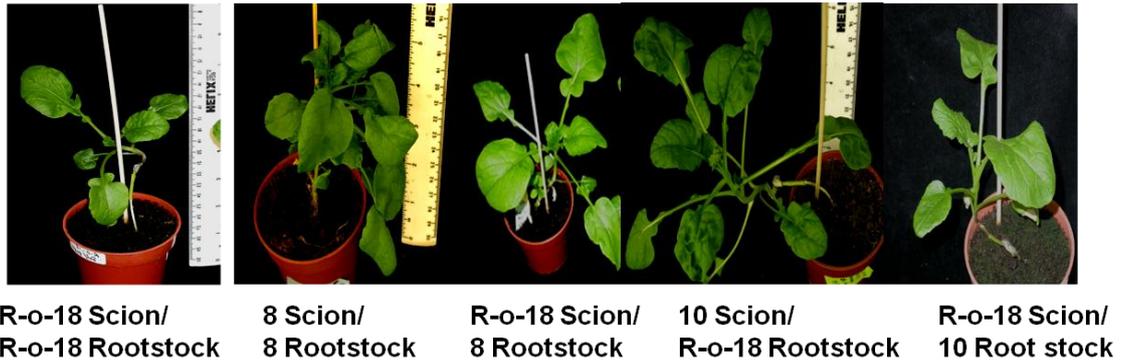


Figure 5.19 a) Phenotype of grafts between different *B. rapa* lines, 4 weeks after grafting.

5.7 Hormonal assays in R-o-18

Hormone response assays developed in *Arabidopsis* provided the opportunity to investigate the effect of hormones on bud outgrowth of *B. rapa*. Attempts were made to adapt the well established *Arabidopsis* hormone response assays to *B. rapa* to examine whether auxin-dependent strigolactone regulation of bud activity is similar or different in this species, under different nutrient regimes.

5.7.1 Split plate assays

The effect of apical auxin in delaying bud outgrowth in *Arabidopsis* in split plate assays have already been shown (Chatfield et al., 2000a). Furthermore, the inability of GR24 alone to bring about bud inhibition, and its ability to enhance the inhibition imposed by apical auxin had been established in *Arabidopsis* (Crawford et al., 2010). Studies in other species such as chrysanthemum gave similar results (Liang et al., 2010). To determine whether *B. rapa* responds in the same way, split plate assays adapted from Chatfield et al (Chatfield et al., 2000a) were performed on isolated nodal segments from R-o-18 plants.

Single node segments were excised from plants grown in glass jars (Weck Canning) in sterile conditions and inserted between agar blocks supplemented with high (9mM) N or low (1.8mM) N in Petri dishes (figure 5.20). Auxin (0.5 μ M NAA) or an equivalent volume of ethanol was supplied via the apical agar block and SL (1 μ M GR24) or equivalent volume of acetone was supplied via the basal agar block as described in Crawford et al (2010) (figure 5.20).

The results of this experiment on high N (figure 5.21) suggested that, as in *Arabidopsis* and chrysanthemum, basally supplied GR24 alone was ineffective at inhibiting bud outgrowth, whereas apically supplied NAA alone was effective at inhibiting bud outgrowth. Combined treatment of GR24 and NAA was able to inhibit bud outgrowth to an even greater extent than auxin alone, but not completely (figure 5.21).

Split plate assays on low N also demonstrated the inability of GR24 alone to inhibit bud outgrowth and elongation, and that the combined treatment of GR24 and NAA affected bud elongation, in addition to causing delay in bud outgrowth (figure 5.22). The NAA alone treatments on low N were lost due to contamination in the plates. These assays confirmed that as in *Arabidopsis*, N limitation alone cannot bring about inhibition of bud outgrowth, as demonstrated by comparing outgrowth of untreated buds on high vs low N.



Figure 5.20 Split plate assays with isolated nodes of R-o-18 between agar blocks.

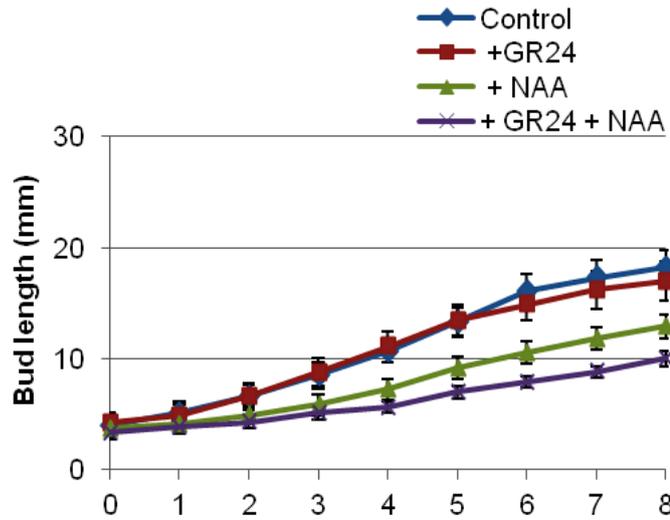


Figure 5.21 Mean bud length of single nodes of R-o-18 on high N (9mM Nitrate) showing the effect of GR24 (1 μ M) and NAA (0.5 μ M) on bud outgrowth. Error bars represent the standard error of the means (n = 10).

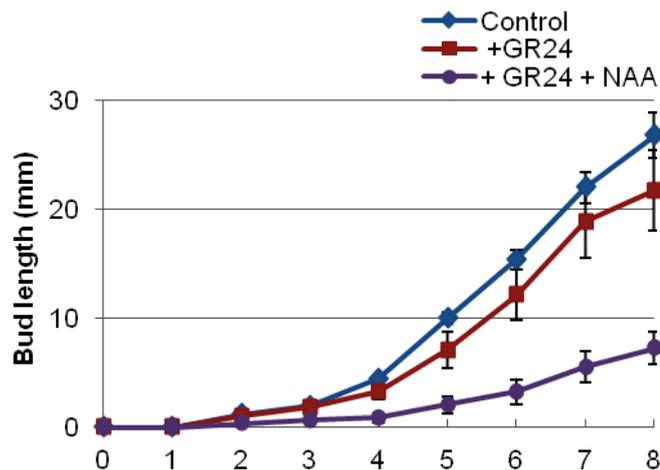


Figure 5.22 Mean bud length of single nodes of R-o-18 on low N (1.8mM Nitrate) showing the effect of GR24 (1 μ M) and combined treatment of GR24 + NAA (0.5 μ M) on bud outgrowth. Error bars represent the standard error of the means (n = 8).

5.7.2 Bud assays on R-o-18

To expand the options for assessing hormone responses in *B. rapa*, Arabidopsis nodal assays described in Chapter 4 were adapted for use with *B. rapa*. Unlike split plate assays, where sterile plants were used, these nodal assays were performed using plants grown in green house. Due to the larger sizes of leaves and branches, a system using 15ml falcon tubes was used in the *B. rapa* bud assay set ups instead of microcentrifuge tubes. Either a propagator lid (for one bud assays) or a humidity chamber, as seen in figure 5.23 d, was used to

maintain humidity and to prevent the buds from drying out. The decapitated apex was sealed with lanolin paste to prevent transpiration loss. An auxin treatment of 1 μ M NAA and an SL treatment of 1 μ M GR24 were used in all bud assays unless otherwise specified.

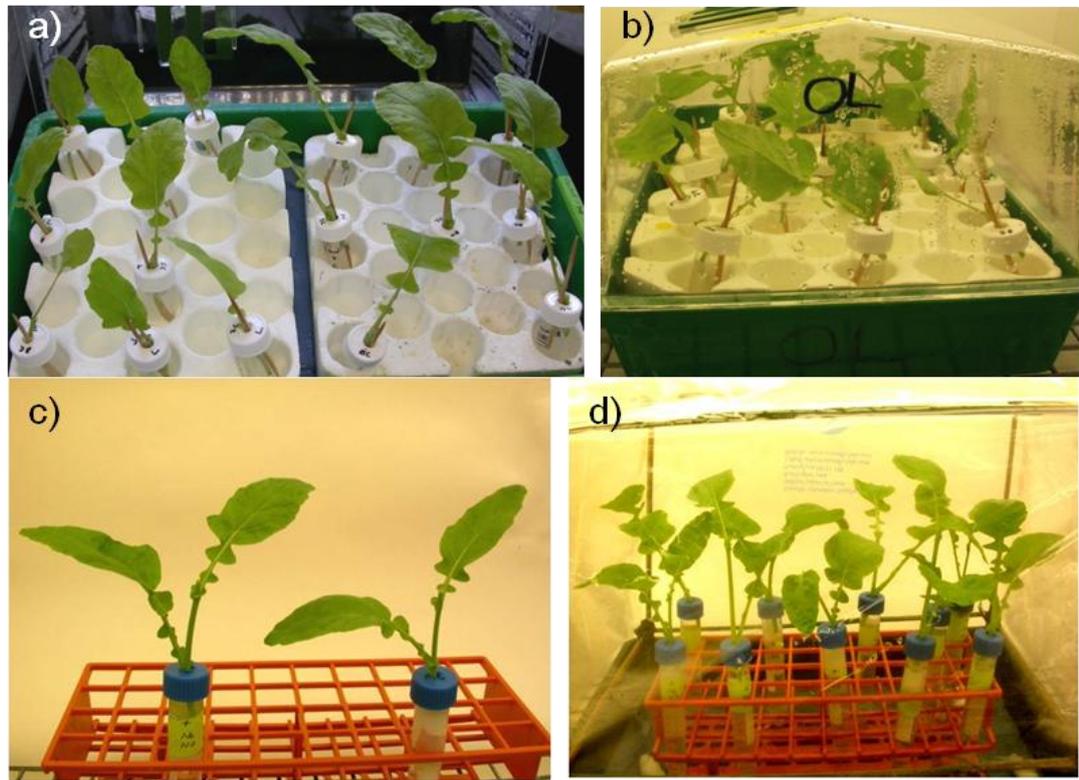


Figure 5.23 a, b *B. rapa* one bud assay set up and c, d *B. rapa* two bud assay set up.

5.7.2.1 One bud assays in *Brassica rapa*

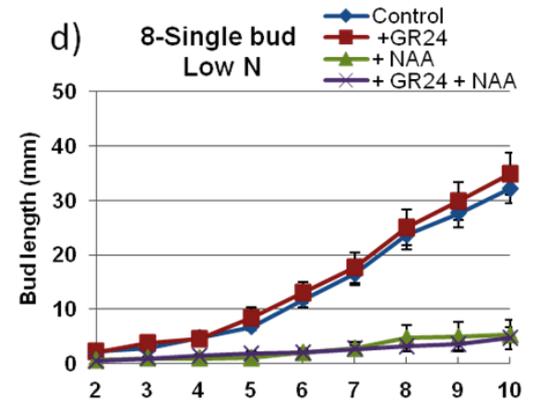
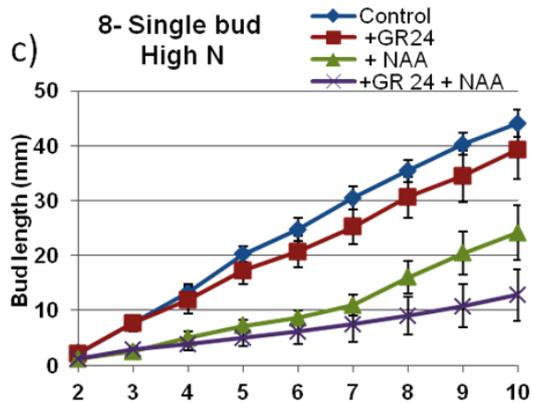
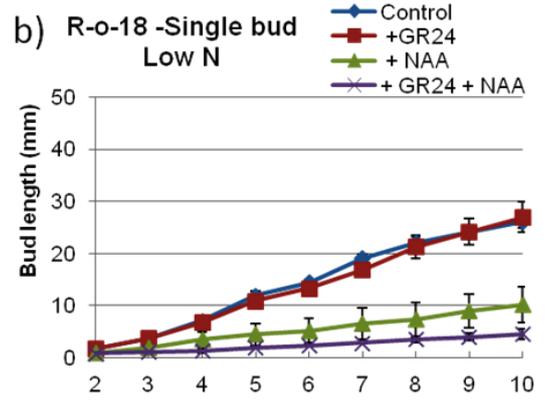
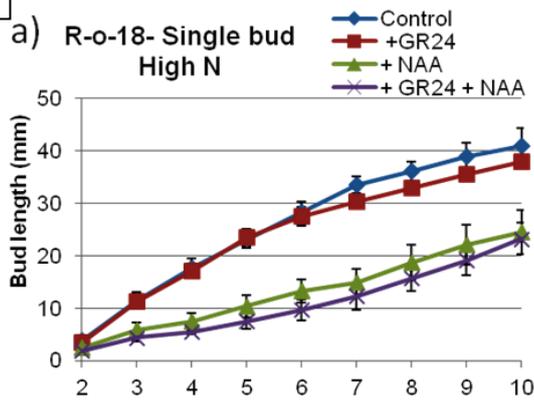
According to the auxin transport canalisation hypothesis for bud regulation, without a competing auxin source, GR24 should have no effect on outgrowth of single buds. Bud assays were performed with both R-o-18 and the branchy mutant line 8 to determine whether this holds true for *B. rapa* (figures 5.24 a, b). The results of one bud assays on high N (figure 5.24 a) confirmed undoubtedly that in R-o-18 both untreated and GR24 treated single buds behave in a similar way to *Arabidopsis* single buds. GR24 alone was ineffective in inhibiting bud outgrowth. Auxin was able to inhibit single buds on high N, but adding basal GR24 in addition to apical auxin did not have any enhanced effect (figure 5.24 a). Bud elongation progressed at a slower rate on low N (figure 5.24 b), but still

the inhibitory effect of NAA was observed. There was also a modest additional effect of GR24 in the GR24 + NAA combination.

Line 8 followed a similar trend to the reference line on both high (figure 5.24 c) and low N (figure 5.24 d). There was no evidence of auxin or SL resistance, as might be expected for a branchy line, rather the inhibitory effect of GR24 + NAA combination was slightly more pronounced in this line. Also the growth of control single buds of line 8 was slightly more vigorous than the reference line on low N.

Among the isolated bud experiments, buds grew more vigorously in this assay compared to the split plate-based assay set up. Also both NAA and the combination of NAA and GR24 were more effective in inhibiting bud outgrowth and delaying the bud outgrowth in this assay set up.

A



B

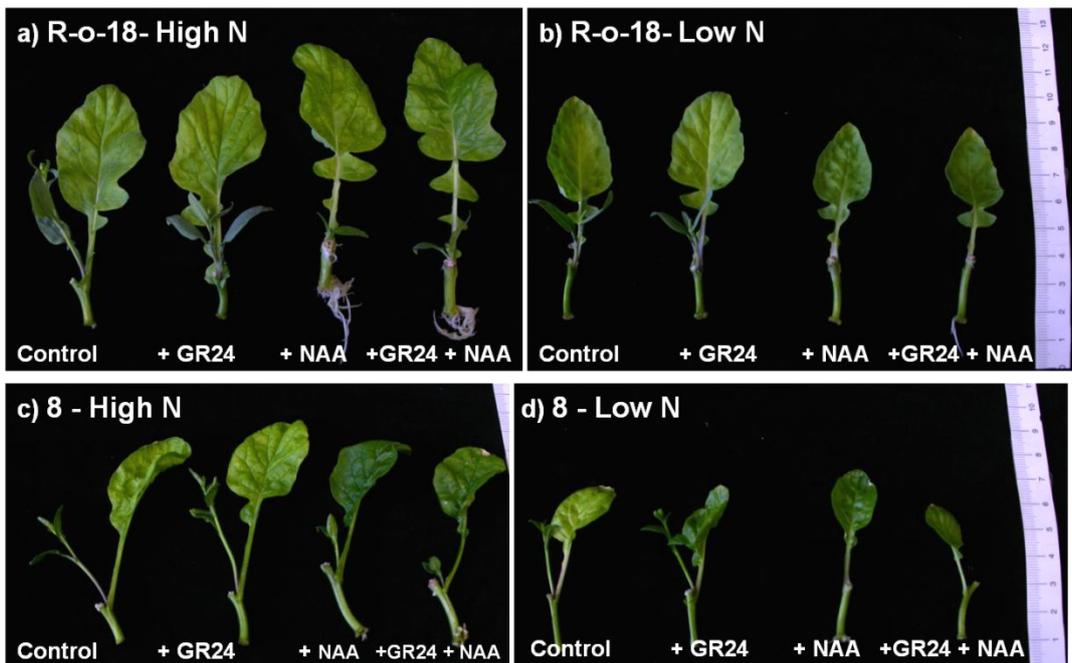


Figure 5.24 A. Bud lengths (mm) of R-o-18 single bud on a) high N b) low N and line 8 on c) high d) low N in one node assays with or without GR24 (1 μ M) and NAA (1 μ M). Means \pm SE of 10 plants are shown. 5.24 B. Bud outgrowth in single node assays of R-o-18 on a) high N and b) low N, line 8 on c) high N and d) low N on day 7.

5.7.2.2 Two bud assays in *Brassica rapa*

Given the difference in response of *B. rapa* buds to GR24, even in the presence of apical auxin, two bud assays were used to assess whether a second bud was a more effective auxin source than an apical auxin application. In two-node experiments with R-o-18 on high N, both the top bud and bottom buds grew to a similar extent (figure 5.25 a). Neither bud responded to basal GR24 application. Apical auxin alone had a mild inhibitory effect on the outgrowth of the top bud, and GR24 treatment did not enhance this effect. None of the hormone treatments had any convincing effect on the bottom bud (figure 5.25 a). The RGIs (figure 5.26 a) for all treatments were similar to the untreated control. On low N (figure 5.25 c), there was a similar overall pattern of bud activity, except that the bottom bud was generally less vigorous. This had an impact on the RGI, which was generally higher than on high N. However, the inhibitory effect of apical NAA on the elongation of the top bud reduced the RGI to values similar to those observed on high N (figure 5.26 b). As in the one node assays, there was no evidence of any effect of GR24, either alone or in combination with auxin.

Just as in R-o-18, the top and bottom buds of line 8 grew to a similar extent and neither bud responded to GR24 treatment (figure 5.25 b). Apical auxin had a more pronounced inhibitory effect on the top bud when compared to R-o-18, but again GR24 did not enhance this effect. On the contrary, the inhibition of the top bud by NAA was apparently alleviated by GR24 treatment. RGI on high N was not affected by any of the treatments except a slight increase for auxin alone (figure 5.26 c). On low N both the top and bottom buds grew to similar extent (figure 5.25 d). As with R-o-18, GR24 alone treatment had no effect on any of the buds. NAA alone had an inhibitory effect on both top and bottom bud on low N but here again, GR24 could not enhance the effect. The similar pattern of bud activity in the top and bottom buds on both high and low N was reflected in similar RGIs for both controls and all the treatments (figure 5.26 c, d).

The striking difference between *Arabidopsis* and *B. rapa* was that, even in the presence of a competing auxin source, *B. rapa* buds generally did not respond to GR24 in either high or low N situations. A higher concentration of 5 μ M GR24 was also tried, and the results were similar to those obtained with 1 μ M GR24.

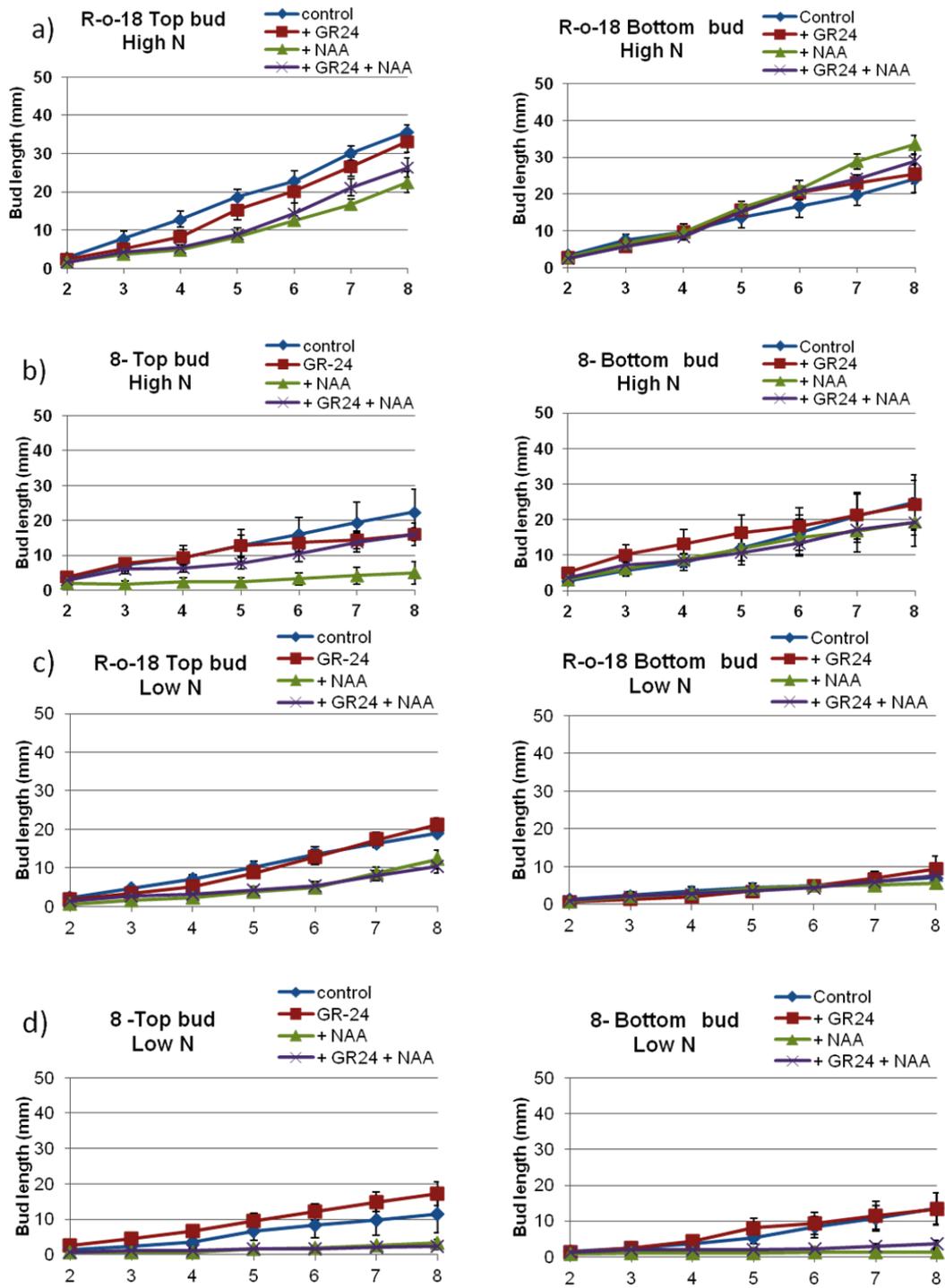


Figure 5.25 Mean bud length (mm) of R-o-18 and line 8 on high (a, b) and low N (c, d) in two node assays with or without GR24 (1 μ M) and NAA (1 μ M). Means \pm SE of 6-8 plants are shown.

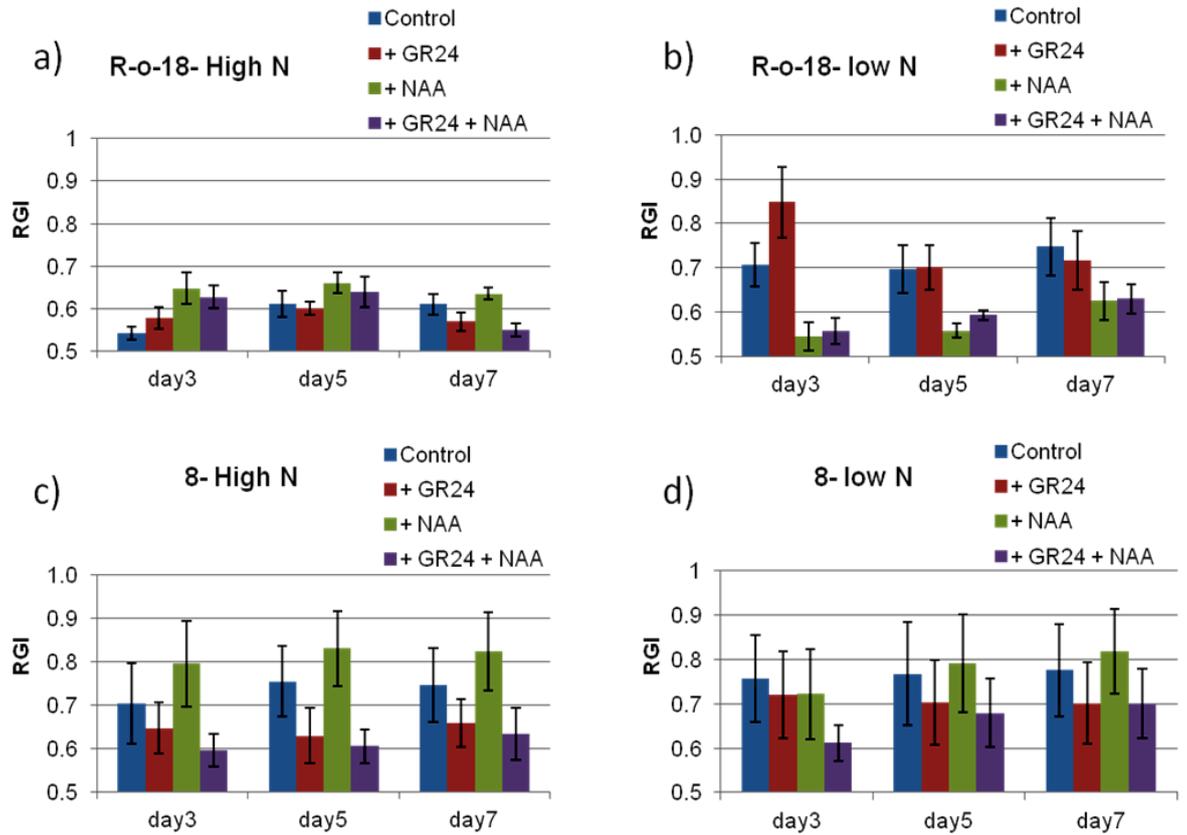


Figure 5.26 Mean Relative growth index of R-o-18 and line 8 on high N (a, c) and low N (b, d) in two node assays with or without GR24 (1 μ M) and NAA (1 μ M). Means \pm SE of 6-8 plants are shown.

5.7.2.3 Effect of SLs on leaf senescence

During the bud assays on high and low N it was observed that basal supply of either 5 μ M or 1 μ M GR24 induced early senescence in some situations. For single node assays, GR24-treated leaves senesced earlier than the untreated controls; but on low N, senescence was rapid in both the GR24 and untreated leaves. In two node assays, GR24 induced early senescence specifically in the bottom leaf, while the top leaf and both leaves of untreated explants stayed greener longer (figure 5.27 c). There was a delay of three to four days between the untreated versus GR24 treated bottom leaves in the induction of senescence.

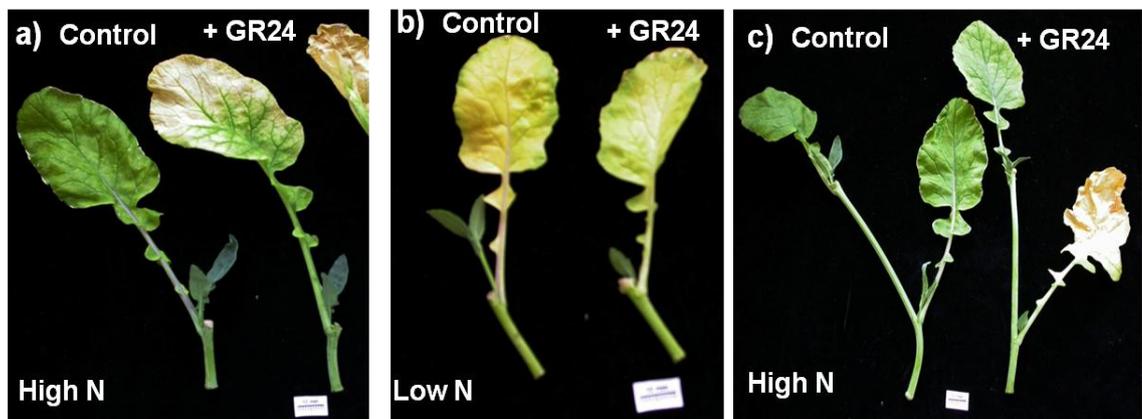


Figure 5.27 Senescence of leaves in control and treated (basal 1 μ M GR24 single buds on a) high N, b) low N c) Bottom leaf senescence in two bud assays with basal application of GR24 (1 μ M) on high N on day 10.

5.8 Tilling in *Brassica rapa*

TILLING (Targeting Induced Local Lesions IN Genomes) is a reverse genetics approach developed by McCallum et al and Colbert et al (Colbert et al., 2001, McCallum et al., 2000). In this technique, mismatches in annealed normal and mutant DNA strands are detected by a mismatch-sensitive nuclease. Ethylmethylsulphonate (EMS) is used to mutagenise seeds, which give rise to plants with random point mutations (Till et al., 2003). The TILLING platform at John Innes Centre available through RevGen UK (<http://revgenuk.jic.ac.uk/>) is based on EMS mutagenised populations of R-o-18 developed by Lars

Ostergaard. This service was used to obtain a *max2* SL signalling mutant in *B. rapa*. This was obtained to test the role of SL in branching in *Brassica rapa*.

Both genomic and cDNA sequence for the *B. rapa* *MAX2* gene from the publically available Chiifu variety (Wang et al., 2011), was sent to RevGen for the TILLING service. A BLAST (Basic Local Alignment Search Tool) search was performed using Arabidopsis *MAX2* to provide an annotation for the Brassica *MAX2* sequence (<http://blast.ncbi.nlm.nih.gov/Blast>, <http://brassicadb.org/brad/>). The Brassica genome browser at <http://brassicadb.org/cgi-bin/gbrowse/cbgdb/?name=Bra016864> showed that the gene had a single exon, as in Arabidopsis, making the genomic and cDNA sequences identical. The sequence of the *B. rapa* *MAX2* ORF was obtained from the Brassica database and was 2.343kb (<http://brassicadb.org/brad/seqFast.php?qinput=Bra016864>). Primers were designed for TILLING (figure 5.28, primers designated by underlining and bold cases) by RevGen based on this sequence, and the TILLING procedure was carried out by RevGen. The complete annotated sequence of *MAX2* genomic DNA, cDNA and protein can be seen in figure 5.28.

An alignment of the *B. rapa* *MAX2* protein sequence with Arabidopsis *MAX2* showed they were very similar. Aligned sequences from *B. rapa* demonstrated more than 85.5% identity to Arabidopsis. Protein sequence alignment of *MAX2* from selected species that had high identities was generated using ClustalW (Larkin et al., 2007) and BioEdit (Hall, 1999). The sequence alignment and the consensus sequence can be viewed in figure 5.29.

Figure 5.29 Alignment of MAX2 protein from selected species showing consensus sequences.

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      10      20      30      40      50      60      70      80      90      100
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
Brassica max2
P.trichocarpa XP_002320412.
P.hybrida AEB97384.1|
A.lyrata |XP_002879997.
A.thaliana|NP_565979.1|
T.halophila dbj|BAJ33992.1|
M.truncatula XP_003607592
P.sativum ABD67495.1|
Clustal Consensus
      .      :      .**      ***      :      :      :      *      ***      *      *      :      :      :      ***      **      **      ***      :      *      .      *

      110     120     130     140     150     160     170     180     190     200
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
Brassica max2
P.trichocarpa XP_002320412.
P.hybrida AEB97384.1|
A.lyrata |XP_002879997.
A.thaliana|NP_565979.1|
T.halophila dbj|BAJ33992.1|
M.truncatula XP_003607592
P.sativum ABD67495.1|
Clustal Consensus
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *

      210     220     230     240     250     260     270     280     290     300
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
Brassica max2
P.trichocarpa XP_002320412.
P.hybrida AEB97384.1|
A.lyrata |XP_002879997.
A.thaliana|NP_565979.1|
T.halophila dbj|BAJ33992.1|
M.truncatula XP_003607592.
P.sativum ABD67495.1|
Clustal Consensus
      *      .      :      :      :      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *

      310     320     330     340     350     360     370     380     390     400
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
Brassica max2
P.trichocarpa XP_002320412.
P.hybrida AEB97384.1|
A.lyrata |XP_002879997.
A.thaliana|NP_565979.1|
T.halophila dbj|BAJ33992.1|
M.truncatula XP_003607592.
P.sativum ABD67495.1|
Clustal Consensus
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *

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On completion of TILLING for a 1.2kb fragment from the C-terminal end of the sequence, M3 seeds of all fertile lines (21 lines) were obtained from RevGen. Out of the 29 mutations identified, 14 were missense mutations, two were nonsense mutations predicted to result in truncations of the protein and the remaining 13 were silent mutations (figure 5.30). Out of the 29 mutants, 17 were G to A transitions and 12 were C to T transitions, consistent with EMS mutagenesis. Among the lines obtained, two lines JI32221 (referred to as 221) and JI31708 (referred to as 708), which were predicted to result in truncation of MAX2, and therefore are likely to be null alleles, were chosen for preliminary analysis (figure 5.30). The mutation report of TILLING for the *MAX2* gene in *B. rapa* with the mutation identified as shown in figure 5.30.

Figure 5.30 Mutation report of TILLING MAX2 gene in *Brassica rapa* showing mutation changes. Two lines selected for preliminary analysis are highlighted in yellow.

Primer Set	Plate Position	Plant Name	Forward Sequencing File	Reverse Sequencing File	Sequence of WT													Mutation Change	Het/Hom	Missense Coding Region	Truncations		Silent				
					-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5				6	7	G/A or C/T	Stop	Splice Site	Non-Coding Region	No Change in AA
MAX2	34:H1	J131614-B	plate34col1_8_f_1111_H04.ab1	plate34col1_8_r_2222_H04.ab1	A	G	A	T	C	A	G	G	A	T	C	A	A	G	A	G/GA	HET					1569G>GA:523R>R/R	
MAX2	37:G5	J131776-A	plate37col5_7_f_1111_G01.ab1	plate37col5_7_r_2222_G01.ab1	A	T	G	T	T	A	A	C	C	A	G	A	G	G	A	C/CT	HET					1818C>CT:606N>N/N	
MAX2	39:G4	J131869-A	plate39col4_7_f_1111_G02.ab1	plate39col4_7_r_2222_G02.ab1	C	T	T	G	A	G	C	G	A	G	C	T	A	G	A	G/GA	HET	1780G>GA:594E>E/K					
MAX2	38:C7	J131830-A	plate38col7_3_f_1111_C03.ab1	plate38col7_3_r_2222_C03.ab1	C	T	C	T	G	C	C	G	A	T	T	T	G	A	G	G/GA	HET	1072G>GA:358D>D/N					
MAX2	36:H2	J131716-B	plate36col2_8_f_1111_H05.ab1	plate36col2_8_r_2222_H05.ab1	T	G	A	G	T	T	C	G	T	A	A	C	A	C	C	C/CT	HET				1513C>CT:505L>L/L		
MAX2	37:F9	J131791-B	plate37col9_6_f_1111_F06.ab1	plate37col9_6_r_2222_F06.ab1	C	T	C	T	G	A	G	A	G	A	C	A	G	A	G	T	G/GA	HET	1456G>GA:486D>D/N				
MAX2	36:A1	J131708-B	plate36col1_1_f_1111_A07.ab1	plate36col1_1_r_2222_A07.ab1	A	C	A	G	T	T	C	C	A	A	G	G	G	G	T	C/CT	HET	982C>CT:328Q>Q/X					
MAX2	46:B2	J132196-B	plate46col2_2_f_1111_B08.ab1	plate46col2_2_r_2222_B08.ab1	T	A	G	A	C	T	G	C	G	G	C	G	A	C	A	C/CT	HET				1677C>CT:559C>C/C		
MAX2	46:C2	J132197-A	plate46col2_3_f_1111_C08.ab1	plate46col2_3_r_2222_C08.ab1	C	T	T	G	A	C	C	G	C	G	C	C	G	C	G	G/GA	HET	1705G>GA:569A>A/T					
MAX2	45:H7	J132171-B	plate45col7_8_f_1111_H09.ab1	plate45col7_8_r_2222_H09.ab1	G	A	C	T	G	C	G	G	C	G	A	C	A	C	A	G/GA	HET	1679G>GA:560G>G/D					
MAX2	45:B3	J132152-B	plate45col3_2_f_1111_B10.ab1	plate45col3_2_r_2222_B10.ab1	G	A	G	C	T	A	G	G	A	C	T	A	A	G	C	G/GA	HET	1625G>GA:542G>G/E					
MAX2	46:E7	J132218-A	plate46col7_5_f_1111_E11.ab1	plate46col7_5_r_2222_E11.ab1	A	A	C	T	T	G	A	G	T	A	T	C	T	G	T	G/GA	HET				1479G>GA:493E>E/E		
MAX2	46:G7	J132219-A	plate46col7_7_f_1111_G11.ab1	plate46col7_7_r_2222_G11.ab1	A	G	A	A	G	C	T	G	C	A	T	A	T	T	G	G/A	HOM				1293G>A:431L>L		
MAX2	46:H7	J132219-B	plate46col7_8_f_1111_H11.ab1	plate46col7_8_r_2222_H11.ab1	A	G	A	A	G	C	T	G	C	A	T	A	T	T	G	G/GA	HET				1293G>GA:431L>L/L		
MAX2	46:C8	J132221-A	plate46col8_3_f_1111_C12.ab1	plate46col8_3_r_2222_C12.ab1	G	A	G	G	A	G	T	C	A	G	A	A	G	A	G	C/CT	HET	1405C>CT:469Q>Q/X					
MAX2	41:G1	J131955-A	plate41col1_7_f_3333_G01.ab1	plate41col1_7_r_4444_G01.ab1	C	T	T	T	A	A	A	G	G	C	G	G	T	T	G	G/GA	HET				1257G>GA:419K>K/K		
MAX2	42:F5	J132018-B	plate42col5_6_f_3333_F02.ab1	plate42col5_6_r_4444_F02.ab1	T	G	A	G	C	C	G	G	A	G	C	T	A	G	G	G/GA	HET	1618G>GA:540E>E/K					
MAX2	42:E2	J132006-A	plate42col2_5_f_3333_E03.ab1	plate42col2_5_r_4444_E03.ab1	G	G	T	T	G	T	T	G	T	C	G	C	T	G	T	G/GA	HET				1047G>GA:349L>L/L		
MAX2	53:C7	J132555-A	plate53col7_3_f_3333_C04.ab1	plate53col7_3_r_4444_C04.ab1	T	T	T	G	C	T	C	C	G	C	C	G	C	A	G	C/CT	HET				996C>CT:332S>S/S		
MAX2	53:G6	J132553-A	plate53col6_7_f_3333_G05.ab1	plate53col6_7_r_4444_G05.ab1	G	G	T	T	T	C	G	C	C	T	T	G	A	C	C	C/CT	HET	1697C>CT:566A>A/V					
MAX2	52:G3	J132493-A	plate52col3_7_f_3333_G06.ab1	plate52col3_7_r_4444_G06.ab1	T	A	G	A	C	T	G	C	G	G	C	G	A	C	A	C/CT	HET				1677C>CT:559C>C/C		
MAX2	54:B5	J132594-B	plate54col5_2_f_3333_B07.ab1	plate54col5_2_r_4444_B07.ab1	T	G	A	G	T	A	T	C	T	G	T	C	T	T	T	C/CT	HET				1483C>CT:495L>L/L		
MAX2	55:B7	J132650-B	plate55col7_2_f_3333_B08.ab1	plate55col7_2_r_4444_B08.ab1	A	A	G	T	A	C	T	C	A	A	C	A	G	A	T	C/T	HOM	1424C>T:475S>L					
MAX2	57:E5	J132741-A	plate57col5_5_f_3333_E09.ab1	plate57col5_5_r_4444_E09.ab1	G	C	G	T	A	G	G	G	T	T	G	T	T	G	T	G/GA	HET				1041G>GA:347G>G/G		
MAX2	63:C1	J133013-A	plate63col1_3_f_3333_C10.ab1	plate63col1_3_r_4444_C10.ab1	T	G	A	G	C	C	G	G	A	G	C	T	A	G	G	G/GA	HET	1618G>GA:540E>E/K					
MAX2	63:D1	J133013-B	plate63col1_4_f_3333_D10.ab1	plate63col1_4_r_4444_D10.ab1	T	G	A	G	C	C	G	G	A	G	C	T	A	G	G	G/GA	HET	1618G>GA:540E>E/K					
MAX2	61:B12	J132960-B	plate61col12_2_f_3333_B11.ab1	plate61col12_2_r_4444_B11.ab1	G	G	G	T	T	C	T	C	C	T	C	T	G	A	A	C/CT	HET	1448C>CT:483S>S/F					
MAX2	61:F12	J132962-B	plate61col12_6_f_3333_F11.ab1	plate61col12_6_r_4444_F11.ab1	G	A	A	G	C	T	G	C	A	T	A	T	T	G	A	C/T	HOM	1294C>T:432H>Y					
MAX2	59:B4	J132831-B	plate59col4_2_f_3333_B12.ab1	plate59col4_2_r_4444_B12.ab1	T	G	G	T	T	C	A	G	A	G	G	A	G	A	A	G/GA	HET	1321G>GA:441E>E/K					

M3 seeds (10 seeds each) derived from heterozygous M2s, from both of the selected lines were planted on F2 compost for initial phenotyping, along with the wild-type R-o-18 line, and the branchy line 8 as controls. Plants were monitored for morphological variations and plants were harvested at maturity and seeds collected for further experiments. Among the plants, several individuals showed pleiotropic phenotypes (figures 5.31 and 5.32) such as chlorosis at seedling stage, short stature (dwarfism), altered branchiness, shorter internodes, altered leaf morphology and sterility. Phenotypes of possible *Brassica rapa max2* homozygous mutants were assessed in comparison to R-o-18. These were documented as photographs (figures 5.31, 5.32, 5.33, 5.34) for future studies. Nine out of the ten seedlings of *max2* line 221 germinated, while all the ten seeds of *max2* line 708 germinated. Three out of nine seedlings of line 221 and two out of ten of line 708 seedlings had chlorotic leaves varying in the degree of chlorosis (figure 5.31). Varying plant heights, from very short to shorter than wild type R-o-18 and bushiness, from bushy to completely unbranched were observed among the mature plants of both the lines (figure 5.32). Several individual plants of both lines showed enhanced branching. In some of the plants, as in *Arabidopsis max2* mutant plants (Stirnberg et al., 2002), branches grew out from all the nodes including the basal-most nodes, resulting in a very bushy adult phenotype as seen in figure 5.33. In the control R-o-18 plants, lateral branch outgrowth followed a basipetal gradient after floral transition and only the more apical nodes produced active branches.

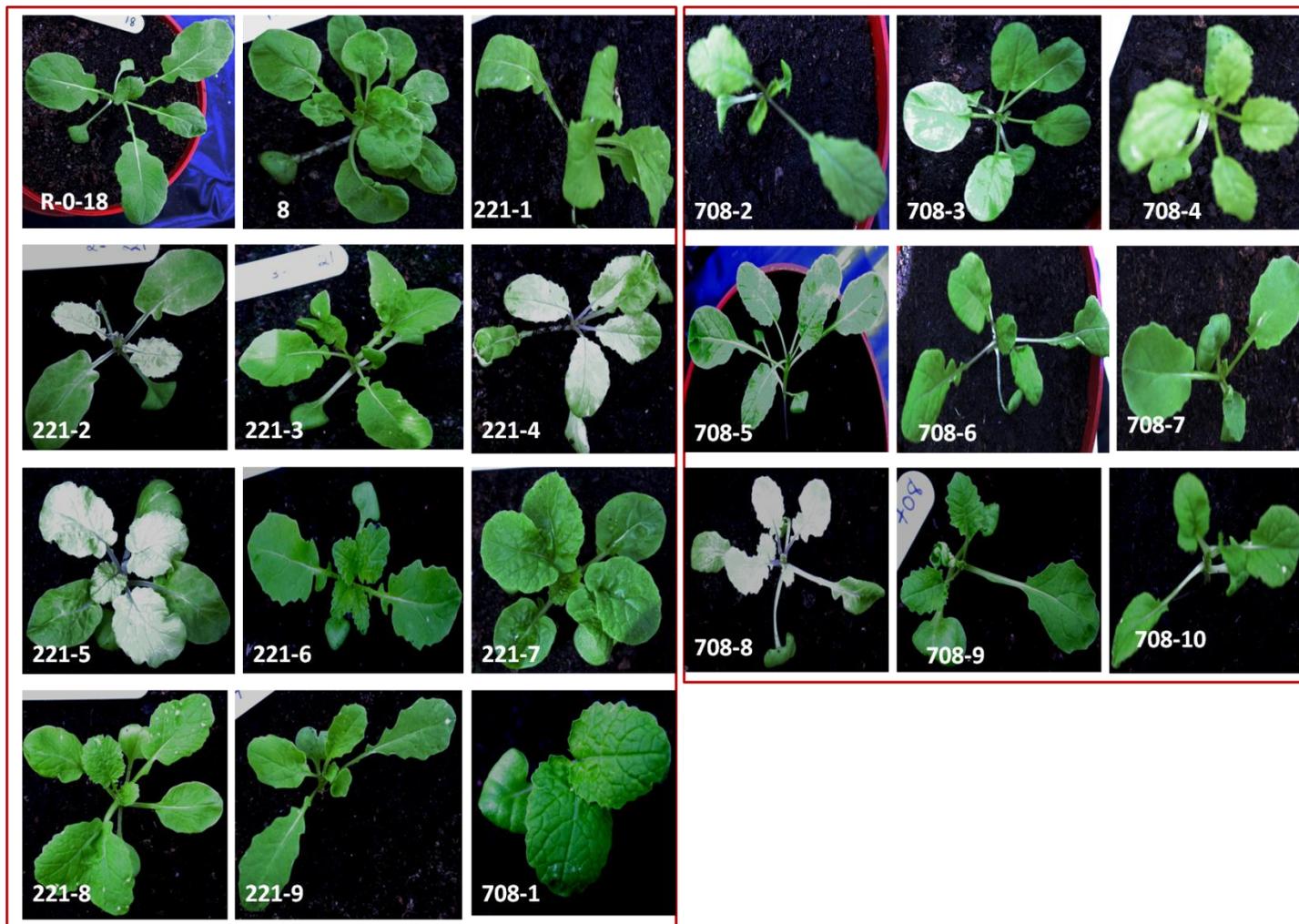


Figure 5.31 Seedling phenotype of reference line R-o-18, line 8 and *max2* (2 week old seedlings).

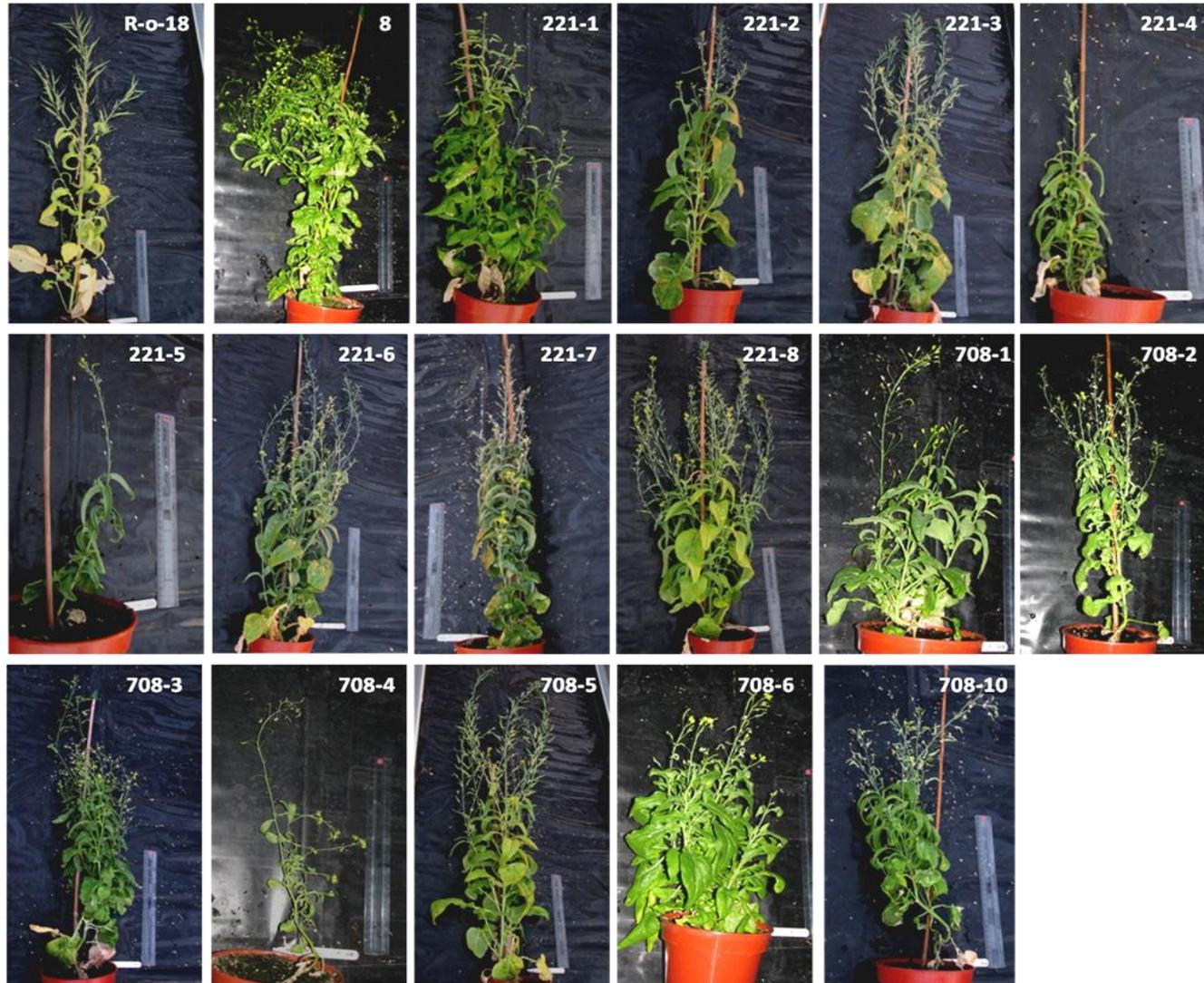


Figure 5.32 Branching phenotype of reference line R-o-18, line 8 and possible *max2* segregants (14 week old plants).

Some individual plants showed unusual phenotypes such as twin shoot apices growing with equal strength (figure 5.34 a), or fasciated stems at the base (figure 5.34 b) with altered phyllotaxy. Similar phenotypes have occasionally been observed in *Arabidopsis max2* mutants, but only in branches, and not the primary shoot (Stirnberg et al., 2002).

In addition to variations in leaf shape, leaf colour was also different, varying from light green to dark green among the plants. As observed in *Arabidopsis max2* mutants (Stirnberg et al., 2002) some plants had rounder leaves and shorter internodes compared to R-o-18. Many plants also showed variations in pod size and had malformed seeds. Many pods did not have seeds and consequently there was reduced fertility. Leaf senescence was also delayed in many lines when compared to R-o-18, similar to *Arabidopsis max2* (Stirnberg et al., 2002). In R-o-18, leaves at the lower nodes were senescing in 14 week old plants as seen in figure 5.32, when compared to many individual plants in the *max2* families, which were still green. Because of the delayed senescence, putative *B. rapa max2* plants continued to keep on making new flowers and siliques for a longer period of time.

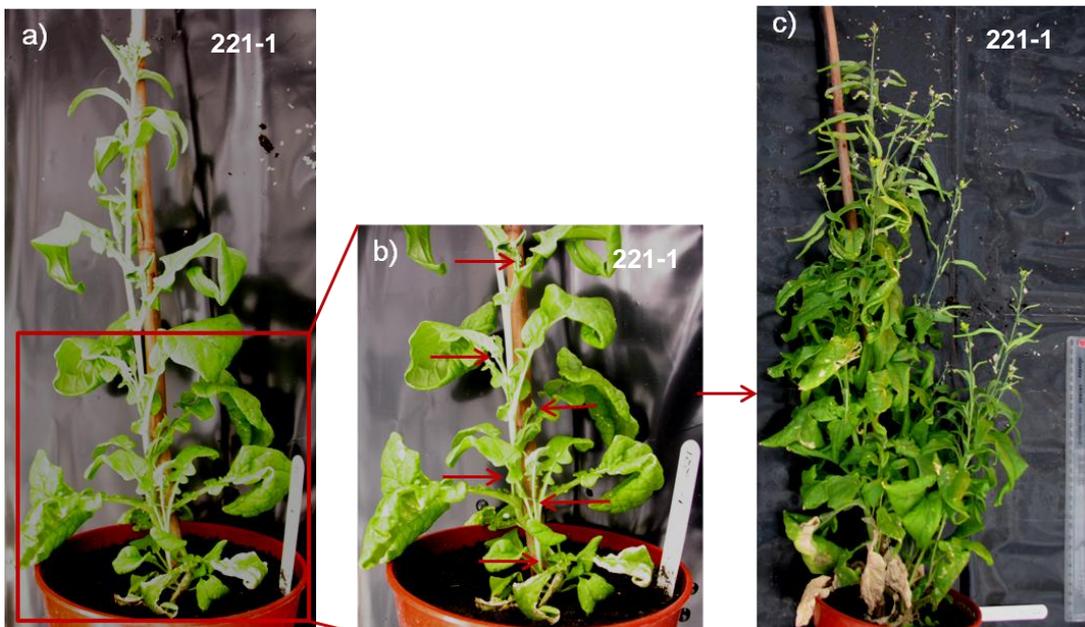


Figure 5.33 Phenotype of an individual bushy plant a), b) branches growing out from all leaf nodes including cotyledonary nodes (6 week old plants) and c) the same plant when flowering was complete (14 week old plant).

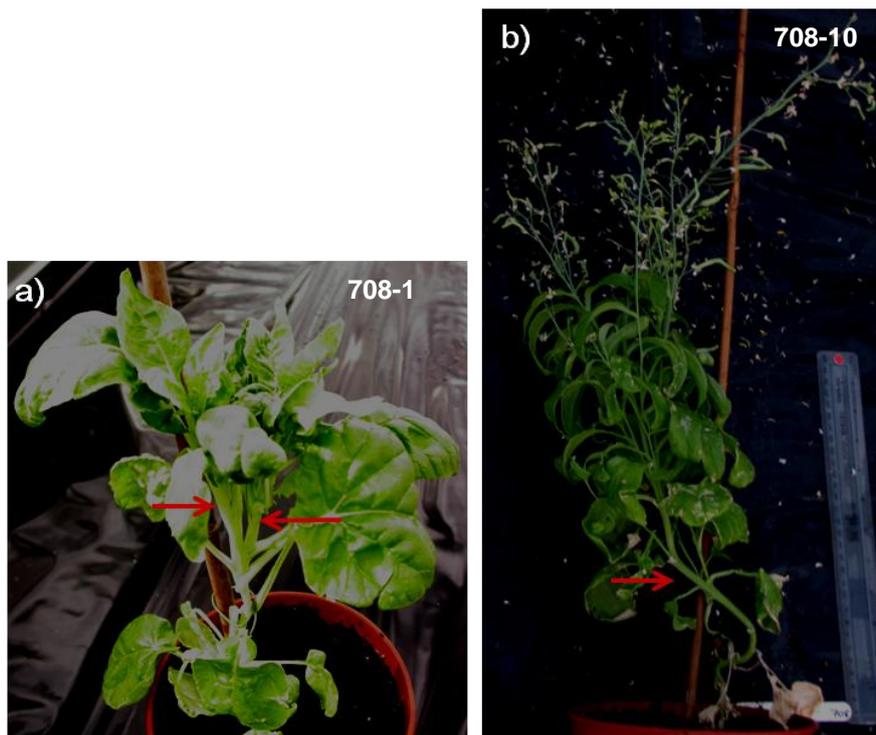


Figure 5.34 Developmental defects in selected plants a) Phenotype of an individual plant with twin shoot apices (4 week old plant) b) with fasciated stem (14 week old plant).

5.9 Discussion

The results in this chapter are preliminary attempts to adapt the hormone and N response analysis from *Arabidopsis* to *Brassica rapa*.

5.9.1 Architecture and basic N response of *Brassica rapa* line R-o-18 is similar to *Arabidopsis*

Preliminary characterisation of the control line R-o-18 showed that the architecture of *Brassica rapa* and *Arabidopsis* were comparable. The branching of *B. rapa* line R-o-18 followed a similar pattern as observed in *Arabidopsis*. Before floral transition there were no visible buds in the leaf axils. After floral transition lateral branches grew out in a basipetal order as in *Arabidopsis* (Hempel and Feldman, 1994). The growing primary shoot of R-o-18 is likely to have a strong inhibitory effect on the bud activation and possibly bud formation, as in vegetative *Arabidopsis* (Grbic and Bleecker, 1996, Stirnberg et al., 1999)

In the basic N response assays using the reference line R-o-18 it was evident that number of branches and yield was considerably reduced when N was limited. This is not surprising, knowing that oil seed rape is a crop relying heavily on N fertilization. Although the number of branches was reduced considerably on low N (figure 5.4 b), the total number of nodes on the primary stem of plants grown on high N and low N were not very different (figure 5.4 c) and there was no substantial difference in internode lengths. The significant reduction in yield on low N and increased yield on high N are similar to the response of Arabidopsis accession Columbia.

5.9.2 EMS mutagenised lines differ in their N response

Comparing the total number of branches among different lines provided evidence that there was difference among lines in their response to N. One of the lines, line 8, which was highly branched on high N loses all its branches on low N, whereas another line, line 10, which had a comparable number of branches to R-o-18 on high N retained up to five branches on low N (figure 5.6). These lines demonstrated that a highly branched line could be either highly responsive to N limitation, or constitutively highly branched.

5.9.3 Decapitation response of *Brassica rapa*

Decapitation studies with R-o-18 at node 4 resulted in activation of buds in the cotyledonary nodes. This might reflect the fact that there were no visible buds in the axils of vegetative plants, and the cotyledonary nodes had the most advanced buds or bud development potential.

When plants on high N were decapitated at higher nodes and the response of different lines compared, it was observed that there was a lack of any substantial differences in decapitation responses on low Vs high N and between the lines (figures 5.10, 5.11). This suggested that decapitation response did not correlate with N response, nor did it correlate with branch number (figures 5.6, 5.10). Although there were relatively few nodes below the decapitation point and many of them activated upon decapitation for all the genotypes, the active branches

inhibited the most basal ones from activation. This response was similar to *Arabidopsis* where all the buds did not activate upon decapitation.

When *B. rapa* plants was decapitated, the most apical branches were longer than the remaining basal branches and changed their angle of growth to replace the primary shoot (figure 5.8). This was a common feature in decapitated plants. This may be speculated as a strategy to maximise light harvest while maintaining yield.

5.9.4 Hormonal response of *Brassica rapa*

Despite the differences in size, most of the hormonal assays were easily adapted to *Brassica rapa*. However, results of hormone response assays were considerably different from *Arabidopsis* in several respects. In two bud assays on high N with the reference line R-o-18 it was evident that buds in untreated plants did not compete with each other as much as in *Arabidopsis* and both buds activated (figure 5.23 a), with resulting low RGIs. Furthermore, *B. rapa* buds were not very sensitive to GR24 even in the presence of a competing auxin source (figure 5.23 a).

Previously mentioned bud assays in *Brassica rapa* suggest the lack of response of buds to GR24. However it is very likely that SLs are acting in the branching control of *B. rapa*. Mutant analysis in different species for orthologous genes in SL pathway have already confirmed that in higher plants, SL regulation of shoot branching is conserved (Sorefan et al., 2003, Snowden et al., 2005, Arite et al., 2007, Johnson et al., 2006). Nonetheless, Arite et al had already suggested that although a conserved mechanism works to regulate shoot branching there could be variations in details of regulation as a consequence of differences in branching habits of different species (Arite et al., 2007). This probably could partially account for the differences between *Arabidopsis* and *Brassica* although the overall architecture was similar. Dun et al (2011) suggested recently that relative CK content may vary between species contributing to the difference in responses to GR24 while reporting on the antagonistic action of SL and CK in controlling bud outgrowth. Given the complexities of the branching regulatory network it

could be concluded that it is not reasonable to expect the same quantitative response in different species to hormones at all stages.

Despite the lack of response of buds to GR24, preliminary evidence for SL regulation of shoot branching in *B. rapa* comes from the *max2* mutants obtained from the TILLING service, which were bushy as in Arabidopsis. Most of the data about the role of SLs in other species relies on mutant studies and not physiological assays so evidence from *B. rapa max2* mutants is reassuring. Also decapitation assays would show if the *max2* mutants make more branches following decapitation as in Arabidopsis, which would suggest the involvement of SLs.

When considering the auxin response of *B.rapa*, in the split plate assay set up, auxin was unable to inhibit single bud outgrowth (on high N) to a similar extent as in Arabidopsis (figure 5.21). 1 μ M NAA was able to inhibit bud outgrowth for 4-7 days in Arabidopsis, whereas it did for 2-3 days in *Brassica rapa*. It is unlikely to get the same length of bud inhibition in two different species so this is not surprising. One might argue that this difference could be due to the difference in initial bud size. It was possible that the Brassica buds might be at a later stage, which reduced auxin response when compared to Arabidopsis. However, there was no valid reason to suppose that the absolute size at which buds were no longer auxin resistant in different species should be the same. On low N (where the bud size was smaller) combined NAA and GR24 treatment delayed bud outgrowth for up to four days (figure 5.22). However, as this experiment lacked an auxin alone control, conclusions about whether the effect was due to auxin alone with no enhanced effect of GR24 could not be reached.

When apical auxin was added to the two bud assay system on high N, only the top bud was inhibited by auxin but not the bottom bud (figure 5.25 a). One possibility is that *B. rapa* buds are relatively auxin resistant. Measuring auxin levels in stems might test this hypothesis. It is likely that although auxin resistant, these buds compete with each other in the same way as in Arabidopsis. Two bud with additional apical auxin can be compared to a three bud situation in Arabidopsis.

Although *B. rapa* appears somewhat auxin resistant and almost completely SL resistant in these assays compared to Arabidopsis, there is evidence of similarity. Low N resulted in a high auxin and high SL situation in Arabidopsis, so intuitively one would expect complete inhibition of the bottom bud in the *Brassica rapa* situation, similar to Arabidopsis. Indeed the bottom bud of R-o-18 was strongly but not completely inhibited on low N (figure 5.25 c) in both control and treated buds. Furthermore, *B. rapa* buds were not very sensitive to GR24 even in the presence of a competing auxin source on high N (figure 5.25 a).

5.9.5 SL and leaf senescence in *Brassica rapa*

In *B. rapa* in general, the leaf senescence effect of SL was much more striking than any bud effect. It is already known that SLs regulate senescence in other species (Woo et al., 2001, Snowden et al., 2005, Yan et al., 2007). Nutrient diversion to younger organs could be enabled by the senescence of older leaves and thus the observed acceleration of senescence in plants grown on low N could be an adaptive response to support continued leaf production. It could be deduced that nutrient limitation alters hormone levels, such as increasing SL, causing a senescence effect. However senescence effect in *B. rapa* needs to be quantified more thoroughly to confirm the preliminary results. Since GR24 was much better at inducing senescence in *B. rapa* than in Arabidopsis, testing this SL response in the *max2* mutant would be more informative than for branching.

5.9.6 Response of mutant line 8 to nutrients and hormones

This chapter also describes attempts to characterise the mutant line 8, which had increased levels of branching. When grown on agar plates on white light, line 8 had shorter hypocotyls than the wild-type R-o-18 (figure 5.15), leading to the initial hypothesis that this mutant might have a reduced response to auxin, as in *axr1* (Lincoln et al., 1990, Leyser et al., 1993). If this were the case then it would open up the possibility of having an auxin signalling mutant in *Brassica rapa*, especially useful to study the interactions between auxin and strigolactones. However results of several assays such as the dark and red light responses did not support this hypothesis.

More importantly, in the two bud assay system line 8 showed slightly increased inhibition by auxin compared to R-o-18 on both high and low N (figure 5.25), in contrast to *Arabidopsis axr1*, which had highly auxin resistant buds. Complete inhibition of top and bottom bud of line 8 on low N could be perhaps because this line was auxin hypersensitive. Another completely speculative possibility is that the altered morphology of line 8 could be ethylene related. The upward curling hyponastic leaves of this mutant was similar to ethylene induced leaf hyponasty in *Arabidopsis* (Benschop et al., 2007). More detailed studies with this mutant might shed some light on the causes of its pleiotropic phenotypes. However, preliminary evidence from the branching phenotypes on high and low N demonstrated that the enhanced branching effect of this mutant disappeared on low N. So it is quite unlikely that this mutant could be directly hormone related.

5.9.7 SL signalling mutant in *Brassica rapa*

Having successfully adapted hormone response assays in *Arabidopsis* to *B. rapa* availability of a *max2* mutant would be a very useful tool to further study mechanisms of MAX pathway and other developmental processes like leaf senescence. Many of the plants in the *max2* mutant families displayed visually detectable alterations in several traits which were consistent with the *max2* phenotypes documented in *Arabidopsis*. However this preliminary analysis was superficial and several other aspects such as timing of branch formation, sensitivity to other hormones etc have not been performed in detail. Also further screening of the next generation, genotyping of individual plants and comparison of phenotypes are necessary for the identification of homozygous lines. Extensive back-crossing is also required to cross away other mutations in the lines.

Once back-crossed homozygous lines are established, the hormone response assay techniques and successful grafting established here, open the way for detailed characterisation of *B. rapa max2* mutants. The TILLING service could also be used to recover SL biosynthetic mutants. The grafting technique established in *B. rapa* has scope for further improvement to increase the grafting success rates and might be useful in characterising branchy mutants.

5.10 Summary

At the start of the study it was assumed that *Arabidopsis* and *Brassica*, having similar architectural traits, might have similar responses to hormones. However, it has become evident that the response of these two species to the hormone SL is different in some aspects which is to be expected. We might deduce from this that, studying the hormone response in *Brassica rapa* might contribute to understanding the regulatory mechanisms and further roles of this hormone. *Arabidopsis* has been a favourite lab model plant for more than two decades and major discoveries made in this species have been translated to its relative crop species in the *Brassica* family. Working with different plant species helps to learn more about the regulatory system and the variation in the system.

Chapter 6
General discussion

The main focus of this thesis was to understand the interactions between genotype and environment in regulating shoot branching. This study aimed to investigate how information from the environmental cue N is integrated into the shoot branching regulatory system of plants and to determine the role of a hormone strigolactone (SL) in this process.

This was addressed by several complementary approaches including N response studies in *Arabidopsis* using MAGIC lines (Chapter 3), hormone response assays in *Arabidopsis* using shoot branching mutants as well as MAGIC lines (Chapter 4), and preliminary attempts to adapt the hormone response assays and N response studies for use on *Brassica rapa*, enabling cross species comparisons (Chapter 5). Findings from these three chapters together have given rise to several conclusions, which provide better insights into shoot branching plasticity and strong evidence for a role for the hormone SL, in N response.

6.1 N affects branching in *Arabidopsis* and *Brassica*

When *Arabidopsis* plants are grown on limited N, they respond by curtailing the basipetal sequence of bud activation. A similar response was observed in *Brassica rapa*. Regulation of branching by N availability is hormonally controlled, as evident from the analysis of bud activation in one bud and two bud assays. This includes a reduction in shoot branching on low N, mediated at least in part through the SL pathway, which allows plants to balance their shoot and root system development in response to N.

6.2 The effect of N on branching is likely to be mediated by hormones

The extreme branching phenotype and limited N response of the SL biosynthetic mutant *max4* suggests that the effect of N limitation on branching is mediated at least in part by SL, and the results from hormone response assays support this idea.

6.2.1 A model of SLs on high Vs low N

SLs regulate PIN1 accumulation on plasma membrane (Crawford et al., 2010) and according to the auxin transport canalization-based model, this can affect shoot branching. (Prusinkiewicz et al., 2009). According to this hypothesis, high SL would enhance competition between buds by reducing the ease with which they can establish auxin transport canalization into the main stem. This is achieved by reducing the sink strength of the main stem for auxin, by reducing auxin transport through the stem, and by dampening the positive feedback loop at the centre of auxin transport canalization, by increasing the rate of PIN removal in canalizing tissues.

Results from the two node assays are consistent with this model, as SL indeed enhances the competition between buds (figure 6.1b). The effect of basal SL in the two bud system is not to inhibit both buds, but to allow one bud to dominate and this could be either the top bud or the bottom bud. For instance, if the top bud activates first and exports auxin to the stem auxin sink, this bud dominates and inhibits the growth of the bottom bud. This, together with the finding that basal SL has no effect on bud growth when only a single bud is present, as in the case of isolated nodal stem segment assays, contradicts the hypothesis that SLs are the second messengers which can themselves directly and locally inhibit bud outgrowth (Brewer et al., 2009).

Strigolactone levels in the roots are high when plants are grown on low N or P (Yoneyama et al., 2007b, Yoneyama et al., 2011). The situation on high N can be considered as a low SL situation, where many buds can activate, and in turn export auxin. This increased auxin export is brought about by increased PIN accumulation, and subsequently leads to increased transcription of SL biosynthetic genes (Crawford et al., 2010), providing a negative feedback loop. In contrast, low N is a high SL situation, and as a consequence of higher SL levels, competition between buds will be increased, as seen in the bud assays. It could be deduced from this that, fewer buds activate on low N and indeed this is seen in the results.

Both low N and GR24 have similar effects on bud activation (figure 6.1); consistent with the idea that the effects of N limitation are mediated by SL. Low N also results in increased auxin levels (V. Ongaro, K. Ljung, unpublished). This increases PIN transcription, but high SL limits PIN accumulation on the plasma membrane. Thus a low N situation results in high auxin levels, with capped PIN accumulation. This can result in reduced shoot branching, but high levels of auxin being delivered to roots, in turn promoting root growth. These combined effects result in a shift in root: shoot ratio on low N.

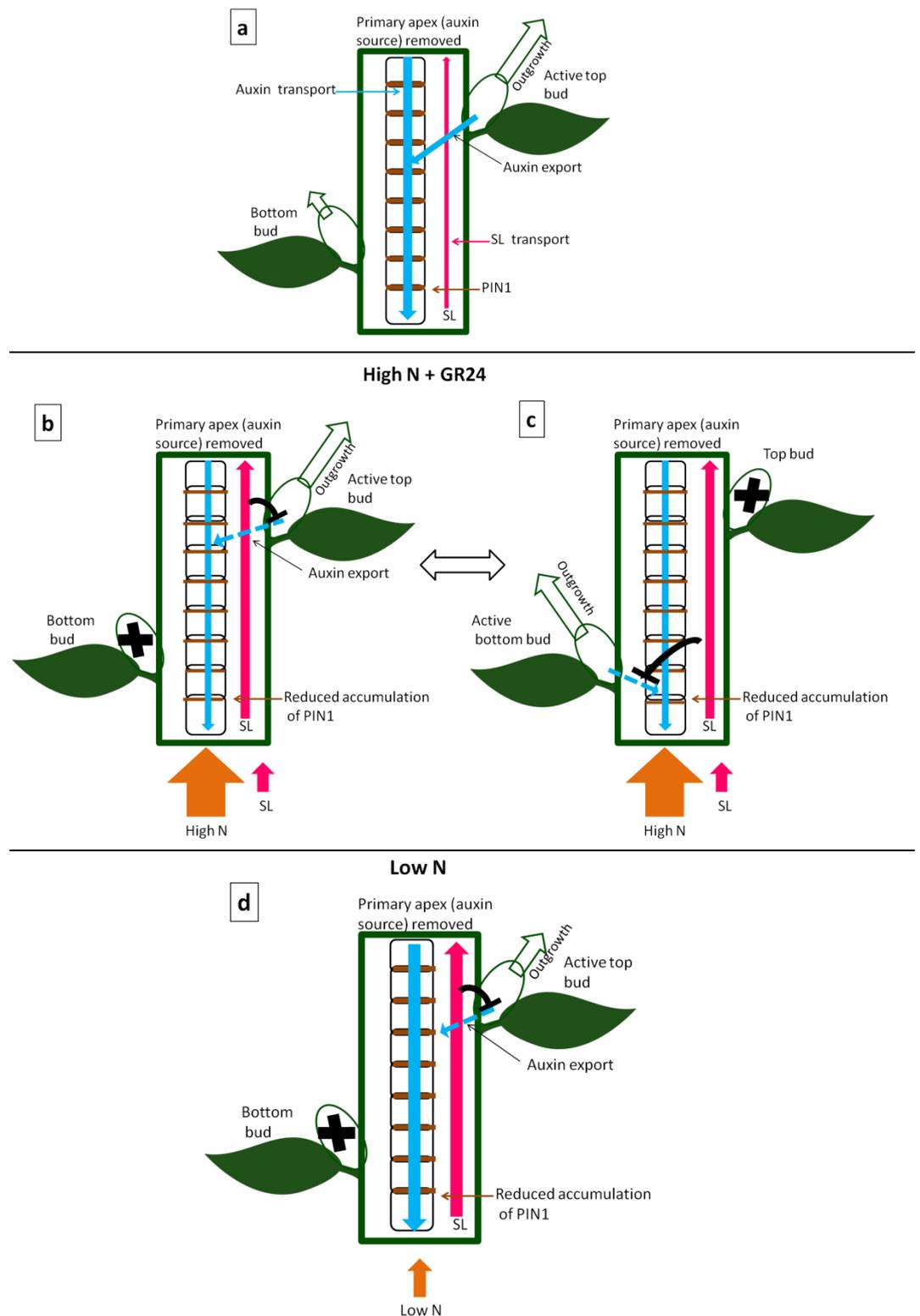


Figure 6.1. Schematic representation of hormone transport and bud activation in two bud assays on high and low N based on the auxin transport canalization model. a) Representation of direction of hormone transport and bud outgrowth in control two bud plants. b), c) SL enhances competition between buds by dampening auxin transport and its canalisation via increased PIN removal from the plasma membrane. Either the top or the bottom bud can be inhibited depending on which bud exports auxin first. d) Low N leads to increased auxin in the stem. Auxin upregulates PIN 1 expression so auxin transport is likely to be increased. But low N also increases SL levels, which reduces PIN1 accumulation. These two effects balance out making low N a high auxin, limited PIN1 accumulation situation. When SL is high, canalisation is harder, (as indicated by the inhibitory arrow) because PINs are removed more rapidly from the plasma membrane, which slows the progress of the positive feedback loop that drives canalisation.

6.3 High branching correlates with high plasticity in the MAGIC lines

The behaviour of SL mutants suggests that reduced SL activity could mediate reduced plasticity in nature, and that low plasticity would therefore be associated with constitutively high branching. Marked variation in branch numbers, branching plasticity and flowering time was evident among the sub set of selected MAGIC lines analysed in this thesis. In these lines high branching correlated with high plasticity and this is opposite to the results observed with *max4*. Low plasticity was correlated with an average branching phenotype on high N. In addition, there was an interesting correlation with flowering time. Relatively early flowering was strongly associated with low plasticity, and relatively late flowering was strongly associated with high plasticity. These results led to the identification of two strategies for adaptation to low N. At one extreme there are the early flowering, low plasticity lines, which when faced with low N do not shift their resource allocation strategy at all. At the other extreme are the later flowering high plasticity lines, which reallocate their resources to roots when faced with low N. The strategy of the low plasticity lines is relatively successful on low N as it results in a reasonable seed yield, but is not as successful on high N, where the lines appear unable to take advantage of the abundant N supply to increase their yield substantially. In contrast, the high plasticity lines are more successful on high N, as they make more effective use of the additional N available, reflected in their higher seed yields. However, the strategy of these lines on low N is comparatively unsuccessful, with poor seed yield.

6.4 The mechanisms underlying MAGIC line strategies are unknown.

Although different lines have different strategies to cope with N limitation, details of molecular basis of the different life history strategies and precise mechanisms need further investigation. Exploiting allelic diversity in natural populations to identify alleles affecting shoot branching plasticity will be useful in this endeavour, as well as providing opportunities for application in plant breeding programmes in future. Preliminary QTL mapping in the MAGIC lines confirms the association of these traits. Co-location of QTL for branch number on low N and a QTL for plasticity, as well as co-location of QTL for plasticity with QTL for flowering time

(P. Kover personal communication, unpublished) supports the observed association between early flowering and plasticity described above and in the long term, offers the opportunity to understand the molecular basis for this association.

These QTL do not provide a link to known hormone-related genes. Hormone responses in the MAGIC lines revealed some evidence for SL resistance in 552 on low N (figures 4.27, 4.28), consistent with its low plasticity. Given that the hormone responses of only two MAGIC lines were analysed, further studies with a wider range of MAGIC lines might be useful.

Ongoing studies in the Leyser lab to exploit natural variation make use of three different populations in parallel. These include 400 MAGIC lines (Kover et al., 2009), a subset of which is used in this thesis, GWA studies (Atwell et al., 2010) using natural accessions and a pseudo domestication population created by artificial selection. Results from this thesis, especially the associations observed between different traits and plasticity will be helpful in comparing the behaviour of MAGIC lines with the natural accessions and artificially selected populations. An instance where this has already proven informative is that comparison of MAGIC lines and natural accessions suggests that among the natural accessions, there are more 471-type lines than 552-type lines, with most natural accessions having few branches on low N (M. de Jong personal communication, unpublished). Lines studied in detail in this thesis, for which the different responses and strategies are characterised, could be used as bench marks or controls for studies with other populations. In addition, these results could be used to make predictions about the behaviour of other populations and to generate new hypotheses, which can be also tested in the ongoing computer modelling studies in the lab.

6.5 Unresolved aspects of the mechanism of SL action

Despite the advances in elucidating the role of SLs in regulating shoot branching, its precise mechanism of action is still unclear. For instance, although it is well established that modulation of auxin transport by SLs is by reducing PIN1 accumulation in xylem parenchyma, the exact mechanism by which this is brought about is unknown (Crawford et al., 2010). Results from Crawford et al

(2010) argue against a transcriptional mode of action, because over-expression of PIN1 does not increase auxin transport or affect branching, therefore suggesting a post transcriptional mode of action. Current and future studies in this direction include analysing the accumulation of PIN1 at the basal plasma membrane of xylem parenchyma cells of Arabidopsis stems grown on high and low N, using wild type plants, and other genotypes, and also pharmacological treatments such as cycloheximide.

Another area where information is lacking is in understanding SL transport within the stem and into the buds. It has been suggested that they are likely to be transported through xylem as SLs have been detected in the xylem sap of Arabidopsis and tomato (Kohlen et al., 2011). Advances in the area of SL transport are ongoing, as illustrated by a very recent discovery in petunia (Kretschmar et al., 2012) demonstrating the involvement of a protein PhPDR1 in SL transport. This protein is highly expressed in roots under P deficiency and is required for SL export into the soil. The role of *PhPDR1* gene in transport to the shoot is less clear, although the knock down lines do have an increased branching phenotype.

Although an increase in SL levels in root exudates of plants grown on both low N and low P conditions (in sorghum- Yoneyama et al., 2007, in rice – Umehara et al 2010) has been reported, much less is known about SL gene expression especially under N limitation when compared to P deficiency, where transcriptional upregulation of the SL biosynthetic genes is well established. Results from this thesis showed that N limitation has no effect on bud outgrowth in the SL signalling mutant *max2* and only a minimal effect on the SL biosynthetic mutant *max4*. Given that there is evidence for SL mediating response to low N, it is very likely that low N will also lead to changes in SL biosynthetic gene expression. This is perhaps the next thing to investigate.

In addition to all the events outside the buds playing a role in regulating shoot branching, processes occurring locally within the bud are also involved in the decision making process of a bud to grow or not to grow. Expression of several bud specific genes from the TCP family including *TB1*, *BRC1* and their homologues in several plant species have been shown to be necessary for bud

inhibition. Among these, a role of Arabidopsis *BRC1* in integrating hormonal and environmental signals has been proposed (Aguilar-Martínez et al., 2007), which has been supported very recently in pea (Braun et al., 2012). Results from the studies in pea showed that the transcription factor PsBRC1 acts downstream of SL to repress bud outgrowth and supported its local site of action in axillary buds. Further studies on local regulation of bud activity are thus important to understand the co-ordinated regulation of bud activity. Connectivity of all phytomers through a common auxin transport pathway perhaps acts as a unifying mechanism ensuring that local regulation in buds is integrated with systemic information such as nutrient sufficiency or limitation. One possibility is that SLs regulate auxin export from the bud, and this auxin export regulates *BRC1* expression.

6.6 Understanding alternative strategies for adaptation to N limitation may be agriculturally useful

Yield stability under low N input is an agricultural priority. Results from this thesis clearly establish a link between yield on low N and branching. For instance, the low plasticity lines were unable to exploit N. So this may not be a favourable strategy from an agricultural perspective. The strategy of high plasticity lines of making use of available N when there is sufficient N and giving high yields is not successful on low N where the yields of these lines are low. Improved agronomic practices could be combined to make the strategy better. For example, this strategy could be used in combination with a single late fertilizer application as it is clear that these lines have the potential to respond to N application.

Furthermore, analysis of branching responses in *B. rapa* revealed many conserved features between Arabidopsis and *B. rapa* branching, suggesting knowledge gained from Arabidopsis will be helpful. This should also aid breeding for shoot system architecture that can deliver improved yield under low N.

6.7 Hormone studies in different plant species can contribute to understanding of different roles of hormones.

Regulation of shoot branching by hormones, especially SL is one area that has been profoundly facilitated by investigations in different plant species such as

Arabidopsis, pea, petunia, rice etc. both from a developmental and evolutionary perspective. Comparison of diverse species contributes to the understanding of the extent to which there is conservation or differences in various aspects of hormonal functions between species. Differences in the behaviour of Arabidopsis (Crawford et al., 2010) and chrysanthemum (Liang et al., 2010) in two node assays have already suggested that there could be species to species differences for example, in which among the two buds is likely to dominate. In Arabidopsis there was no strong preference for top or bottom bud, although this varies between experiments, whereas in chrysanthemum the bottom bud was the one that was always inhibited. Nevertheless there are strong similarities in that in both species SL inhibited buds only in the presence of a competing auxin source, suggesting that many aspects are conserved across the species.

Hormone response assays in Arabidopsis and *B. rapa* showed that SLs enhanced competition between buds in both the species, although to varying degrees. *B. rapa* appears to be less sensitive to GR24, the synthetic form of SL used in the assays. In contrast, *B. rapa* appears to be more sensitive to SL-mediated senescence. SL deficient mutants in many species show delayed senescence (Yan et al., 2007, Stirnberg et al., 2002), but there are very few reports of SL-induced senescence. This effect was clear in *B. rapa*. The likely changes in SL levels on low N may contribute to the senescence associated with N deprivation. Leaf senescence observed on low N is comparable to leaf senescence observed with addition of SL into the system on high N (figure 5.27). This result is also consistent with the predicted lower levels of CK on low N, since CK is well known to delay senescence (Gan and Amasino, 1996, Gan and Amasino, 1997).

Availability of the *B. rapa* TILLING resource enabled to obtain a *max2* mutant in this species. Preliminary analysis suggested that *B. rapa max2* lines were more branchy and displayed delayed senescence, as has been reported for the *max2/ore9* mutant in Arabidopsis (Stirnberg et al., 2002, Yan et al., 2007) *dad1* in petunia (Snowden et al., 2005) and *D3* mutant in rice (Yan et al., 2007). In future, the TILLING service could be used to obtain SL biosynthetic mutants for further enhancing cross species comparison studies of the diverse roles of SL. In

particular, *B. rapa* may be especially useful for studies of SL-mediated senescence, which is easily visible, stronger and scorable.

6.8 Future prospects

The fundamental question of how the environment interacts with the genetic constitution of plants to make crucial developmental decisions is an intriguing, as well as a challenging one. Findings in this thesis contribute to enhancing current understanding of the shoot branching regulatory mechanisms, in addition to giving rise to new questions and clues which need to be fitted together in order to complete the overall shoot branching puzzle. Study of various aspects of shoot branching which started almost a century ago is set to continue for many more years, providing clues to the still mysterious aspects of branch regulation. To this end, diverse approaches such as computational modelling, physiological experiments, molecular genetics, cell biology, exploitation of natural variation, genomics etc. may assist advancement in understanding of the unknowns.

Appendix 1 - 19 parental lines and their geographical location

stock number	Accession	Origin
N6643	Bur-0	Ireland
N6660	Can-0	Canary Isles
N6673	Col-0	USA
N6674	Ct-1	Italy
N6688	Edi-0	Scotland
N6736	Hi-0	Netherlands
N6762	Kn-0	Lithuania
NW20	Ler-0	Germany
N1380	Mt-0	Libya
N6805	No-0	Germany
N6824	Oy-0	Norway
N6839	Pa-0	Germany
N6850	Rsch-4	Russia
N6857	Sf-2	Spain
N6874	Tsu-0	Japan
N6889	Wil-2	Russia
N6891	Ws-0	Russia
N6897	Wu-0	Germany
N6902	Zu-0	Germany

Source: NASC Website

Appendix 2 - MAX2 genomic sequence

atctcctctcttagatttgcgcttctcATGGCTTCCACTACTCTCTCCGACCTCCCTGACGTCATCTTATCCACCATTTCCTCTCTCGTATCCGATCCCGAGCTCGCAACTCTCTCTCCCTCGTCTCTCACAAATTCCTCGCTCTCGAACGATCCACTCGCTCTCACCTCACTATCCGTGGCAACGCTCGTGATCTTCCCTCGTCCCCGACTGTTTTCCGATCAATCTCACATCTCGATCTCTTTCTCTCCCCATGGGGTCACACTCTTCTCGCTTCTCTCCCAATCGATCACCAGAACCTTCTCGCTCTCCGTCTCAAATCTGTTTTCCCTTTCGTGAGTCTCTAAACGTCTACACAGATCTCCGAGCTCTCTCGAGCTTCTACTTCCTCAATGGCCGAGAATTCGCCACATCAAGCTCTCCGATGGCATCAACGAGCTTCTCAGATCCCTACCGGTGGCGATTTTGTTCCTATTTTTGAACACTGTGGTGGTTTTCTTGAGTCTTTAGATCTCTCCAACCTCTATCACTGGACTGAAGACTTACCTCCTGTGCTTCTCCGCTATGCTGACGTGGCGGCGAGGCTTACACGGTTAGATCTCTTGACGGCGTCGTTACCCGAGGGATACAAATCAAGCGAAATCGTTAGTATCACAAATCTTGCCCTAATTTGAAGACTTTTTCGTGATGCTTGTACGTTGATCCGAGATACTTTGAATTCGTCCGAGACGAGACTCTCTCCGCCGTAGCTACCAGTCCCCTAAGTTAACGCTTCTACACATGGTGGACACAGCTTCGTTGGCGAATCCTAGAGCTATTCAGGTACGGAAGCTGGAGATTCAGCTGTCACGGCGGGGACGCTAATTGAAGTTTTCTCAGGTTTACCGAATCTAGAGGAGCTGGTCTTGACGTAGGAAAGGATGTGAAGCATAGTGGTGTAGCTTTAGAGGCATTGAATTCTAAATGCAAGAAAGTTAAGAGTATTGAAGCTAGGACAGTTCCAAGGTGTTTGCTCTGCTACAGAATGGAGGAGGCTCGACGTGTGGCTTTATGTGGAGGATTGCAGTCGTTGTCGATTAAGAATTCGGCGATTTGACTGATATGGGTTGGTGGCTATAGGGAGAGGATGTTGTAAGTTGACTACGTTTGAGATTCAAGGGTGTGAGAATGTAACAGTGGATGGACTAAGAACAATGGTTAGTCTTCGGAGTAAGACTTTGACTGATGTGAGAATCTTTGCTGCAAGAATCTTGACACAGCTGCTTCTTTAAAGGCAATTGAGCCGATTTGTGATCGGATCAAGAGACTGCATATAGACTGTGTGGTCTGGTTCAGAGGACGAGGAGGTAGAAGGAAGAGTGGAAGTACTAGTGGGCTGACCAAGAAGAGGAGGATGATGGTTACGAGAGGAGCCAGAAGAGGTGCAAGTATTCATTGAGGAAGAACAAGCTGCTCAACTAGTGTGATGGAATGGATTCTGTTGCAAGATAGAGTATGGGAGAAACTGGAGTATCTATCTTTATGGATCAATGTTGGAGAATTTTTGACGCCATTACCTATGACAGGACTAGATGACTGTCCGAATTTGGAAGAGATTAGGATCAAGATAGAAGGAGATTGCAGAGGTAAACGCAGGCCAGCCAGCCAGAGTTTGGGTTAAGTTGTCTCGCTCTTACCCAAAGCTCTCAAAGATGCAGTTAGATTGCGGGGACACAATCGGTTTCGCACTGACCGCACCGCAATGCAGATGGATTTGAGTTTATGGGAAAGATTCTTCTTGACCGGAATTGGAAGCTTGAGCTTGAGCGAGCTTGATTATTGGCCACCACAGGATAGAGATGTTAACAGAGGAGTCTCTCGCTTCTGGAGCAGGTCTGTTACAAGAGTGCCTGACTTTGAGGAAGCTGTTCCATCCATGGAACAGCTCATGAGCATTTCATGAACTTTTTGTTGAGAATCCCAAACCTAAGGGATGTACAGCTTAGAGCAGACTATTATCCGGCGCCGGAGAACGATATGAGCACAGAGATGAGAGTTGGTTCGTGTAGCCGATTCGAGGACCAATTGAACAGCCGCAAATCATTGACTGAaactgaagagtgagttacctacactattatatctgtattgactcagaaactggccattttattgtatgtcaagagtgtttataatggttaagaggaaaggacaaagactataatgtcgatgattaatgatatacataaacaataatccattttataaatgataaaactcaatgaagaagccggagagatctggagtacaaactca

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