

Identification of a Gene Underlying a Major Fruit Texture QTL in Tomato

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ABSTRACT

Controlling fruit softening in tomato (*Solanum lycopersicum*), in the absence of a negative impact on other ripening events, is a major challenge, but vital to maintain good shelf life. This work was focused on identification of the gene underlying a major texture quantitative trait locus (QTL) in tomato to provide a mechanism for selectively controlling fruit texture in the absence of effects on other quality attributes. A texture QTL was mapped to tomato chromosome 3 in a previous study using the Zamir *Solanum pennellii* introgression lines (IL) (Eshed and Zamir, 1995). Fine mapping within IL3-4 revealed a 1 Mb region harbouring the texture QTL. Within this region was a gene encoding a pectate lyase that was highly expressed in the soft M82 parent, but lowly expressed in the firm fruit of the IL3-4 parent. This PL (*Solyc03g111690*) was nominated as the QTL candidate gene and the effect of modulating its expression was tested in transgenic plants using an RNAi approach. Fully red ripe RNAi PL fruits were substantially firmer than wild type, but ripened normally with respect to colour, sugars, acids and the development of flavour volatiles. PL was necessary for cell wall pectin solubilisation and depolymerisation, linked to cell-to-cell adhesion, and played a much more significant role in softening than previously characterised cell wall modifying enzymes. The GM approaches and availability of natural variation described in this thesis provide methods to enhance texture and extend shelf life in tomato without compromising fruit quality.

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Table of Contents

1	INTRODUCTION	1
1.1	The Tomato.....	1
1.1.1	The importance of tomato in the diet.....	2
1.1.2	Tomato as a model system to understand fruit ripening ...	3
1.2	Molecular Biology and Biochemistry of Tomato Ripening	4
1.2.1	Ethylene and Ripening.....	4
1.2.2	Transcriptional Control of Ripening.....	14
1.2.3	Changes in colour, flavour and aroma.....	19
1.3	Changes in texture and cell wall remodelling.....	23
1.4	Cell Wall Remodelling Enzymes	29
1.4.1	Cellulose degrading enzymes.....	30
1.4.2	Hemicellulose Degrading Enzymes	32
1.4.3	Pectin Degrading Enzymes.....	36
1.5	Identification of QTL for fruit texture.....	42
1.5.1	Introgression Lines	42
1.6	Aims and Objectives	48
2	General Materials and Methods	50
2.1	Plant Material and Growing Conditions	50
2.2	Measurement of Fruit Colour.....	52
2.3	Genomic DNA Extraction	55
2.4	Primers.....	57
2.5	Polymerase Chain Reaction (PCR).....	57
2.6	Purification and Sequencing of PCR Products	58
2.7	RNA Extraction	59
2.8	Quantitative RT-PCR.....	60
2.9	Statistical Analysis	61
3	Fine Mapping of Fruit Texture QTL on Tomato Chromosome 3	63
3.1	Introduction	63
3.2	Material and Methods.....	67
3.2.1	Plant Materials and Development of QTL-NILs.....	67
3.2.2	Phenotypic Analysis	67
3.2.3	Genotyping of QTL-NIL lines.....	68
3.3	Results and Discussion.....	71
3.3.1	Fine mapping the texture QTL	71
3.3.2	Investigating the expression of the QTL candidate gene .	77
3.3.3	The texture QTL candidate gene is a pectate lyase (PL)..	80

3.4	Conclusion	81
4	QTL Candidate Gene Validation – Generation of Transgenic PL Lines and Analysis of their Fruit Texture	83
4.1	Introduction	83
4.2	Materials and Methods	84
4.2.1	Gateway Cloning	85
4.2.2	Amplification of PL PCR product and Gateway Cloning	85
4.2.3	Identification of transgenic lines	91
4.2.4	Identification of single copy homozygous lines	92
4.2.5	qPCR determination of PL gene expression	94
4.2.6	Determination of PL enzyme activity.....	94
4.2.7	Analysis fruit texture and shelf life	95
4.3	Results and Discussion.....	96
4.3.1	Analysis of the PL transgenic lines.....	100
4.3.2	Identification of Homozygous lines	101
4.3.3	PL gene expression	103
4.3.4	PL enzyme activity	106
4.3.5	Texture Measurement on fruit from homozygous PL::RNAi lines	109
4.4	Conclusion	114
5	Effect of PL Silencing on Fruit Metabolism and Ripening Related Gene Expression	116
5.1	Introduction	116
5.2	Materials and Methods	118
5.2.1	Extraction and analysis of carotenoids	118
5.2.2	Extraction and analysis of intermediary metabolites	119
5.2.3	Tomato Aroma Analysis by GC-MS	120
5.2.4	Ethylene Measurement	122
5.2.5	RNA sequencing analysis	122
5.3	Results and Discussion.....	124
5.3.1	Metabolite analysis	124
5.3.2	RNA-Seq analysis and specifically expression of cell..... wall Structure related genes	133
5.4	Conclusion	137
6	Mechanism of Action of PL in Tomato Fruit Softening.....	140
6.1	Introduction	140
6.2	Material and Methods.....	141
6.2.1	Plant Material	142

6.2.2	Alcohol Insoluble Solids Production (AIS) as crude cell wall material (CWM)	142
6.2.3	Fractionation of tomato CWM	142
6.2.4	Uronic Acid Assay	143
6.2.5	Determination of pectin molecular weights	143
6.2.6	Tissue Fixation, embedding and sectioning	144
6.3	Results and Discussion.....	146
6.3.1	Determination of solubility	146
6.3.2	Determination of MW of soluble pectin	149
6.3.3	Changes in cell wall structure as determined by light	151
	Microscopy	151
6.3.4	Crosses between PL:RNAi and a line where PG has been silenced using an antisense approach	153
6.4	Conclusion	155
7	General Discussion	157
7.1	PL in transgenic fruits and its mechanism of action	161
7.2	Conclusion	170
8	References	172
9	Appendix	187
9.1	Speed DNA Extraction Buffer	187
9.2	Primers used for genotype analysis, and transgenic plant ...	188
9.3	PCR-DIG probe synthesis	189
9.4	Tomato Transformation Protocol.....	190
9.4.1	Seed Germination	191
9.4.2	Agrobacterium Incubation	191
9.4.3	Co-cultivation.....	191
9.4.4	Induction.....	191
9.4.5	Elongation	192
9.4.6	Rooting	192
9.5	Southern Blotting	193
9.6	Identification of homozygous lines	195
9.7	Carotenoids	197
9.8	Sugars.....	198
9.9	PL-PCA analysis (GCMS&Carotenoids)	199
9.10	PL-PCA analysis (GCMS&Carotenoids)	200
9.11	Pectate lyase sequences.....	201
9.11.1	Protein sequence of PL	201
9.11.2	Gene sequence of PL.....	202

9.11.3 PL into entry clone	203
9.11.4 PL into destination vector.....	204
9.12 Gene models underlying 1 Mb region	205
9.13 QTL Mapping markers.....	208
9.14 Texture Measurement of Transgenic Lines	214



List of Figures

Figure 1.1 Ethylene Biosynthesis in Plants (Yang Cycle) (Wang <i>et al.</i> , 2002)	6
Figure 1.2 Expression of ACS and ACO genes associated with system 1 and system 2.....	9
Figure 1.3 Three-state model for receptor signaling.....	11
Figure 1.4 Model for the regulation of ripening in climacteric fruits showing the importance of DNA methylation.	18
Figure 1.5 Carotenoid Biosynthesis Pathway in plants.	20
Figure 1.6 Cartoon to show possible structure of the primary cell wall of higher plants.....	25
Figure 1.7 Schematic structure of cellulose and its degradation with cellulolytic enzymes.....	32
Figure 1.8 Schematic structure of three hemicelluloses, xylan, galacto(gluco)mannan, and xyloglucan, with hemicellulolytic enzymes.	35
Figure 1.9 Schematic diagram of α -1,4-polygalacturonic acid cleavage by a β -elimination mechanism.	40
Figure 1.10 Genome introgressions on the tomato chromosomes of the 76 <i>S.pennellii</i> ILs.	45
Figure 2.1 A 6-mm transverse section was cut from each fruit, and the maximum load.....	52
Figure 2.2 A diagram of the Hunter L*, a*, b* colour space.....	54
Figure 3.1 Cartoon of chromosome 3 with IL3-4 and markers which are within the IL at each end.	65
Figure 3.2 The population of informative recombinants identified with markers.....	69
Figure 3.3 (A) Maximum load values for inner and outer pericarp of fruits from parental lines M82 and 3-4 and for the F1.....	72
Figure 3.4 Maximum load (N) for pericarp of the recombinant individuals (A) outer and (B) inner pericarp.	73

Figure 3.5 Example of genotype and phenotype data map.....	74
Figure 3.6 Fine mapping of IL3-4 texture QTL to a 1.05 Mb region delineated by markers G390 and G660.....	76
Figure 3.7 <i>Solyc03g111690.2</i> PL gene expression in M82 and IL 3-4 at different developmental stages.	78
Figure 3.8 Relative expression of <i>Solyc03g111690</i>	79
Figure 4.1 Flowchart describing steps involved in cloning of the PL gene fragment by Gateway cloning.....	91
Figure 4.2 The gene specific PL PCR product (2G111690.2) showed by an arrow was chosen to be used for Gateway Cloning due to its appropriate size (300bp).....	97
Figure 4.3 Cloning of PL gene fragment in to Gateway vector pK7GWIWG2(1)	98
Figure 4.4 Gene construct has been inserted into 35S-pK7GWIWG2(1) destination vector to create expression clone..	99
Figure 4.5 Lane 1 to 13: Transgenic lines containing the 35S::PL RNAi gene construct.....	101
Figure 4.6 Twelve T ₁ seeds from the T5 PL line were sown and seedlings used to identify of homozygous, heterozygous and azygous plants.	103
Figure 4.7 (A) Comparison of PL gene expression during fruit development and ripening	105
Figure 4.8 PL enzyme activity in acetone powders prepared from Br+7 red ripe fruit pericarp of Ailsa Craig and PL RNAi (5-5) line.	108
Figure 4.9 Changes in fruit pericarp firmness during ripening as a result of RNAi silencing of PL.	111
Figure 4.10 Azygous control (5-6) and PL:RNAi transgenic (5-5) fruits after storage at 20 °C 14 days.	113
Figure 5.1 Fruit characteristics with respect to weight, colour index and brix measures in red ripe fruits	126
Figure 5.2 Analysis of carotenoid precursors, carotenoids and tocopherol composition of PL::RNAi and azygous fruits harvested at 7 days post breaker.	128
Figure 5.3 Analysis of sugar and organic acid composition of PL::RNAi and azygous fruits harvested at 7 days post breaker.....	129
Figure 5.4 The concentration of each compound per sample was calculated in ppm (mg/kg). I.....	130

Figure 5.5 Ethylene evolution from fruit of 'Ailsa Craig', PL:RNAi (5-5) and azygous (5-6) lines at breaker+4, breaker+7 stages.....	132
Figure 5.6 A comparison of the transcriptome of the PL:RNAi and control fruits by RNASeq.	135
Figure 6.1 Fractionation of cell wall material from mature green (MG), breaker (Br) and breaker +7(Br+7) fruit of azygous (5-6) control and PL: RNAi (5-5).	148
Figure 6.2 Pericarp cell walls from Azygous (5-6) and PL:RNAi (5-5) lines at Br+7 stage.....	152
Figure 6.3 The effect of crossing a PL:RNAi line with a PG antisense on outer and inner pericarp firmness at breaker+7.....	154
Figure 7.1 As cells expand, the corners of three-way junctions take much of the stress in keeping cells stuck firmly to their neighbours	162
Figure 7.2 Down-regulation of cell wall related genes and their effects on fruit softening..	165
Figure 7.3 Cellulose microfibrils (blue bars) linked with hemicellulose (yellow lines) within a matrix of pectin (light brown).	169
Figure 9.1 Lane 1: Plasmid control without DIG labelled. Lane 2: Genomic DNA DIG labelled. Genomic DNA DIG labelled product was slightly higher than plasmid control because of the DIG label.	193
Figure 9.2 DNA was digested by SacI Lane 1: Transgenic line 2 (T2), Lane 2: T5, Lane 3: T6, Lane 4: T8, Lane 5 :T10, Lane 6: AC, Lane 7: Plasmid Control. M: Marker.....	193
Figure 9.3 Separating DNA on gel Lane 1: Transgenic line 2 (T2), Lane 2: T5, Lane 3: T6, Lane 4: T8, Lane 5: T10, Lane 6: AC Control, M: Marker.....	194
Figure 9.4 Lane 1: AC, Lane 2: Transgenic 1 (T1), Lane 3: T5, Lane 4: T6, Lane 5: T8, Lane 6: T10	194

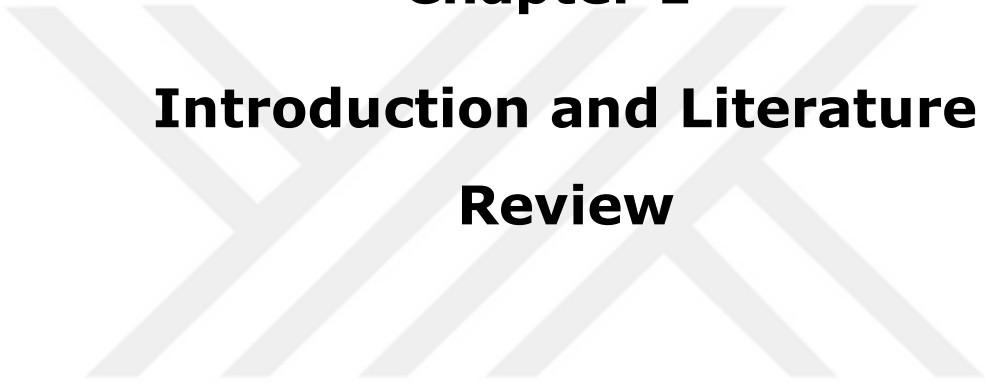
List of Tables

Table 1.1 Patterns of expression for a subset of the major flavor volatiles during fruit maturation..	23
Table 1.2 Survey of pericarp firmness-associated QTLs in the ripe fruit of <i>S.pennellii</i> introgression lines.	47
Table 5.1 Six key compounds were selected from the identified compounds and listed in the table.	130
Table 6.1 Weight average molecular weight of water, CDTA and carbonate soluble pectins extracted from cell wall material	150
Table 7.1 A comparison of 1.5 kb of the PL regulatory region between <i>S.lycopersicum</i> and <i>S.pennellii</i>	160



LIST OF SYMBOLS AND ABBREVIATIONS

ABA	Absciscic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	1-aminocyclopropane-1-carboxylate synthase
Br+	breaker plus
Ca ²⁺	calcium ions
cDNA	complementary DNA
CTR	constitutive triple response
CWM	cell wall material
dH ₂ O	dionised water
ELF	elongation factor
ER	endoplasmic reticulum
ERF	ethylene-response factor
EST	expressed sequences tag
HEMA	2-hydroxyethyl methacrylate
HG	homoglacturonan
IAA	Indole-3-acetic acid
LR	London resin
MG	mature green
M-ML V	Moloney murine leukemia virus
NIL	near isogenic lines
PCR	polymerase chain reaction
PE	pectin esterase
PG	polygalacturonase
PL	pectate lyase
PME	pectin methylesterase
PSY	phytoene synthase
RNAi	RNA interference
TBG	beta-galactosidase
QTL	quantitative trait loci



Chapter 1

Introduction and Literature Review

1 INTRODUCTION

1.1 The Tomato

The cultivated tomato *Solanum lycopersicum*, is the second most widely grown and consumed fruit both as raw or processed forms (Foolad, 2007). Botanically, the tomato is described as a berry that belongs to the nightshade family Solanaceae. This family consists of 96 genera and over 3000 species in three sub-families (Knapp *et al.*, 2004). Worldwide production of fresh market and processed tomatoes has steadily increased during the last ten years and reached annual production of about 162 million tons in 2012 (United Nations Food and Agriculture Organization (FAO) statistics; and a net value of over \$55 billion (Vincent *et al.*, 2013). China, USA, Turkey, India, Egypt and Italy produce half of the all tomatoes worldwide (USDA-FAS, 2005).

All tomato species are diploid ($2n=24$), with 12 chromosomes as the haploid complement. There are 13 species including the cultivated tomato (*Solanum lycopersicum*). In recent years, limited genetic diversity in *S. lycopersicum* has begun to be supplemented in breeding programmes by harnessing wild tomato species variation such as tolerance to abiotic stress, diseases resistance and high fruit quality (Foolad, 2007). Particularly, 76 near isogenic lines (NIL) created from *S. lycopersicum* M82 x *S. pennellii* cross have been subjected to physiological, agronomic, biochemical and molecular phenotyping (Eshed and Zamir, 1995).

During the 20th century, investigators have made extensive germplasm collections, including numerous natural and induced mutants and interspecific populations. Many of these are maintained at the Tomato Genetics Resource Centre in Davis, California (TGRC, <http://tgrc.ucdavis.edu/>). In addition to this, an exhaustive mutant population has been developed (<http://zamir.sgn.cornell.edu/mutants/>), which is an isogenic tomato 'mutation library' containing a total of 13 000 M₂ families (Menda *et al.*, 2004).

1.1.1 The importance of tomato in the diet

Fruits are important for a healthy diet, providing vitamins such as A, C, E, K and bioactive compounds including carotenoids such as lycopene and various polyphenols which are associated with a reduced risk of certain cancers and heart diseases (Bhowmik *et al.*, 2012; Wei and Giovannucci, 2012). Desirable characteristics such as flavour, shelf life, processing qualities and nutritional value determine quality of fruit.

The importance of fruit in a healthy diet is a major driver for governments to encourage consumers to eat more of these products. However fruit generally have a short post-harvest life. The control of fruit ripening is of substantial commercial significance. In tomato, non-ripening mutations are widely used in the heterozygous form in elite varieties to slow the rate of ripening and therefore extend shelf-life. A major disadvantage of using this approach is that it often delays all aspects of ripening. A more effective approach would be to target individual ripening processes for instance extend shelf-life of fruits by

slowing softening in the absence of detrimental effects on colour and flavour. To do this in a rational way we need to understand the biological basis of fruit ripening.

1.1.2 Tomato as a model system to understand fruit ripening

In addition to tomato's economic importance and worldwide cultivation, it has several features that make it a convenient plant for experimental purposes (Carvalho, 2011). These include efficient sexual hybridization, ease of culture under a wide range of environments, ease of controlled pollination and hybridization, diploid species with a haploid set of 12 chromosomes and moderately compact genome (c.900 Mb) (c. : the value shows the amount of DNA contained, in picograms, within a haploid nucleus (e.g. a gamete)) combined with a marker-saturated genetic linkage map and genome sequence (Tomato Genome Consortium, 2012) encoding approximately 35000 genes that are largely sequestered in contiguous euchromatic regions. There is an extensive literature on the biochemistry and molecular biology of ripening and many well characterised monogenic mutants and quantitative trait loci (QTL). Tomato is also readily amenable to genetic transformation allowing rapid and effective validation of ripening candidate genes.

1.2 Molecular Biology and Biochemistry of Tomato Ripening

1.2.1 Ethylene and Ripening

Fruit ripening is an important physiological process for plants. It is the terminal stage of development in which matured seeds are released and in fleshy fruits is the normal mechanism for seed dissemination. Ripening of fleshy fruits represents a highly programmed and tightly controlled developmental process leading to textural modifications, enhanced colour, as well as increased accumulation of sugars, acids and volatile compounds culminating in a diverse array of tastes and smells that vary among species (Klee and Giovannoni, 2011). Ripening fruits can be divided into two classes; (1) climacteric fruit such as apple and banana are characterised by a ripening-associated increase in respiration and in ethylene production; and (2) non-climacteric fruit such as strawberry and grape are characterised by the lack of ethylene-associated respiratory peak and the signalling pathways which are not yet elucidated (e.g. other hormones, developmental factors, etc.).

Ethylene is a gaseous hormone. Its synthesis occurs in three main steps (Yang, 1985). **1)** Methionine is converted to S-adenosyl-L-methionine (SAM), catalysed by the enzyme SAM synthase, **2)** Formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM via ACC synthase (there are at least 12 ACS genes in the tomato genome with specific spatial and temporal expression (Seymour *et al.*, 2013). **3)** The conversion of ACC to ethylene, catalysed by ACC oxidase (ACO is encoded by at least seven genes in tomato LeACO1-7) (Figure 1.1).



development of non-climacteric fruit. System 2 is specific to climacteric fruit and refers to the autocatalytic ethylene production that occurs during ripening (Bapat *et al.*, 2010).

Plant ethylene receptors are related to bacterial two component regulators and are disulphide linked dimers; endoplasmic reticulum (ER) associated integral membrane proteins with protein kinase activities. Genetic analysis has shown that the receptors act as negative regulators of the ethylene response pathway in tomatoes (Hua and Meyerowitz, 1998). Ethylene responses are actively suppressed by receptors in the absence of the hormone. Mechanism of ethylene perception is described as the receptors in a functional 'on' in the absence of ethylene and converted to 'off' state in the presence of ethylene (Klee and Giovannoni, 2011). Binding of ethylene to its receptors is the first step in ethylene signal transduction.

To date, seven ethylene receptor genes have been identified in the tomato genome (*LeETR1*, *LeETR2*, *NR LeETR3*, *LeETR4*, *LeETR5*, *LeETR6* and *LeETR7*). They have been classified into subfamily 1 and subfamily 2 depending on gene and protein structures. Whereas the subfamily 1

members have the highest similarity to histidine kinases and are more critical for ethylene signalling, the subfamily 2 members have diverged and acquired serine kinase activities (Moussatche and Klee, 2004). When the expression of subfamily 2 genes, *LeETR4* or *LeETR6*, is reduced by antisense transgenes, this results in considerably increased ethylene sensitivity. Antisense plants with greatly reduced expression of these two receptors ripen significantly earlier. The expression of *NR* increases significantly at the onset of ripening. Together these results show that *LeETR4*, 6 and *NR* are of most significance with respect to fruit ripening (Kevany *et al.*, 2007) (Figure 1.2).

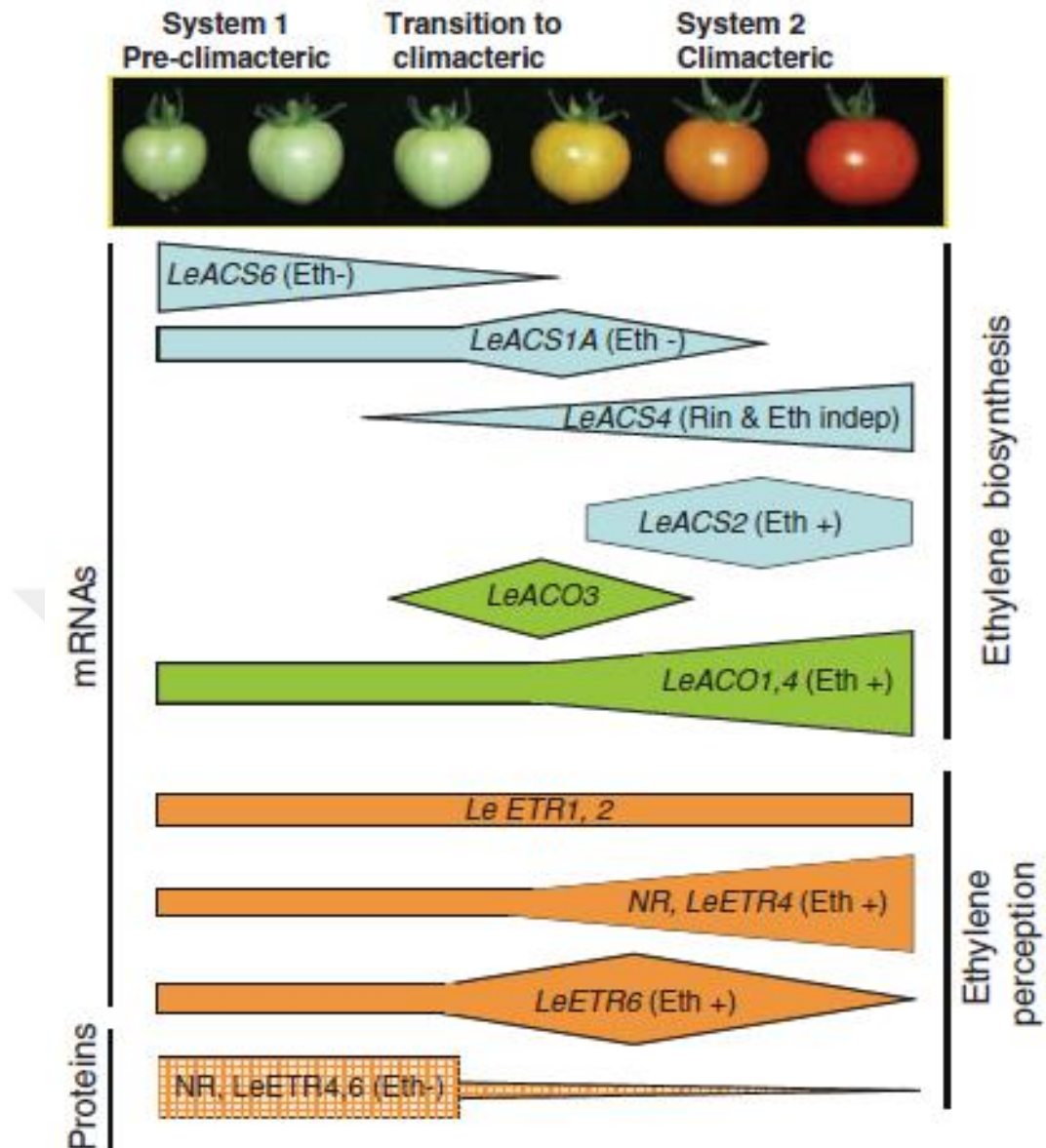


Figure 1.2 Expression of ACS and ACO genes associated with system 1 and system 2 ethylene syntheses in tomato development and ripening. Auto-inhibition of ethylene synthesis during system 1 ethylene is synthesized by LeACS1A, 6 and LeACO1,3,4. At the transition state, LeACS4 is induced; a large increase of autocatalytic ethylene starts, resulting in negative feedback on System 1. LeACO1,4 and LeACS2,4 are responsible for the high autocatalytic ethylene production via System 2. LeACS, *Solanum lycopersicum* ACC synthase; LeACO, *Solanum lycopersicum* ACC oxi-dase; LeETR and NR, ethylene receptors. Eth+ and Eth- refer to the stimulation and repression, respectively of gene or protein expression (adapted from Bouzayen, 2010).

The *CONSTITUTIVE TRIPLE RESPONSE* (CTR1) gene that was originally identified in *Arabidopsis*, encodes a protein that is part of the ethylene

signal transduction pathway. It has high amino acid sequence similarity to the mammalian serine/threonine MAP kinase kinase kinase (MAP3K) and acts as a negative regulator of ethylene responses (Barry and Giovannoni, 2007). CTR1, major component of ethylene signalling (Figure 1.3), is present in the same location as the ETR1 ethylene receptor and interacts preferentially with the ETR1 receptor (Gao *et al.*, 2003). Mutations in CTR1 which abolish these interactions cause CTR1 to be non-functional (Lacey and Binder, 2014). In tomato, four CTR1 homologous have been identified (tCTR1-4) whose function is to suppress the ethylene signalling pathway.

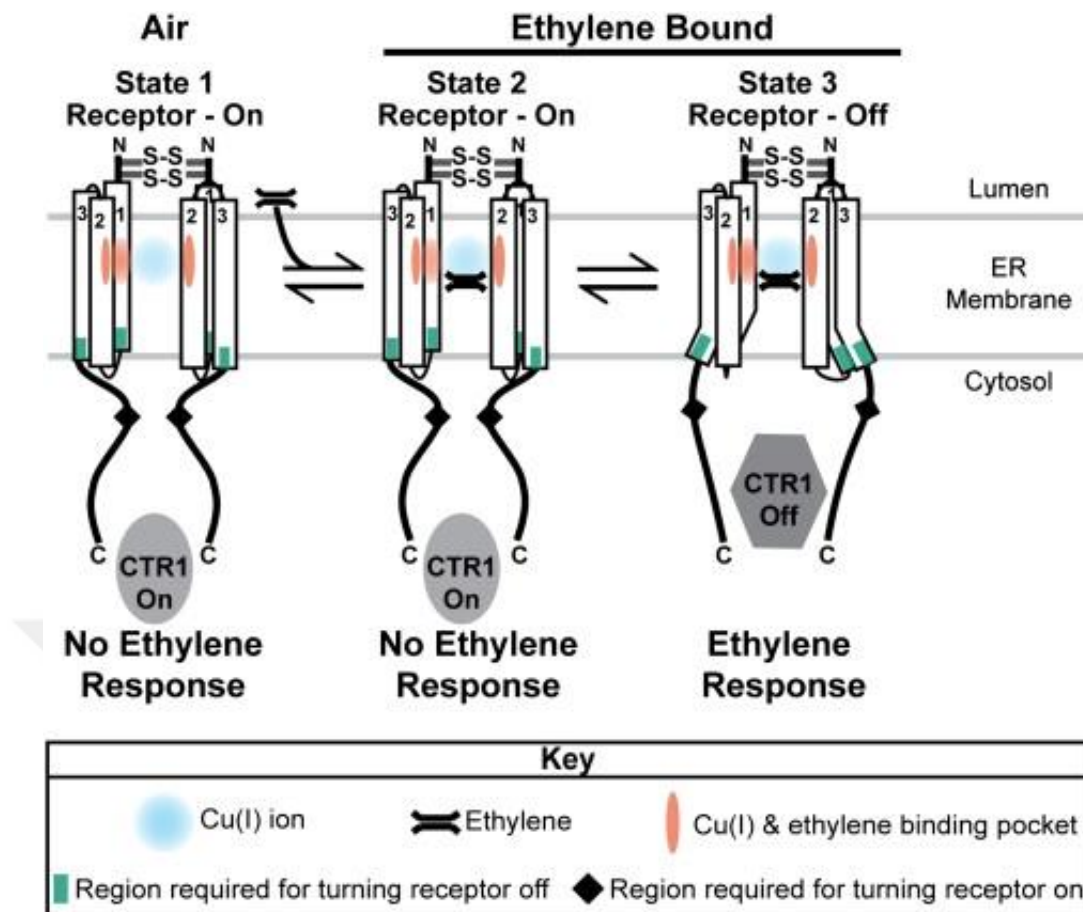


Figure 1.3 Three-state model for receptor signaling. The ethylene binding domain of ETR1 is shown as a homodimer stabilized with two disulfide bonds at the N-terminus. Each monomer contains three transmembrane α -helices numbered 1 through 3. Areas that affect ethylene signaling as defined by mutational studies are shown. These include regions important for copper/ethylene binding, turning off the receptor, and maintaining the receptor in the signaling state. In air, the receptor favours State 1. When ethylene binds there is an intermediate conformation where the receptor is still transmitting signal (State 2). While ethylene is bound, the receptor is in equilibrium with State 3 where the receptor is turned off. This leads to diminished activity of the negative regulator, CTR1, causing release of inhibition to downstream signaling (adapted from Lacey and Binder, 2014).

Upon ethylene binding, the receptors transmit the signal to the CTR1 protein kinase inhibiting its ability to phosphorylate EIN2 (ETHYLENE

INSENSITIVE2), a membrane spanning protein also present on the ER. Inhibition of EIN2 phosphorylation causes the C-terminal end of EIN2 to translocate to the nucleus. This C-terminal peptide targets EIN3 and EIN3-like proteins (EILs), where the EIN2 C-end leads to the stabilization of the EIN3/EIL proteins and prevents them from degradation. These proteins now interact with the *ETHYLENE RESPONSE FACTOR* (ERF) genes that target downstream effectors of the ethylene signalling (Merchante *et al.*, 2013). There are four tomato EILs which are functionally redundant (Tiemen *et al.*, 2001), however it has been suggested that overexpression of LeEIL1 in the ripening mutant NR only restored expression of some ethylene-regulated genes (Chen *et al.*, 2004).

Green Ripe (*Gr*) and its allele, *Nr-2*, are loci that give non-ripening phenotypes when mutated and where the underlying genes are also part of the ethylene signalling system. Their phenotypic similarity to *Nr* suggested that they might be receptor mutants. Their non-ripening phenotype is the result of reduced ethylene responsiveness in fruit tissues. Nevertheless, ethylene is slightly reduced in non-fruit tissues. *GR/NR-2* locus was isolated by positional cloning. 334 bp of identical deletion in *Gr/Gr* and *Nr-2/Nr-2* genotypes were revealed by DNA sequencing. Molecular analysis exposed that the *Gr/Nr-2* deletion causes ectopic expression of *GR*, consistent with a dominant gain of function mutation (Barry and Giovannoni, 2006). *Gr* locus encodes a conserved protein of unknown biochemical function which is associated with ethylene signalling as it does not have sequence homology with *Nr*

(*Never-ripe*), ethylene receptor, which was described before (Giovannoni and Barry, 2006). Although overexpression of *GR* in transgenic plants generated the *Gr* mutant phenotype, plants did not display whole plant ethylene insensitivity (Barry and Giovannoni, 2006).

There are several other plant hormones apart from ethylene that play roles during ripening processes. Evidence indicates that auxin acts as an inhibitor of ripening in tomato and other fruits (Seymour *et al.*, 2013).

In tomato, while level of free IAA, the most abundant plant hormone of the auxin class, decreases prior to the onset of ripening, the levels of the 'inactive' IAA amino acid Asp which is generated from IAA by IAA-amido synthetase is upregulated at the onset of ripening (Seymour *et al.*, 2013).

Another ripening regulating hormone is abscisic acid (ABA). Recent evidence on the role of ABA indicates that it acts with ethylene to promote ripening. *SINCE1* gene codes an enzyme which catalyses a key step in ABA biosynthesis, and suppression of this gene reduces transcription of several major ripening related cell wall enzymes, polygalacturonase (PG) and pectin methylesterase (PME), enhanced fruit shelf life and lower softening (Seymour *et al.*, 2013). Zinc finger transcription factor (*SIZFP2*) involved in ABA biosynthesis is overexpressed in tomato, and resulted in more branches, early flowering and delayed fruit ripening. Down regulation of the same gene caused problem in fruit set, accelerated fruit set and inhibited seed germination (Weng *et al.*, 2015).

1.2.2 Transcriptional Control of Ripening

A number of tomato mutants have been characterised that provide insights into the transcriptional control of ripening in tomato and other fleshy fruits beyond but alongside ethylene biosynthesis and signal transduction. These mutations include, *ripening inhibitor (rin)* (Robinson and Tomes 1968), *nonripening (nor)* (Tigchelaar *et al.*, 1978), and *colourless nonripening (Cnr)* (Thompson *et al.*, 1999). They block the ripening process; produce fruit that remain firm and green for an extended period and that are also deficient in production of ripening ethylene. Furthermore, they do not ripen in response to exogenous ethylene (Manning *et al.*, 2006).

The *rin* locus encodes a MADS box transcription factor, *LeMADS-RIN* (Vrebalov *et al.*, 2002). The *MADS-RIN* gene lies on chromosome 5 adjacent to the tomato *APETALA1*, *MACROCALYX (MC)*. The *rin* mutation causes spontaneous deletion in the C terminus of the *MADS-RIN* and approximately 1kb of the sequence separating *MADS-RIN* from *MC*. The original *rin* allele is used in tomatoes for extending shelf life (Kopeliovitch *et al.*, 1979). It is introgressed into elite backgrounds and then used to generate F₁ hybrids. A single non-working copy of *rin* yields fruit characterized by firmness and long shelf-life, but some aspects of flavour development are lost as reduced levels of normal *rin* transcript lower all aspects of ripening such as ethylene level, carotenoid and sugar accumulation. The *Cnr* mutant is an epigenetic change which alters the promoter methylation of a gene encoding a putative SQUAMOSA

promoter-binding (SBP) protein which results inhibition of ripening related genes (Osorio et al., 2011).

Recently it has been shown by using chromatin immunoprecipitation (ChIP), that the *RIN* gene product is needed to induce the expression of genes involved in cell wall modification, carotenoid accumulation and ethylene synthesis. It has been shown that *RIN* contributes to at least 67 metabolic pathways by direct transcriptional regulation of 241 genes (Fujisawa et al., 2011). A strawberry cDNA library has been screened with *MADS-RIN* cDNA and yielded a homologous gene whose expression was elevated during ripening. These results may indicate that transcriptional control of ripening is conserved among climacteric and non-climacteric fruits (Seymour et al., 2010).

The gene at the *Cnr* locus encodes a transcription factor of the *SQUAMOSA PROMOTER BINDING PROTEIN* family (SBP). The mutation has arisen as a result of the hypermethylation of several normally unmethylated cytosines in the *LeSPL-CNR* promoter (Manning et al., 2006). Bisulfite sequencing of the *CNR* mutant allele showed hypermethylation of cytosine in a region 2.4 kb upstream of the predicted ATG start site. This hypermethylation state correlated with a drastic reduction of *CNR* gene expression. Additionally, in normal tomato fruit (cv. Liberto) development, the promoter of *CNR* appears to be demethylated in a specific region just prior to the onset of ripening (Seymour et al., 2008).

There is evidence that *in vivo* RIN cannot be recruited to target loci in the absence of CNR (Martell and Giovannoni, 2011). Although there is no evidence of a direct interaction between the RIN and CNR proteins, it cannot be excluded that CNR and RIN are part of the same protein complex. Alternatively, CNR might be necessary for the expression of a cofactor needed for proper DNA binding activity of the RIN-containing complex. As a member of SBP family of transcription factors, CNR plays important roles in the regulation of transcription of certain MADS-box genes. *TDR4* belongs to the SQUAMOSA class of MADS-box gene, a class of gene known to be regulated by SBP-box proteins, of which CNR is a member (Martel *et al.*, 2011). The *Cnr* mutation, like *rin* affects all aspects of ripening.

The discovery of an epigenetic basis for the *Cnr* mutation (Manning *et al.*, 2006) lead to the hypothesis that DNA methylation contributes to the regulation of fruit ripening (Figure 1.4) (Seymour *et al.*, 2008). In order to test this hypothesis, Zhong *et al.*, (2013) exposed tomatoes to the methyltransferase inhibitor 5-azacytidine. They predicted that this would inhibit maintenance methylation and could lead to early ripening. Interestingly the tomato fruits ripened treated with 5- azacytidine ripened prematurely. They performed whole-genome bisulfite sequencing on fruit in different developmental stages. They discovered 52,095 ripening related differentially methylated regions (representing 1% of the genome) and observed that binding sites in ripening-related genes that are targets for the RIN protein are localised in the demethylated regions and binding occurs in concert with demethylation

(Figure 1.4). The master regulators of ripening, which include RIN and CNR are responsible for controlling the expression of genes that affect changes in the fruit phenotype leading to ripening.



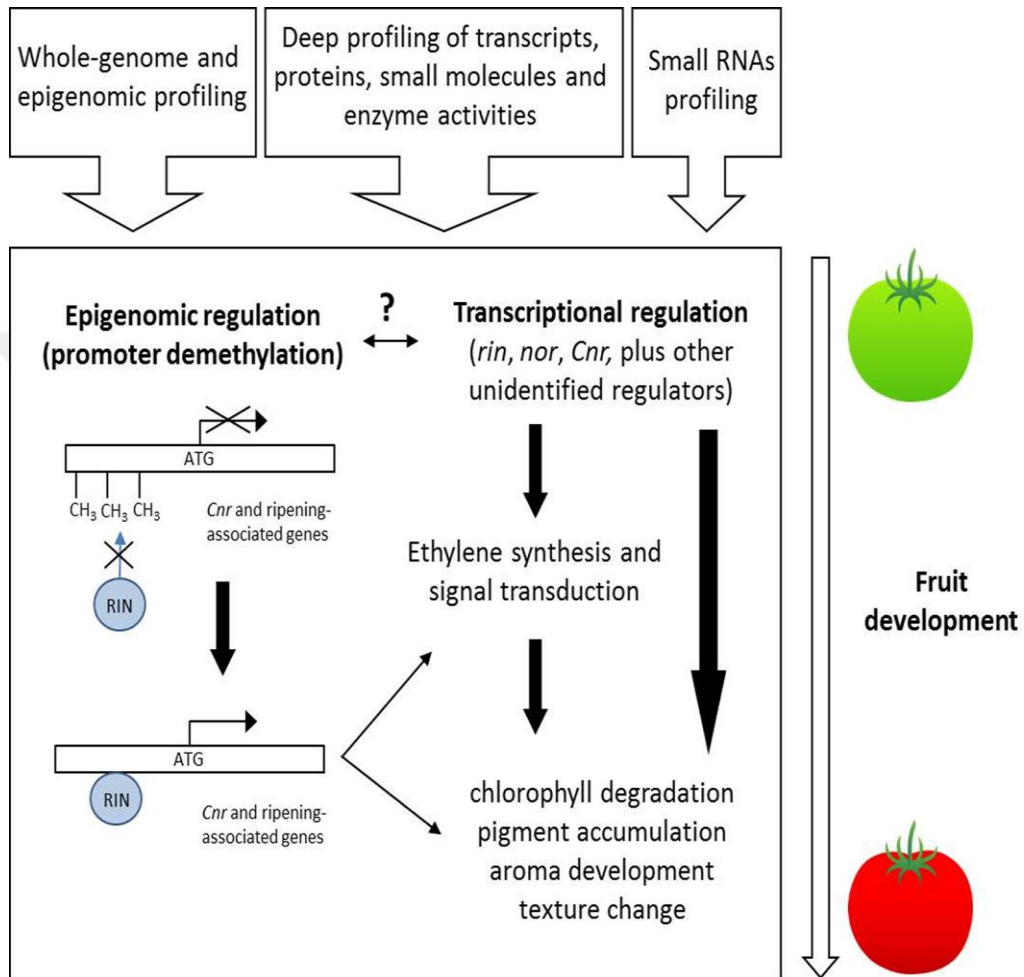


Figure 1.4 Model for the regulation of ripening in climacteric fruits showing the importance of DNA methylation. The contribution of systems profiling approaches (shown at the top) will help identify novel regulatory genes and elucidate the interplay between epigenomic remodeling and transcriptional regulation involved during the ripening process (From Osorio *et al.*, 2013).

1.2.3 Changes in colour, flavour and aroma

The colour change of fleshy fruit is an indicator of ripening, and is tightly controlled by ripening regulators. The final colour of tomato is regulated by pigment types and environmental conditions. Carotenoids, especially lycopene and β -carotene, are the principle ripe fruit pigments of tomato. The carotenoid biosynthetic pathway in plants is shown in (Figure 1.5Figure 1.5). Gene expression for the rate limiting step in the carotenoid pathway, i. e phytoene synthase (PSY), is regulated by ethylene (Lee *et al.*, 2012). Deposition of lycopene and β -carotene, which are associated with the change from green to red, causes characteristic pigmentation of red ripe tomato fruit. At the breaker stage of tomato ripening, lycopene begins to accumulate and its concentration increases 500 fold in ripe fruits (Alexander and Grierson, 2002). More than 20 genes that play roles in type, amount and distributions of tomato fruit carotenoids have been characterised (Labate *et al.* 2007).

In order to understand the genetic basis of quantitative variation of tomato fruit colour, quantitative trait locus (QTL) mapping studies have been carried out using intraspecific and interspecific populations. Numerous colour QTLs which are mapped to all 12 chromosomes were discovered (Liu *et al.*, 2003). For example, for the fruit colour QTLs mapped at the bottom of chromosome 4, IL4-4, carrying introgression for this region from *S.pennellii*, increased the intensity of the red colour up to 80% for IL4-4 (Monforte *et al.*, 2001). Inspection of these QTL genomic regions failed to reveal genes linked to carotenoid biosynthesis indicating that the QTL reveal as yet unknown regulators of this process.

Flavour and aroma are important parameters for tomato fruit. The flavour of fruit is generally determined from tens to hundreds of constituents, but only 30 are present at concentrations exceeding 1nl/L (Buttery, 1993).

The ratio between organic acids and sugars which represent 60% of tomato dry matter play a significant role in the overall flavour of fruit. Sugars establish around 60% of total soluble solids fraction. Sugar and related contents such as fructose and glucose have been analysed in a number of QTL studies involving eight different species (Liedl *et al.*, 2013). Wild species alleles enhanced tomato sugar content in most of the QTLs. The best example for this, *brix 9-2-5*, which increased sugar yield by more than 20%, was isolated by map based cloning (Fridman *et al.*, 2000). The *lin5* gene which encodes for an apoplastic invertase was responsible for the QTL. Total acid content is also important for its

contribution to flavour of tomato. Several QTL mapping studies have been carried out to identify the source of variation of altering acid content (Liedl *et al.*, 2013). The organic acids that contribute to tomato fruit flavour are citric, malic and ascorbic acid.

Volatile aroma compounds are generated by the breakdown of essential amino acids, fatty acids and carotenoids compounds (Goff and Klee, 2006).

There are approximately 400 volatile compounds (Osorio and Alisdair, 2013). However, only 15-20 of those volatile compounds are sufficiently present have an impact on human perception in tomato flavour (Baldwin *et al.*, 2000). Principle contributors to the ripe flavour are *cis*-3-hexanal, *cis*-3-hexanol, 3-hexanal, 3-methylbutanal, 6-methyl-5-hepten-2-one, 1-pentan-3-one, *trans*-2-hexanal, methyl salicylate, 2-isobutylthiazole and β -ionone (Buttery and Ling, 1993). Some of these volatile contribute positively, like sweetness, while others contribute negatively, such as astringency and bitterness, to tomato flavour (Baldwin *et al.*, 1998). Volatile compound have been studied by QTL mappings. Saliba Colombani *et al.*, (2001) measured 18 volatiles and identified 29 QTLs which are localised on chromosome 4, 8 and 9.

Table 1.1 Patterns of expression for a subset of the major flavour volatiles during fruit maturation. FA: fatty acid, BCAA: branched chain amino acid, Phe: phenylalanine. Levels of volatile accumulation are normalized to mature green (MG) and shown for various ripening stages: breaker (Br), turning (Tu), fully ripe (Red) (Klee and Giovannoni, 2011).

Volatile	Precursor	MG	Br	Tu	Red
<i>cis</i> -2-penten-1-ol	FA	1X	1X	2X	10X
<i>trans</i> -2-pentenal	FA	1X	1X	1X	10X
<i>cis</i> -3-hexenal	FA	1X	1X	1X	10X
<i>trans</i> -2-hexenal	FA	1X	1X	2X	5X
<i>trans</i> -2-heptenal	FA	1X	1X	2.5X	2.5X
β -ionone	Carotenoid	1X	1X	1X	10X
6-methyl-5-hepten-2-one	Carotenoid	1X	1X	1X	10X
geranylacetone	Carotenoid	1X	1X	1X	10X
2-methylbutanal	BCAA	1X	1.3X	1.5X	1.5X
2-methyl-1-butanol	BCAA	1X	1X	10X	10X
3-methylbutanal	BCAA	1X	3X	5X	4X
3-methyl-1-butanol	BCAA	1X	2X	10X	4X
isovaleronitrile	BCAA	1X	1X	1X	10X
isobutyl acetate	BCAA	1X	1X	1X	1X
isobutylthiazole	BCAA	1X	1X	1X	1X
phenylacetaldehyde	Phe	1X	1X	1X	1X
2-phenylethanol	Phe	1X	1.3X	1.5X	1.5X
benzaldehyde	Phe	1X	1X	1X	10X
methylbenzoate	Phe	1X	1X	3X	2X

1.3 Changes in texture and cell wall remodelling

Alteration in tomato fruit texture is one of the most important components of the ripening process and greatly influences consumer acceptability (Seymour *et al.*, 2002). They not only make the fruit soft enough to consume, but more importantly influence shelf-life in the supply chain. Too rapid softening can lead to susceptibility to post harvest pathogens and short shelf life leading to high levels of waste. Texture changes are a product of several events including changes in water loss through the fruit cuticle, but the main driver for changes in texture is thought to be highly controlled remodelling of the cell walls of the fruit pericarp (Isaacson *et al.*, 2009).

The primary cell wall of flowering plants is composed of polysaccharides and proteins, the polysaccharide fraction can be divided into three major components cellulose, hemicelluloses and pectin that are all modified during fruit ripening (Figure 1.6).

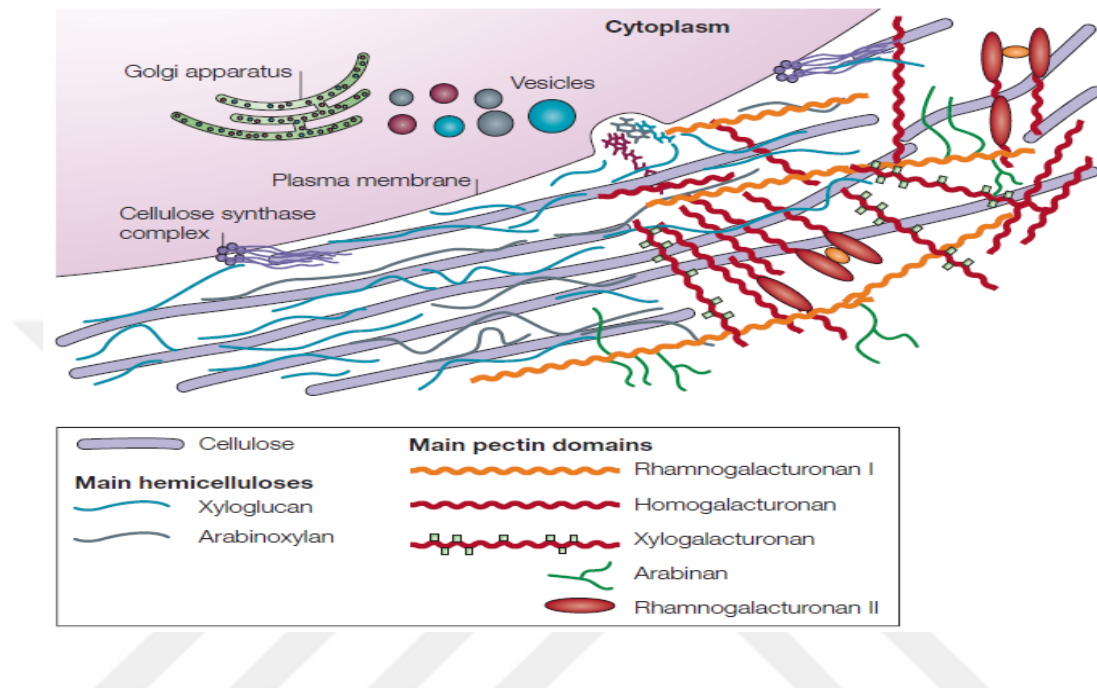


Figure 1.6 Cartoon to show possible structure of the primary cell wall of higher plants. Hemicelluloses and pectins are synthesised in the Golgi apparatus and are deposited to the wall surface by vesicles, whereas cellulose microfibrils are synthesised by hexameric complexes in the plasma membrane (Cosgrove, 2005)

Cellulose is composed of unbranched 1,4- β -D linked glucan chains that associate with other cellulose chains by hydrogen bonding and Van der Waals forces (Nishiyama *et al.*, 2002). Cellulose is the main load-bearing polymer of the primary and secondary cell wall because length and crystallinity of cellulose microfibrils are important factors of the physical characteristics of the cell wall (Lodish *et al.*, 2000). The cellulose chains are synthesised by cellulose synthase complexes that contain multiple cellulose synthase (CesA) subunits which form a rosette structure (Ann Ha *et al.*, 1998). The role of the cellulose matrix is to provide the basis of the strong support structure that makes up the total wall matrix.

Hemicelluloses are neutral or weakly acidic, composed predominantly by xyloglucan which possesses a 1,4- β -D glucan backbone (like cellulose) but has numerous regularly spaced xylose side chains. Other related polymers include xylans, arabinoxylans and mannans. The hemicelluloses are richer in secondary walls than primary ones of both monocot and dicot species (Lodish *et al.*, 2000)

Xyloglucan (XG) is the most abundant and studied polysaccharide in dicot primary walls (Cosgrove, 1997). XG has a backbone structure of β -(1,4)-D-glucopyranose residues. This backbone does not vary among plant species and only a limited number of different side chains have been reported (Cosgrove, 1997). XG has primarily role in a structural capacity in the cellulose-xyloglucan network of the plant cell wall, because XG has strong binding to cellulose. The strength of the

interaction results from the strong non-covalent and additive interaction of hydrogen bonds between XG molecules and cellulose microfibrils (Scheller and Ulvskov, 2010).

Xylan polysaccharides comprise linear chains of β -(1,4)-D-Xylp residues and may be found as arabinoxylan (AX), glucuronoarabin-oxylan (GAX), glucuronoxylan (GX), or the unsubstituted homoxylan (Chesson *et al.*, 1983). The mannans which are structurally important parts of the cell wall as well as an important source of storage polysaccharides.

The exact role of the hemicellulose components on the cell wall is still a matter of debate (Schröder *et al.*, 2009). However they are thought to be involved in linking separate cell cellulose microfibrils and also they may interact directly with the pectin matrix through covalent (bonds). These linkages between matrix polysaccharides are thought of as wall maturation phenomena occurring by transglycosylation in the wall. However, XG and RG-1 may be linked already in the Golgi (Scheller and Ulvskov, 2010). The cellulose/ hemicellulose network is embedded within a jelly like pectin matrix.

Pectic polysaccharides are some of the most complex polymers to exist in nature and make up around % 30-50 of the content of plant cell walls (Cosgrove and Jarvis, 2012). They are a class of heteroglycans that are frequently found in the middle lamellae of plant cells (Lodish *et al.*, 2000). There is strong evidence that pectins play an important role in intercellular adhesion and controlling the porosity of the wall (Willats

et al., 2001). There are a wide range of pectic polymers. The general structure of these is characterized by relatively high proportions of galacturonic acid (GalA) residues and can be unbranched and linear poly 1,4- α -D-GalA, homogalacturonan, or with a homogalacturonan backbone and single xylogalacturonan (Xyl) side chains (Atmodjo *et al.*, 2013). These side chains are composed of numerous neutral sugars including arabinose and galactose (Figure 1.6). The galacturonic acid rich polymers can be classified into several structurally distinct regions.

Homogalacturonan (HG) This is a polymer of α -1,4-linked-D-galacturonosyl uronic acid (GalA) which composes of greater than 60% of pectins in the plant cell wall (Ridley *et al.* 2001). HG residues are often thought be methyl-esterified at the C-6 carboxyl or acetylated at the O-2 or O-3. The degree of methylesterification varies depending on the source and tissue developmental stage. HG would be expected to be negatively charged in the unmethylated C-6 of GalA residues. These negatively charged residues interact with Ca^{2+} to form a stable gel (Liners *et al.*, 1989). This structure of the HG-calcium complex is sometimes stated as the egg-box model (Grant *et al.*, 1973). This gelling ability of pectin has been used by food industry as stabiliser and jam making.

Rhamnogalacturonan I (RG-I) is the second major component of pectic polysaccharides. RG-I has a backbone repeating units of $[-\alpha\text{-D-GalpA-1,2-}\alpha\text{-L-Rhap-1,4-}]_n$ which can be released from commercial

pectin by controlled acid hydrolysis. The rhamnosyl residues are normally substituted at O-4 with neutral sugars side chains which are mainly composed of galactosyl and/or arabinosyl residues. The GalA residues of RGI may lack methyl-esters and, therefore RGI is not degraded under β -eliminative conditions. The GalA residues are often O-acetylated on position O2 and/or O-3 in the RG-I backbone (Liepman *et al.*, 2007).

Rhamnogalacturonan II (RG-II) is a highly conserved and found in nearly all higher plants and can be released by endo-polygalacturonase action. This conservation of RG-II structure across the plants indicates that RG-II must play an important role in cell walls, although its precise role is unknown (Caffall and Mohnen, 2009). The basic structure of RG-II is similar to HG ((1-4)- α -D-GalpA) but containing clusters of four different side chains with unusual sugar residues, such as apiose, aceric acid, 3deoxy-lyxo-2-heptulosaric acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO). These side chains have a precisely defined structure and are attached to a HG fragment of approximately nine GalA residues, of which some are methyl-esterified (Ridley *et al.*, 2001). RG-II can complex together with boron which is shown as an essential microelement in plant growth (Whittington, 1959).

1.4 Cell Wall Remodelling Enzymes

In fleshy fruits, softening is thought to mainly be a result of cell wall disassembly and a major part of the cell wall remodelling involves pectin degradation (Brummel and Harpster, 2001). Recent studies have shown

that fruit species have different degrees of remodelling of the pectins and various other cell wall components, and there are even differences between cultivars of the same fruit species.

1.4.1 Cellulose degrading enzymes

Cellulase enzymes are mixture of various enzymes act synergistically on the plant fibre to degrade cellulose completely (Kuhad and Singh, 2007). Cellulose degrading-enzymes provide a key opportunity for achieving tremendous benefits of biofuel production (Shahzadi et al., 2014). At least three types of hydrologic enzymes (Valjamae, 2002) play roles in the formation of soluble sugars from cellulose (Figure 1.7)

Endoglucanases (EC 3.2.1.4) break the β -1,4-linkages of glucan polymer in small chains and, cellodextrins, cellobiose and glucose are released. These enzymes are efficiently active against carboxymethyl cellulose, but cannot degrade crystalline cellulose.

Exoglucanases (EC 3.2.1.74) break the β -1,4-linkages from the non-reducing end of the cellulose chain releasing a cellobiose unit. Unlike endoglucanases, exoglucanases are very active against crystalline cellulose.

β -glucosidases (EC 3.2.1.21) catalyse the hydrolysis of cellobiose into glucose residues. These enzymes are active against cellobiose, esculin (one of the best known glycosides which can be extracted from the bark of the horse chestnut tree) and salicin (is an alcoholic β -

glucoside which is commonly found bark of populus and poplar species) releasing aromatic residues and glucose (Peng *et al.*, 2014).

The modifications of cellulose fractions during ripening in fruits would be expected to have a dramatic effect on softening. Schematic structure of cellulose with cellulolytic enzymes is shown in (Figure 1.7). However, the cellulose content of the cell wall does not change during ripening (Brummel, 2006). It is important to note that, the exact substrate for cellulase *in vivo* remains to be demonstrated. Hatfield and Nevins (1986) suggested that xyloglucans might be native substrates for cellulases, however purified avocado cellulase could not degrade purified xyloglucans. Tomato fruits have 2 cellulases, *Cel1* and *Cel2*, expressed during fruit ripening. These genes were suppressed individually and simultaneously, however, there was no obvious change in firmness (Lashbrook *et al.*, 1998) (Brummel *et al.*, 1999a). Interestingly, over expression of *Cel2* in the *rin* mutant caused partially softening but not ripening (Flors *et al.*, 2007). Similar results to these in tomato have been observed for strawberry with suppression of *Cel1* in strawberry fruits having no significant effect on fruit firmness (Woolley *et al.*, 2001).

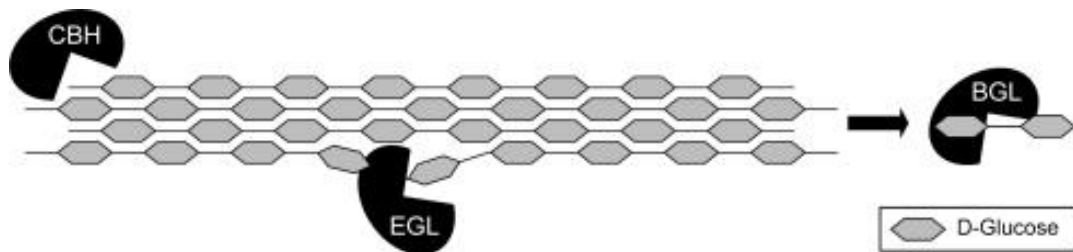


Figure 1.7 Schematic structure of cellulose and its degradation with cellulolytic enzymes. BGL β -glucosidase, CBH cellobiohydrolase, EGL β -1,4-endoglucanase (van den Brink and Vries, 2011).

1.4.2 Hemicellulose Degrading Enzymes

Hemicellulose requires several different enzymes because of its heterogeneous structure. Hemicellulases which belongs to the glycanhydrolases family (EC 3.2.1) are frequently classified according to their action on distinct substrates. The typical hemicellulose is

hydrolysed by a specific set of committed carbohydrate-active enzymes, D-xylan-glucosidase, L-arabinase and a D-mannan enzyme (Vries and Visser, 2001). Schematic structure of hemicelluloses and their enzymes are shown in (Figure1.8).

Xylan is the most abundant hemicellulose (Nowrousian, 2014) and also the most common resources in nature besides cellulose. Therefore, β -D-1,4-endo-exo cutting xylanase has been extensively studied.

β -1,4-endoxylanases belong to Glycoside Hydrolase (GH) family 10 and 11 which differ from each other in substrate specificity. The GH families are being updated thorough years, which contains 101 families in the latest identification (Glycoside Hydrolase family classification, CAZY (<http://www.cazy.org/Glycoside-Hydrolases.html>)). A classification of glycoside hydrolases in families based on amino acid sequence similarities has been proposed a few years ago. Because there is a direct relationship between sequence and folding similarities The GH10 endoxylanases degrade linear chains of 1,4-linked D-xylose residues and, but also xylan backbones with a high degree of substitutions and have a broader substrate specificity then GH11 endoxylanases (Pollet *et al.*, 2010).

Xyloglucanases also referred as xyloglucan-active endoglucanases belong to GH families 12 and 74. These two families of enzymes are active on different residues of hemicellulose, for instance GH74 xyloglucanase cleave at branched glucose residues and prefers

xylogluco-oligosaccharides containing more than six glucose residues; however members of GH12 xyloglucanase cannot cleave at this residue (Desmet *et al.*, 2007).



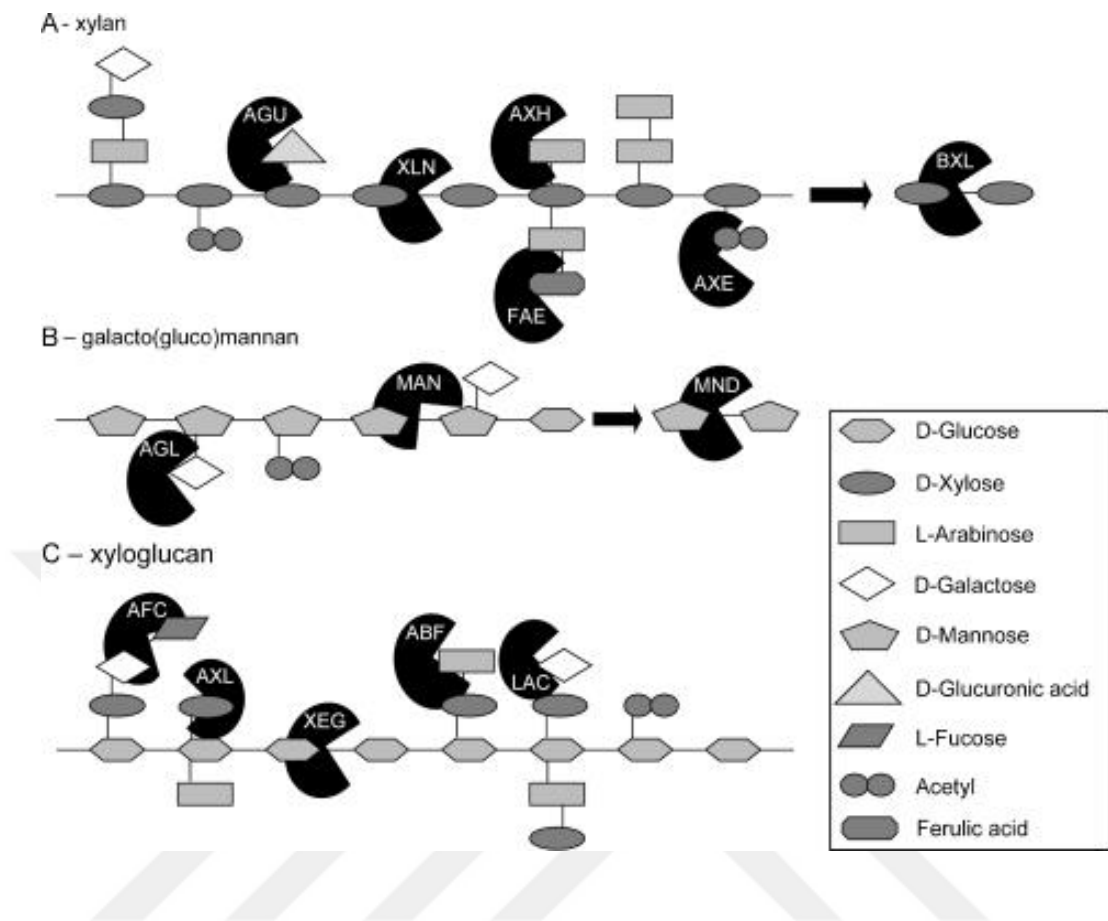


Figure1.8 Schematic structure of three hemicelluloses, xylan, galacto(gluco)mannan, and xyloglucan, with hemicellulolytic enzymes. ABF α -arabinofuranosidase, AFC α -fucosidase, AGL α -1,4-galactosidase, AGU α -glucuronidase, AXE acetyl (xylan) esterase, AXH arabinoxylan α -arabinofuranohydrolase, AXL α -xylosidase, BXL β -1,4-xylosidase, FAE feruloyl esterase, LAC β -1,4-galactosidase, MAN β -1,4-endomannanase, MND β -1,4-mannosidase, XEG xyloglucan-active β -1,4-endoglucanase, XLN β -1,4-endoxylanase

Endomannanases belong to GH families 5 and 26 and have roles in degradation of mannan polysaccharides. The GH5 endomannanases show substrate specificity towards manno-oligosaccharides with more than three D-mannose residues (Do *et al.*, 2009).

The glycans associated with hemicellulose domains undergo depolymerisation during the ripening of tomato. Therefore, these enzymes have been targets for genetic manipulation. Tomato fruit showed ten *XTH* genes during development which were detected at all stages (Miedes and Lorences, 2009). From those, *LeEXGT1* (Asada *et al.*, 1999) and *LeXETB1* (de Silva *et al.*, 1994) suppressions did not have an effect on the texture of ripe fruit.

Expansins Cell wall modifying proteins called expansins are known to cause cell wall loosening. These proteins disrupt non-covalent linkages such as hydrogen bonds at the cellulose-hemicellulose interface, particularly xyloglucan (Martel and Giovannoni, 2007). According to the Tomato Genome Consortium (2012) out of seven expansins isoforms, only *LeExp1* is fruit specific and ripening related, with three expansins precursors (*EXPA3*, *EXPA4*, *EXPA5*) expressed in developing fruit prior to the onset of ripening. Suppression of *LeExp1* mRNA, leads to slightly reduced softening during ripening (Brummel, 2001). However, overexpression of the same gene leads to much more rapid fruit softening and depolymerisation of matrix glucan due to increasing substrate susceptibility to cell wall hydrolases later on in ripening (Brummel *et al.*, 1999b).

1.4.3 Pectin Degrading Enzymes

Polygalacturonase (PG) (EC3.2.1.15) is a major enzyme responsible for pectin degradation in ripe fruits and especially in tomato. PG activity is not present at the mature green tomato fruit stage, but there are

increasing levels of PG messenger RNA and enzyme activity during ripening (Hobson and Grierson, 1993). The *rin* and *Cnr* mutants fail to soften and have low PG activity (Eriksson *et al.*, 2004). However, antisense repression of PG in the ripening tomato fruit (Smith *et al.*, 1988) results in very limited effects on fruit softening (Smith *et al.*, 1990).

Pectin methyl esterase (PME or PE, EC3.1.1.11) catalyses the demethylesterification of cell wall polygalacturonans, generates carboxyl groups and releases free methanol in the cell wall. PME could act to help remodel the cell wall in two ways. Plant PME isoforms act to convert methoxyl groups into carboxyl groups on the polygalacturonic acid chain and releasing both methanol and protons. They do this in (1) a block-wise or (2) linear fashion on homogalacturonan (HG). In the latter case, blocks of free carboxyl groups that could interact with Ca^{2+} to form an 'egg box' structure, and which could also prevent attack from endo-PG or PL are generated. However, a more random removal of methyl groups from GalA may create sites for the subsequent action of depolymerising enzymes such as PL and PG, and hence facilitate wall loosening (Wen *et al.*, 2012).

In tomato, analysis of the genome sequence has revealed 5 cell wall structure related PE genes that are expressed during fruit development and ripening (The Tomato Genome Consortium, 2012 Supplementary Table 12). PE1, encoded by *PMEU1* is ubiquitously expressed in fruit and

other vegetative tissues. The PE2 isoform is fruit specific and represents the dominant isoform that accumulates during fruit ripening. When a PE2 antisense cDNA fragment was transformed into tomato, the fruit showed no major differences in the rate of softening (Hall *et al.*, 1993). Targeting of *PMEU1* by RNAi in tomato resulted in fruits that showed more rapid softening suggesting that the role of this gene product was related to the formation of calcium junction zones to stabilize the remodelled cell wall (Phan *et al.*, 2007).

Galactanase (EC 3.2.1.89) in ripening fruit, studies have shown a 4-5 fold increase in exo-galactanase activity with a corresponding increase in free galactose (Smith *et al.*, 2002). These enzymes probably degrade the galactosyl-rich side chains of RG1. At least seven tomato β -galactosidase (*TBG*) genes (six are known to be expressed during ripening) have been identified to be expressed during fruit development (Smith and Gross, 2000). The expression of *TBG1* was suppressed to 10% of control levels, but no changes in texture or cell wall composition were detected in transgenic fruit (Carey *et al.*, 2001). The function of *TBG3* was also investigated by antisense suppression. However, the resulting ripe fruits were not significantly firmer than controls. Suppression of the *TBG4* gene by antisense RNA produced 40% firmer fruits than controls. Fruits from the antisense line also had the highest wall galactosyl content, demonstrating an involvement of this gene product in cell wall modification and fruit softening in tomato (Smith *et al.*, 2002).

Pectate lyases (PL) (EC 4.2.2.2) randomly cleaves $\beta(1-4)$ linkages between galacturonosyl residues, but it does this not by hydrolysis but by β -elimination generating 4,5-unsaturated oligogalacturonates (Rodriguez *et al.*, 2002) (Figure 1.9).



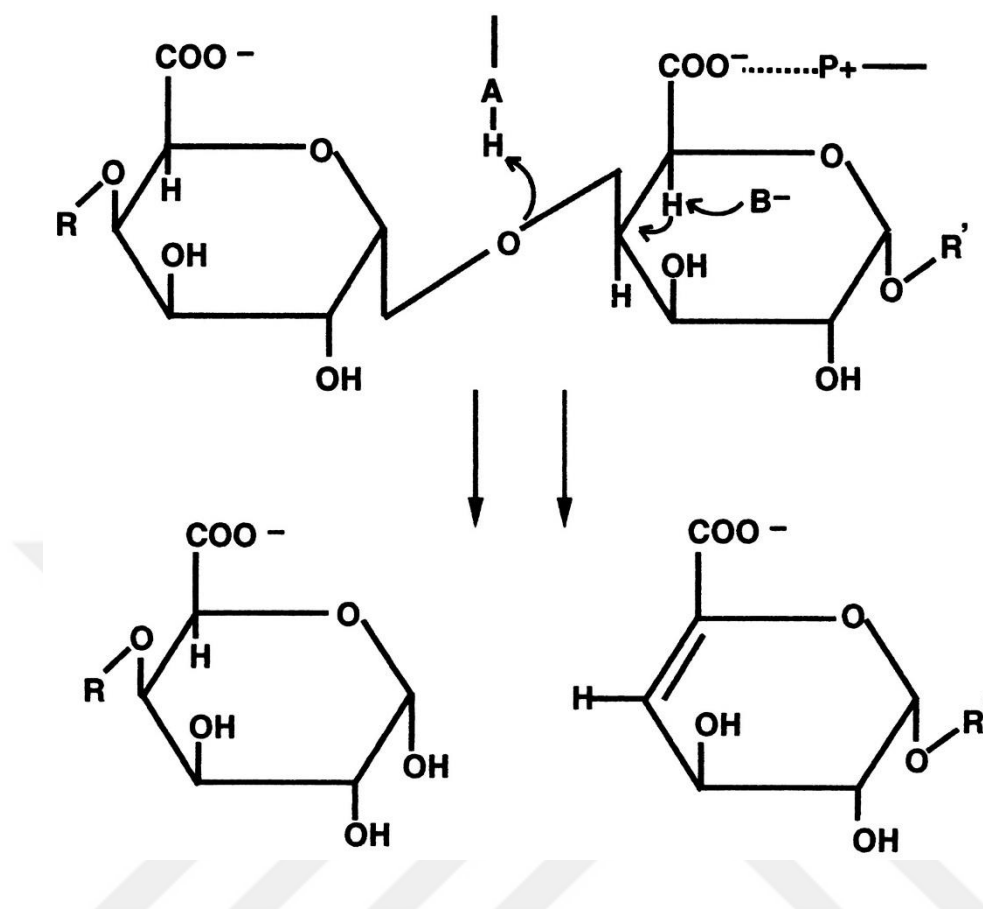


Figure 1.9 Schematic diagram of α -1,4-polygalacturonic acid cleavage by a β -elimination mechanism. The pectate lyases are expected to contribute a minimum of three groups to the catalytic mechanism: P^+ , which neutralizes the charge on the carboxylic acid group; B, a general base that abstracts the proton from C-5; and A, which is involved in the transfer of the final proton to the glycosidic oxygen, leaving a double bond between C-4 and C-5. (Herron *et al.*, 2000)

Most plant tissues can be macerated due to action of these enzymes which are often produced by plant pathogenic bacteria. PL sequences are known to be expressed in a wide variety of plant tissues and organs including in pistils (Kulikauskas and McCormick, 1997), anthers, pollen and developing treachery elements (Wing *et al.*, 1989). Elongating and

differentiating *Zinnia* cell cultures and Japanese cedar pollen allergen has been identified as having PL activity (Domingo *et al.*, 1998). PLs are also expressed in ripening fruits such as the strawberry, banana and grape (Rodriguez *et al.*, 2002). In tomato five PL genes show fruit-related expression (Tomato Genome Consortium, 2012). Attempts to measure the presence of PL activity in tomato have been largely unsuccessful (Besford and Hobson, 1972). However, the tomato EST programme suggests a high level of PL-like gene expression in ripe tomato fruits (<http://www.tigr.org/tdb/lgi/index.html>) and this is supported by RNAseq data from the genome project (Tomato Genome Consortium, 2012).

Current data indicates that a number of gene products including PG, Exp1 and β -gal must be working together to influence fruit texture and other factors must also be involved. For example, transgenic tomatoes with suppressed *LeExp1* and *LePG* simultaneously were significantly firmer than controls throughout whole ripening period and less susceptible to deterioration during long term storage. At the red ripe stage, the transgenic fruits were 20% firmer than control fruits. (Powell *et al.* 2003). However they still softened to a considerable extent, so more gene products than just PG and Exp1 must be involved. It has proved difficult to select the appropriate genes to target from the many that are expressed during ripening simply based on gene expression data as PG is highly expressed but there are no significant effects on texture when PG is silenced in transgenic tomato fruits. One way to test the role of these cell wall enzymes in fruit softening is to look for their

association with fruit firmness QTLs and this is most easily done in tomato where well characterised genetic mapping populations exist.

1.5 Identification of QTL for fruit texture

Identification of genes that control complex traits such as fruit texture has proved difficult using approaches based on expression data alone because of the large numbers of genes involved and therefore the difficulty of selecting the “right” candidates. Tomato quality traits including changes in fruit texture are therefore quantitative, that is the effects are controlled by several genes, which are more or less affected by the environment. Many important traits have been detected in tomatoes by QTL mapping (Lippman *et al.*, 2007). The genes underlying these QTLs can be characterized by genetics without prior knowledge of the protein products that they encode through positional cloning (Frary *et al.*, 2000) often helped by a candidate gene approach based on expression information on genes in the mapping interval. A major advance has been the development of suitable mapping populations, such as introgression lines allows the ‘Mendelisation’ of QTL and permits both the mapping and resolution of traits, and the advent of the tomato genome sequence, both of which allow efficient map-based cloning.

1.5.1 Introgression Lines

The conventional way of generating a QTL mapping population involves genotyping segregating populations based on a cross between two lines, however epistatic interactions in recombinant inbred lines (RILs) can make mapping difficult to fully define and it is difficult to characterize

individual loci. Moreover, the resolution of RILs is often limited to approximately 10-20 cMs, unless the population is very large, which is insufficient to nominate candidate genes under a QTL with sufficient precision because of the large number of genes in the mapping interval. Moreover, traits that have a minor effect on the phenotype may not be detected due to overshadowing effects of major QTLs which cause an incorrect estimation of the number of QTLs responsible for the trait (Tanksley, 1993).

Wild species relatives provide beneficial genetic variation for the breeding of modern cultivars in which natural biodiversity has been lost during domestication. In tomato several inbred lines have been created following the crossing of *S. lycopersicum* with wild relatives from the so-called *esculentum complex* (Steinhauser *et al.*, 2011). These populations include those developed by the Zamir lab (Eshed and Zamir, 1995) a set of 76 introgression lines (ILs) that are nearly isogenic to the *S. lycopersicum* parent produced through a succession of backcrosses (Figure 1.10). Each of these lines carries a single genetically defined chromosome segment of the *S. pennellii* genome, containing an estimated 200 to 1000 genes in a genetic background of *S. lycopersicum*. In other words, the 76 ILs cover the entire *S. pennellii* genome (Eshed and Zamir, 1995). A number of traits of interest from wild relatives are provided by small introgressions while reducing the probability of losing the desirable traits of elite tomato lines (Giovannoni, 2006).



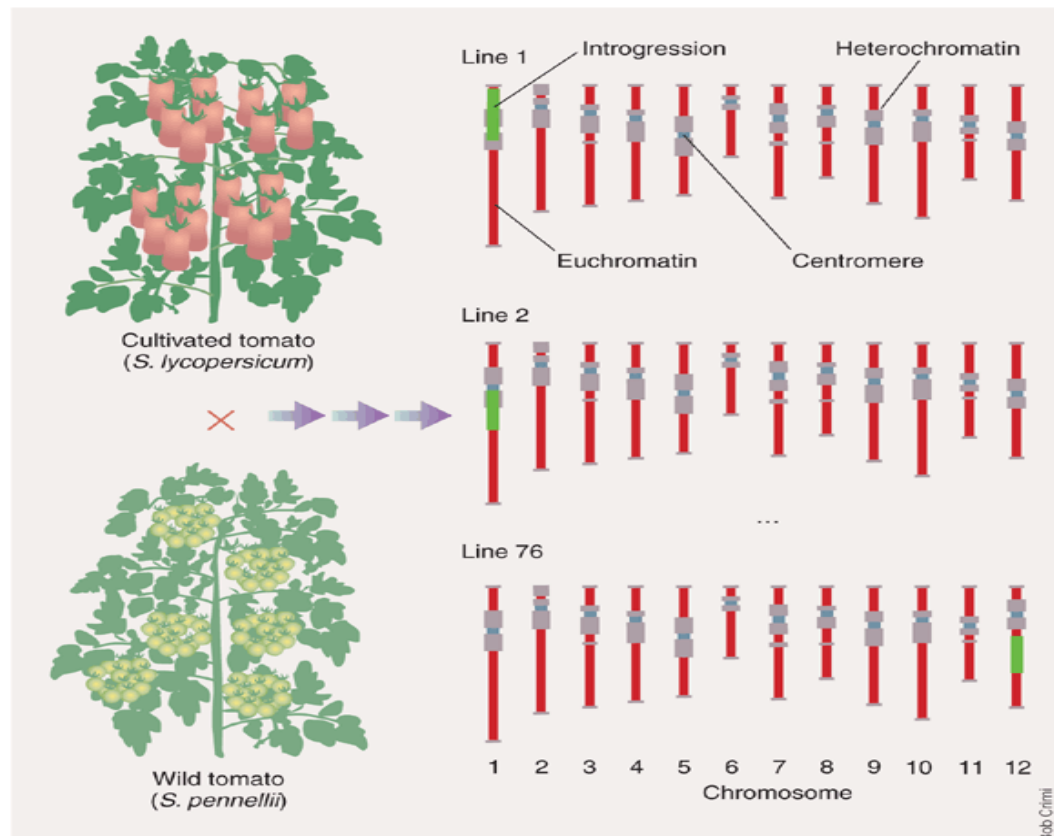


Figure 1.10 Genome introgressions on the tomato chromosomes of the 76 *S.pennellii* ILs, which are nearly isogenic to each other and differ only for the marked introgressed chromosome segments. (Giovannoni, 2006).

The *S.pennellii* IL population has been used for cloning, plant breeding and interspecific QTL identification. Over 2700 QTLs relating to important crop traits have been identified (Lippman *et al.*, 2007). A Major QTL for fruit weight, *fw* 2.2, maps to the same position on chromosome 2. This was the first crop QTL to be cloned using a map-

based approach (Frary *et al.*, 2000). The gene underlying this QTL is involved in the control of cell division and accounts for a major proportion of fruit weight variation in tomato. QTL related to fruit texture have proved more difficult to identify.

Texture is a sensory property and is recognised as a combination of different features, most likely dependent on the anatomical properties of the primary cell wall. Instrumental measurements are generally used to determine firmness related to mechanical properties of tissue (Chaib *et al.*, 2006).

In previous studies, recombinant inbred lines (RILs) from an intraspecific cross between cherry tomato line Cervil with good aroma intensity, and a common tomato line Levovil with classical taste were used to track texture QTL on chromosomes 4 and 9 (Chaib *et al.*, 2006). In addition Fulton and colleagues (2000) using a *S. parviflorum* x *S. lycopersicum* cross identified 12 firmness QTLs across the tomato genome. However the mapping precision was relatively low for all these studies with QTL resolved only to large chromosomal regions and no candidate genes were nominated.

Recent work has led to the discovery of the QTLs for texture that lie on IL2-3 and IL2-4 on chromosome 2. Five distinct regions were located on this firmness QTLs, QTL 2.1 to 2.5 which covers 8.6 Mb region, and an effect on a distal region of IL 2-4 that was non-overlapping with IL 2-3. Each region within this combinatorial locus was mapped to a physical location by using genetic markers. The region contains three

PME genes underlying QTL2.5 and an ethylene response factor (ERF) underlying QTL2.2. These were nominated as QTL candidate genes (Chapman *et al.*, 2012).

Previous work in the Seymour lab using the *S. pennellii* ILs indicated that there was also a strong texture QTL on tomato chromosome 3. The texture QTL on IL 3-4 had a much larger effect on firmness than IL 2-3 and IL 2-4 with fruits being 2 to 3 x firmer than those from M82 cultivated line when red ripe (Poole and Seymour, unpublished). QTLs on both chromosomes have shown indications that they are to some extent independent of environmental influences, as they have been observed in plants grown in the United Kingdom (Table 1.2).

Table 1.2 Survey of pericarp firmness-associated QTLs in the ripe fruit of *S.pennellii* introgression lines ILs harbouring fruit firmness QTLs were grown under field and glasshouse conditions. Maximum load was the force required to penetrate the pericarp tissue at 10 mm min⁻¹.

Firmness Test	Growth Environment, Location, and Date	IL containing Pericarp Firmness-Associated QTLs	<i>P</i>
---------------	--	---	----------

Maximum load, outer pericarp	Glasshouse, United Kingdom, 2006	IL 2-3 IL 3-4	<0.01 <0.001
Maximum load, inner pericarp	Glasshouse, United Kingdom, 2006	IL 2-3 IL 3-4	<0.001 <0.001

The texture QTL on tomato chromosome 3 is an ideal candidate for positional cloning as it has a major effect on fruit firmness and IL3-4 fruit appear to ripen normally although with a much firmer texture.



1.6 Aims and Objectives

The overall aim of present work was to identify the gene underlying the texture QTL on the *S. pennellii* IL 3-4 and test the ability of the candidate gene(s) to modify fruit texture in transgenic plants. The specific objectives were:

1. Resolve the QTL to a 0.5 cM interval on IL3-4

2. Nominate the candidate texture related genes under the QTL and investigate its expression by qRT-PCR.
3. Test the function of the candidate gene(s) in transgenic tomato plants.
4. Investigate the mechanism of action of the candidate gene to better understand the molecular control of fruit firmness during ripening.



Chapter 2

General Materials and Methods



2 General Materials and Methods

2.1 Plant Material and Growing Conditions

Tomato, *Solanum lycopersicum*, cv.M82 recurrent parent and the *Solanum pennellii* LA716 introgression lines (Eshed and Zamir, 1995) were grown under standard glasshouse conditions (16 hours day length, day temperature of 20 °C, and night temperature of 18 °C). Plants were grown in Pro C2 coarse potting compost (Levington) in 7.5 L pots.

Irrigation was supplemented with Vitax 214. Parental lines used throughout the main study were *S.lycopersicum* 'M82' and *S.pennellii* IL3-4 (seeds provided from Dani Zamir, The Hebrew University of Jerusalem). Fruits from each line were tagged at the breaker stage and harvested after 7 days at the red ripe stage.

Phenotypic analysis was carried out on both parental lines, recombinant QTL-NILs and transgenic lines. Fruit mechanical properties were investigated using probe penetration tests on 6 mm equatorial sections. Inner (cells between the vascular boundary and the endodermis), and outer pericarp (below the skin before the boundary) measurements were recorded by using a Lloyd Instrument LF plus machine (Chapman *et al.*, 2012). All tests were carried out using a 1.6 mm flat head cylindrical probe mounted on a 10N load cell (Figure 2.1).

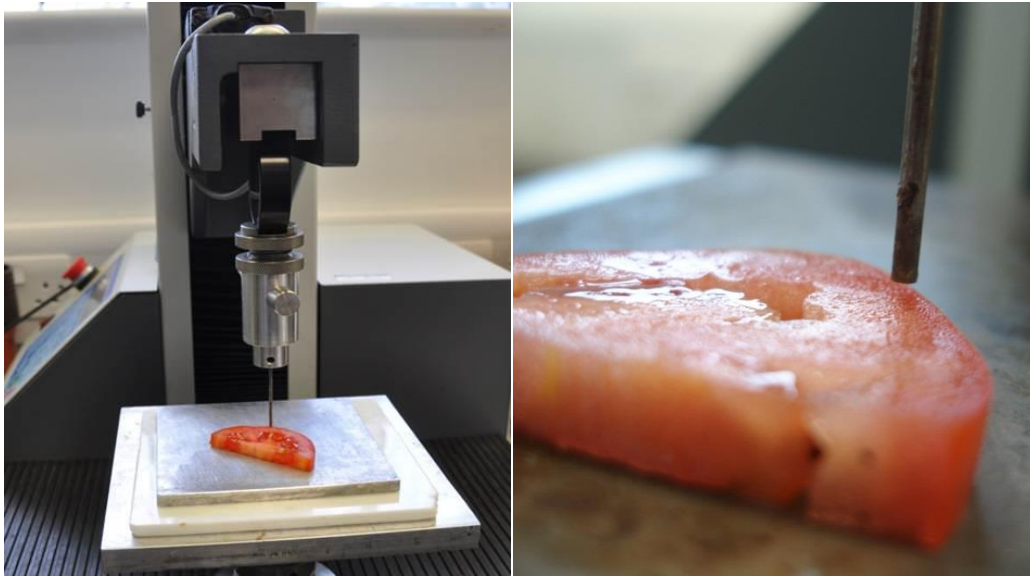


Figure 2.1 A 6-mm transverse section was cut from each fruit, and the maximum load (the force required to penetrate the pericarp tissue at 10 mm min^{-1}) to a 4 mm depth was measured using a Lloyd Instrument LF plus machine equipped with a 10-N load cell and 1.6-mm flat-head cylindrical probe.

2.2 Measurement of Fruit Colour

Texture tests were accompanied by colorimetric data measured by Chroma meter CR-400 taken three different reading for tomato colour. (L^* , a^* , and b^*). These refer to the Hunter L^* , a^* , b^* colour scale

(Figure 2.2). As three reading were taken for L^* , a^* and b^* for each tomato fruit, a mean average was created for each scale. The data was then put through Genstat 10th edition to see whether any differences in quality parameter colour.



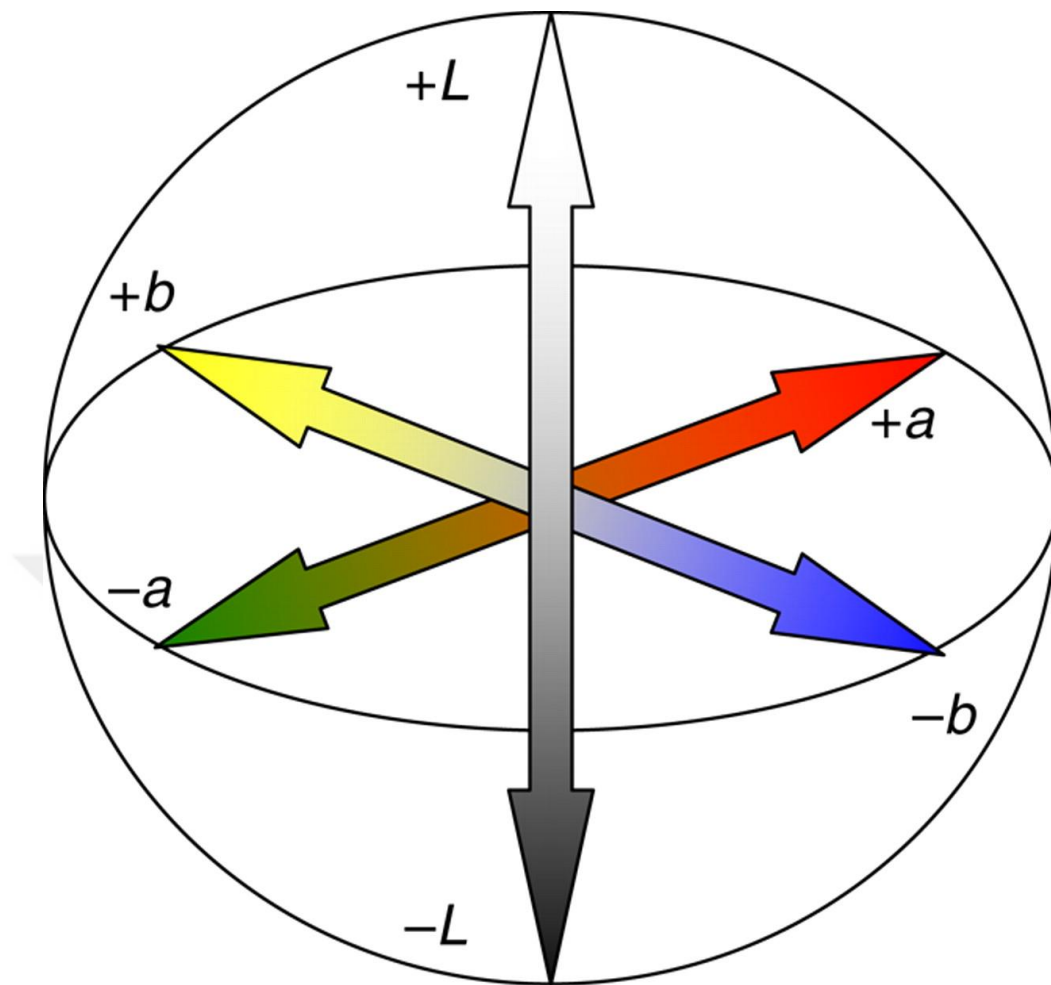


Figure 2.2 A diagram of the Hunter L^* , a^* , b^* colour space. The maximum for L^* is 100, white, and the minimum for L^* is zero, black. The a^* and b^* axes do not have specific limits. Positive a^* is red, negative a^* is green. Positive b^* is yellow and negative b^* is blue. From <http://hunterlab.com/appnotes/>.

After all colour measurements were taken, colour index were calculated by formula.

$$\text{Colour Index (CI)} = \frac{2000a^*}{L^* \sqrt{(a^{*2}+b^{*2})}}$$

Weight of tomatoes was also recorded as another quality parameter.

2.3 Genomic DNA Extraction

Method 1 for small numbers of samples

Tomato DNA extraction was undertaken using the DNeasy Plant Mini Kit (QIAGEN) according to manufacturer's instructions. Samples of fresh leaves (100 mg) were collected and frozen in tubes in liquid nitrogen. The leaves were rigorously ground with a plastic pestle within eppendorf tube. Then 400 μ L of AP1 lysis buffer was pipetted into each tube and then 4 μ L of RNase was added. The tubes were mixed to remove any clumps by using vortex mixer VM20. The tubes were then placed in an incubator (Techne DRI-block DB-2A) for 10 minutes at 65 °C and mixed 3-4 times during incubation by inverting the tube. Then 130 μ L of AP2 precipitation buffer was added to each tube, mixed and incubated on ice for five minutes to precipitate proteins, polysaccharides. The samples were then centrifuged (Eppendorf Centrifuge 5514D) for five minutes at 16168g. The flow-through, which was approximately 450 μ L, was transferred into a new 1.5 mL tube and mixed with 675 μ L of AP3/E neutralization buffer 650 μ L of the mixture was pipetted into a DNeasy Mini Spin Column and centrifuged for one minute at 5919g. The flow-through was discarded and collection tube was reused with remaining sample to repeat this step. The flow-through and collection tube were discarded and DNeasy Mini Spin Column was placed into a new 2 mL collection tube. 500 μ L of AW wash buffer was added to the column and centrifuged for one minute at 5919g. The flow-through was discarded and this step was repeated with centrifugation for two minutes at 16168 g. Finally, the DNeasy Mini Spin Column was transferred to a 2 mL micro-

centrifuge tube and 50 μ L of AE elution buffer was pipetted onto the DNeasy membrane. The tubes were incubated for five minutes at room temperature and then centrifuged for one minute at 5919g. The DNeasy mini spin column was then placed to a new 2 mL centrifuge tube and last step was repeated and a second sample collected. The quantity and quality of DNA was always checked by NanoDrop (Labtech) which measures nucleic acid concentration at 260nm and purity using the 260/280 ratio.

Method 2 for high throughput analysis

In some cases DNA was extracted from fresh leaves by a 'speed DNA extraction method' due to the large number of plant samples that needed to be analysed.

Fresh leaves were collected in 96 well plates and stored on ice during collection. Then a 3mm stainless steel ball was placed into each well. 600 μ L of Speed DNA extraction buffer (Appendix 9.1) was added at room temperature and immediately shaken for 5 minutes in a shaker (Mixer mill, 8000). The plate was centrifuged at 4032 g for 20 minutes (Allegra 25R centrifuge). After this 300 μ L of supernatant was transferred with a long narrow pipette tips to a fresh plate (Thermofisher storage plate) containing 300 μ L of 100 % isopropanol, mixed and incubated for 5 minutes at room temperature. The plate was centrifuged again at 4032 g for 20 minutes. Supernatant was then removed by aspiration from the corner of the well. After aspiration, 100 μ L of 70 % ethanol was added and mixed well by shaking vigorously for 5 minutes.

Then the plate was centrifuged at 4032g for 20 minutes. The supernatant was removed by aspiration and the pellet was dried under desiccation for 15 minutes. Finally, DNA was re-suspended in 0.1 X TE buffer (50 μ L) for 3 hours at 4 °C.

2.4 Primers

Primers were designed using the Primer 3 programme (<http://bioinfo.ut.ee/primer3-0.4.0/>) and oligonucleotides were synthesised by Eurofins MWG Operon (Anzingerstr. 7a, 85560 Ebersberg, Germany). Primers were diluted according to manufacturer's instructions and stored at -20°C. 100 pmol mL⁻¹ of stock concentration primers were diluted to 10 pmol mL⁻¹ by 10X TE buffer. 20 μ L of stock primer and 180 μ L of water were mixed and used as working solutions.

2.5 Polymerase Chain Reaction (PCR)

PCR reactions were performed using a Techne TC-412 PCR machine. Each reaction tube contained a total reaction mixture of 20 μ L composed of 11.5 μ L of water, 2 μ L of 10 x PCR buffer (Fermantes), 0.5 μ L of dNTP 10mM, 0.5 μ L of the forward and reverse primer (MWG), 2.5 μ L of cresol dye (1%) and 0.5 μ L of Taq polymerase (5U/ μ L). Unless otherwise stated, the genomic DNA template used was from a sample of tomato DNA extracted from leaf tissue. The PCR programme that was used, unless otherwise stated was a denaturing step of 95°C for 3 min, 35

cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 1 min, and a final extension step of 10 min at 72°C. PCR products were kept at 4°C.

PCR was used to verify the primers were successful in amplifying the parental M82 and target IL 3-4 DNA and also a heterozygotes sample. As the same protocol was used for both parents a master mix was initially created consisting of 11.5 µL of water, 2µL of 10 x PCR buffer, 0.5µL of dNTP 10mM, 0.5µL of the forward and reverse primer, 2.5 µL of cresol and 0.5µL of Taq polymerase. 18µL of the master mix and 2µL of DNA was placed in a 0.2mL PCR tube.

Following PCR, the product was run on 1% agarose gel, dyed with ethidium bromide by electrophoresis. The gel was then photographed under UV light by using INgenius™ Syngene Bio Imaging system and GeneSnap™ software.

2.6 Purification and Sequencing of PCR Products

Following the manufacturer's instructions, QIAquick PCR Purification Kit (Qiagen). This involved taking 20 µL of the PCR reaction that was then placed in individual 1.5 mL tubes and 70 µL of buffer PB binding buffer was then added. The mixture was mixed by pipetting and then placed in a QIAquick column and centrifuged for 60 seconds at 15682g. The flow through was discarded and 0.75 mL of buffer PE was added to the column. The column was then centrifuged again for 60 seconds at 15682g. The flow through was discarded and centrifuged again for an additional minute. The QIAquick column was then placed in a new 1.5mL

microcentrifuge tube. The DNA was eluted by the addition of 30µL of EB elution buffer into the QIAquick column. The column was left to stand for a few minutes and centrifuged. The 1.5 mL microcentrifuge tube was then placed in a freezer at -20°C.

2.7 RNA Extraction

RNA extraction was carried out according to RiboPure™ RNA Purification Kit (Life Technologies). Frozen pericarp tissue was ground with a coffee grinder in liquid nitrogen. A 100 mg of sample was mixed with 1 mL of trizol reagent (Life Technologies) and vortexed vigorously, and then left for 5min at room temperature (RT). The samples were then centrifuged at 15682 g for 10 minutes at 4 °C. 900 µL of supernatant was transferred into new 1.5 mL of microcentrifuge tube, 200 µL of chloroform was then added and vortexed vigorously. After incubation for 5 minutes at room temperature, samples were centrifuged at 15682 g for 10 minutes at 4 °C. 400 µL of the upper layer was taken into a new tube, and mixed with 200 µL of 100 % of ethanol and transferred into RNeasy Column (RiboPure™ RNA Purification Kit, Life Technologies). The samples were then centrifuged at 15682 g for 30 seconds at room temperature and flow through discarded. The columns washed 2 times by 500 µL of wash solution, centrifuged for 30 second at the same speed. Finally, the RNA is eluted from the column with 50 µL of elution buffer. The RNA amount and purity were determined via a nanodrop™ spectrophotometer (Thermo Scientific) comparing the concentration values obtained at 260 and 280 nm.

2.8 Quantitative RT-PCR

RNA was diluted to give 100 ng/μL using RNAase free water (Promega) and used in the following reaction. Annealing step: 5 μL total RNA, 1 μL random primers (Promega) and 9 μL RNase free water (Promega) were incubated at 70°C for 5 minutes using a Technie TC-412 PCR machine and then transferred directly onto ice. Reverse Transcriptase reaction; 5 μL 5x MMLV Reverse Transcriptase buffer, 1.25 μL dNTPs, 0.5 μL RNase inhibitor, 1 μL MMLV reverse Transcriptase and 2.25 μL RNase free water (Promega) were added to the annealed RNA and incubated for 10 minutes at room temperature, then for 60 minutes at 42°C using a Technie TC-42 PCR machine. 75 μL of dH₂O was then added to the cDNA sample and stored at 20°C until required. 'No RNA' and 'no Reverse Transcriptase' controls were included, with the RNA or reverse transcriptase replaced with RNase free water.

Expression levels of PL from fruit pericarp in lines M82, IL 3-4, and PL transgenic lines were validated using TaqMan quantitative RT-PCR to amplify gene specific products. Primers and dual-labelled fluorescent probes (5'FAM and 3'TAMRA) were designed using Primer3 (<http://frodo.wi.mit.edu/>).

The PCR contained a 5 μL cDNA pool, 7.5 μL of 2X LightCycler480 Probe Master (Roche Applied Science), 10 mM forward primer, 10 mM reverse primer, and 10 mM probe in a final volume of 15 μL. Three biological replicates per cDNA pool were used for PL and an internal standard, elongation factor (EF). Standard curves for each gene were run concurrently. TaqMan quantitative RT-PCR was run on a LightCycler480

System (Roche Applied Science), and PCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 50 s, and 72°C for 1 s, and a final cooling step of 40°C for 10 min. Standard curves were used to calculate relative mRNA concentrations from Crossing Point (the point at which the fluorescent signal is statistically significantly above background to the specific fluorescence threshold) values using absolute quantification with LightCycler480 software release 1.5 (Roche Applied Science) and normalized to the reference gene EF (primer sequences in Appendix 9.2).

2.9 Statistical Analysis

Maximum load data from the 2011 and 2012 recombinant experiments were analysed separately for the inner and outer pericarp using restricted maximum likelihood (REML; Patterson and Thompson, 1971) after log transformation to stabilize the residual variance. All analyses were carried out using the statistical analysis package GenStat (VSN International). The data from both years were analysed jointly. Plant line was taken as the only fixed factor, while year, the number of the truss from which the fruit was harvested, the position in the glasshouse of the plant from which the fruit was harvested, the remaining variability between plants, and the remaining variability between fruit and between the two measurements on each fruit were all taken as random factors. For the analysis of inner pericarp data, the variance component of the

plant-to-plant variability was allowed to vary between years. For the analysis of outer pericarp data, the variance component of the truss was allowed to vary between years. The line means from this analysis were used in QTL mapping.



Chapter 3

Fine Mapping of a Texture QTL on Tomato Chromosome 3

3 Fine Mapping of Fruit Texture QTL on Tomato Chromosome 3

3.1 Introduction

Recent work in the Seymour lab with the Zamir introgression lines had indicated the presence of a major QTL for fruit firmness on chromosome 3, IL3-4 (not published).

Fine mapping of this chromosome 3 QTL was begun prior to the start of this project by backcrossing the *S.pennellii* IL3-4 to the M82 parent. The resulting F₁ generation was allowed to self-pollinate and the F₂ lines were used to resolve more precisely the map position of the texture QTL. This was undertaken by screening 1000 F₂ lines with SSR markers (TG599

and CT243) defining the ends of the IL3-4 introgression (Figure 3.1). Individuals showing recombination events within the introgression were then selected. Recombinant individuals were selfed and homozygous lines selected for texture phenotyping to delineate a small mapping interval for the texture QTL.



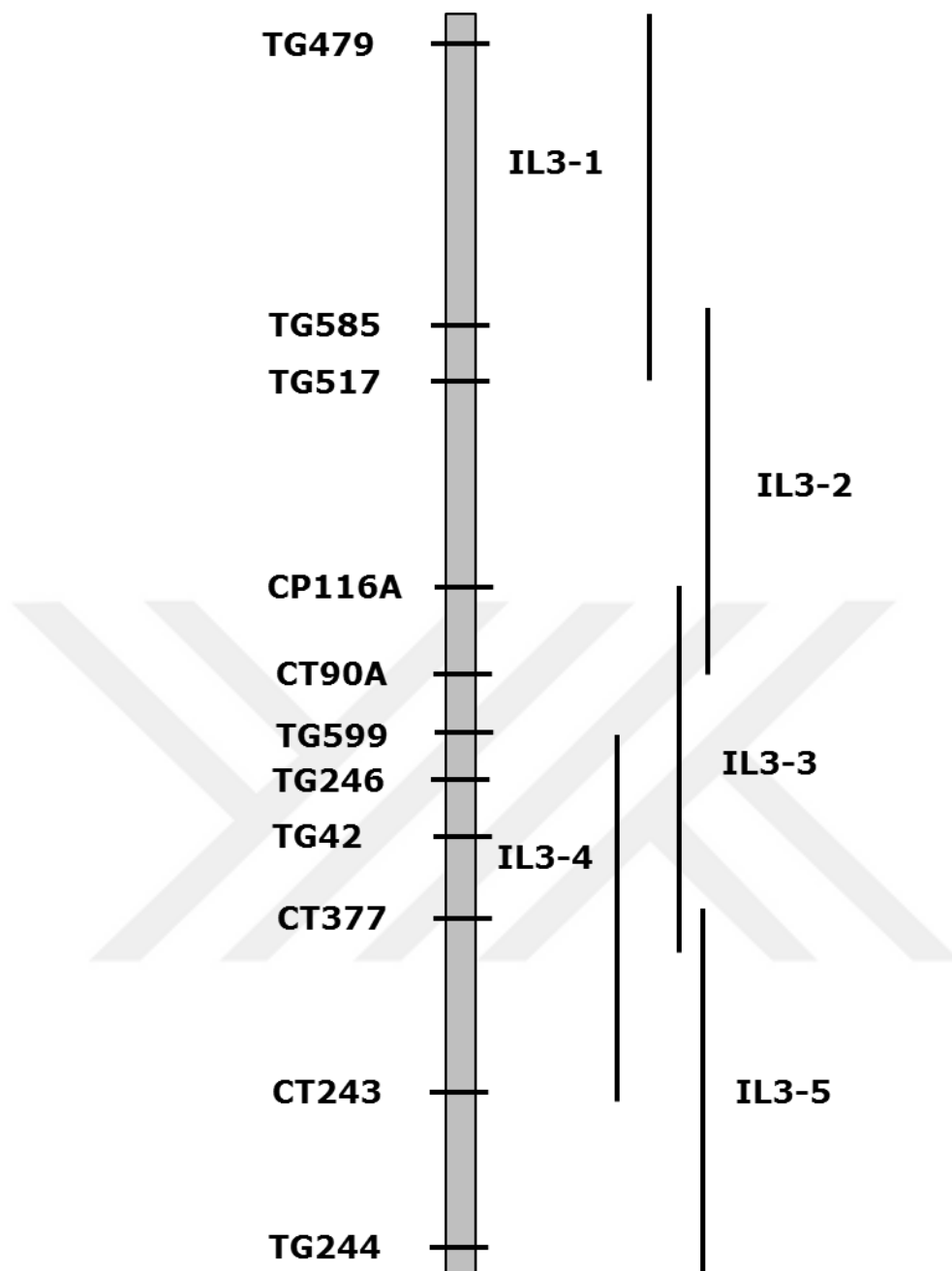


Figure 3.1 Cartoon of chromosome 3 with IL3-4 and markers which are within the IL at each end.

The texture QTL was mapped to a 2.1 Mb interval between the RFLP markers TG599 and TG42. The firmness QTL was associated with normally ripening fruits with respect to colour development (M.Poole, unpublished). This QTL was therefore chosen for further study in the current project. This chapter describes the use of marker information available from the tomato genome sequence and further F₂ population to fine map the chromosome 3 texture QTL.



3.2 Material and Methods

3.2.1 Plant Materials and Development of QTL-NILs

A cross was made between the *S.pennellii* introgression line IL3-4 and M82 and the F₁ progeny were self-pollinated. A total of 2000 F₂ seedlings were then genotyped using markers (TG599 and TG42) flanking the IL 3-4 region to identify recombination events. A total of 96 informative recombinant individuals were identified. Recombinants were grown under standard glasshouse conditions.

QTL-NILs (Quantitative Trait Locus Near Isogenic Lines) were generated from the F₂ recombinant population by selfing of heterozygous individuals and marker based selection of homozygous lines. These QTL-NILs were then used for the fine mapping of the texture QTL. At least four fruits per line were tagged at breaker and harvested seven days later in preparation for the texture tests.

For development of the QTL-NILs, a total of 420 F₃ plants derived from the cross between *S. lycopersicum* and *S.pennellii* near isogenic line IL3-4 were planted.

3.2.2 Phenotypic Analysis

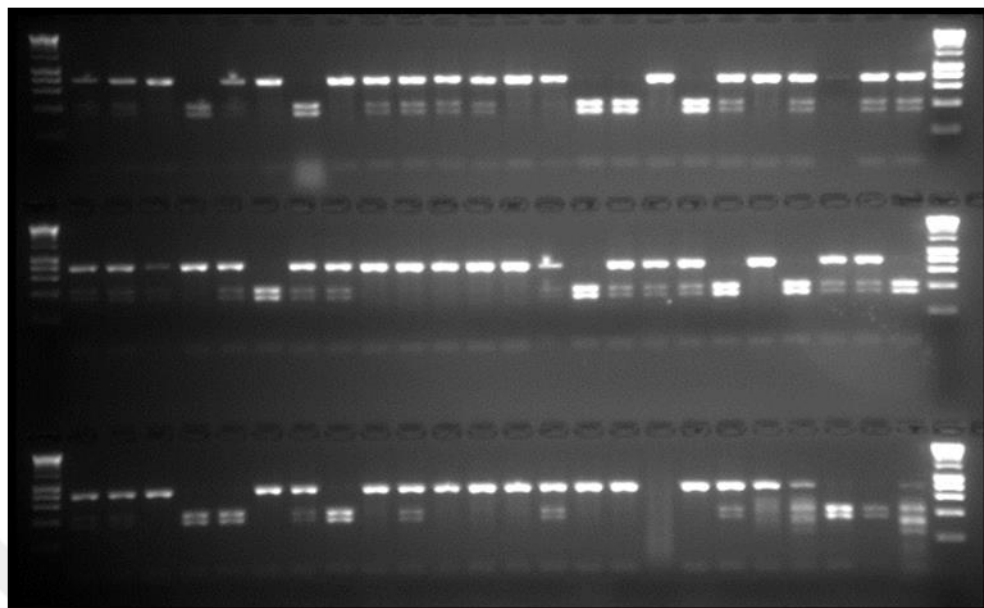
Phenotypic analysis focus on assessment of fruit firmness and colour and was carried out on parental lines, recombinant F₂ and QTL-NILs as described in Chapter 2.

3.2.3 Genotyping of QTL-NIL lines

Plant DNA was extracted from leaf material using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (Chapter 2). Primers were designed to chromosome 3 RFLP marker sequences and bacterial artificial chromosome end sequences obtained from the Sol Genomics Network Website (<http://www.sgn.cornell.edu/>) using primer3 (<http://frodo.wi.mit.edu/>; (Supplemental Table). Markers were tested on parental varieties *S. lycopersicum* 'M82' and *S. pennellii* IL 3-4.

From QTL mapping experiments in the Seymour lab (Uluisik MSc project, unpublished) indicated that the texture QTL was located near the marker TG246. Markers were therefore designed to genomic regions that encompassed this marker and spanned chromosome 3 from position 56029307 (G390) to position 61001222 (G970). Marker intervals were around 50 kb apart (Appendix 9.13). The genome sequence at www.solgenomics.net (v2.40) was used as a basis for these markers with product sizes were between 800-1000 to allow sites for unique restriction enzymes to be identified permitting the development of Cleaved Amplified Polymorphic Sequence (CAPS) markers based on the protocols from (http://solgenomics.net/tools/caps_designer/caps_input.pl). Additional markers, ST3025A, ST1025 and ST1257, were also used from the Syngenta SSR platform. Results with marker G970 are given as an example (Figure 3.2) and others are in Appendix 9.13.

markers 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

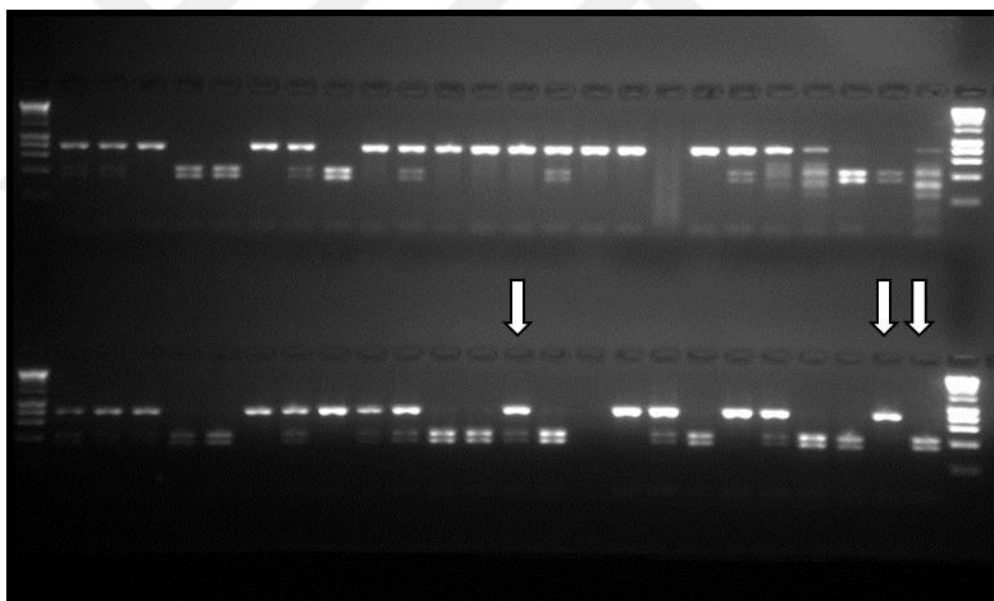


Figure 3.2 The population of informative recombinants identified with markers made to the ends of the IL3-4 introgression was then screened with internal markers, e.g G970 cut by DpnII. The bands from each line were compared to the parental bands. **Sample 13**: Heterozygous, **Sample 23**: M82 (parental line), **Sample 24**: IL3-4 (parental line)

All CAPS markers were tested on the parental DNA. Digestion reactions were prepared as, 5 μ L of PCR product, 12.5 μ L of enzyme buffer, 6.5 μ L of dH₂O, and 1 μ L of digestion enzyme (1,000 units). The reactions were incubated at 37 °C for 2 hours, and the reaction was then deactivated at 65 °C for 20 minutes. 10 μ L of the sample was loaded on a 2% gel and visualised under UV light. Once the CAPS markers were shown to be successful on the parents, they were then used on the whole population.



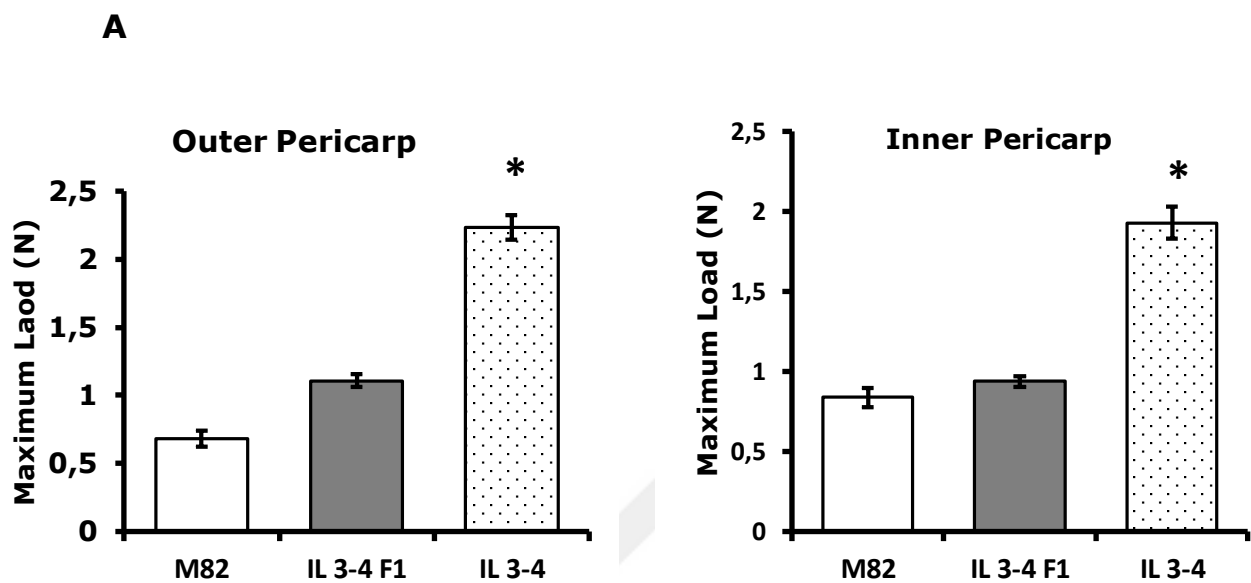
3.3 Results and Discussion

3.3.1 Fine mapping the texture QTL

Measurement of the maximum load on pericarp samples for the red ripe fruits of the parental lines M82 and IL3-4 are shown (Figure 3.3A). The differences between the lines for both outer and inner pericarp was highly significant ($P < 0.001$) in both cases with IL3-4 fruit having much firmer pericarp tissues. There were no significant ($P > 0.05$) differences between the fruits with respect to colour or weight (Figure 3.3B).

Fruit of the IL3-4 line ripened normally with respect to colour and weight. There were no significant differences between M82 and IL3-4 for colour index ($P = 0.492$) and weight ($P = 0.132$). The data indicated that red ripe fruits of IL3-4 were on average 3 times more maximum load than M82. The texture of the F_1 fruits indicates the effect of fruit firmness is additive and two copies of the *S.pennellii* allele will be needed to achieve the maximum increase in fruit firmness.

QTL-NILs were also screened for fruit firmness (Figure 3.4). This screen revealed lines with fruit that were both firmer and softer than the IL3-4 parent. The genotypes of the QTL-NILs are shown in Figure 3.4 and where fruit had a firm texture phenotype this is indicated. The texture data combined with the marker information allowed us to map the firmness effect to a 1.05 Mb interval delineated by markers G390 and G660 (Figure 3.5).

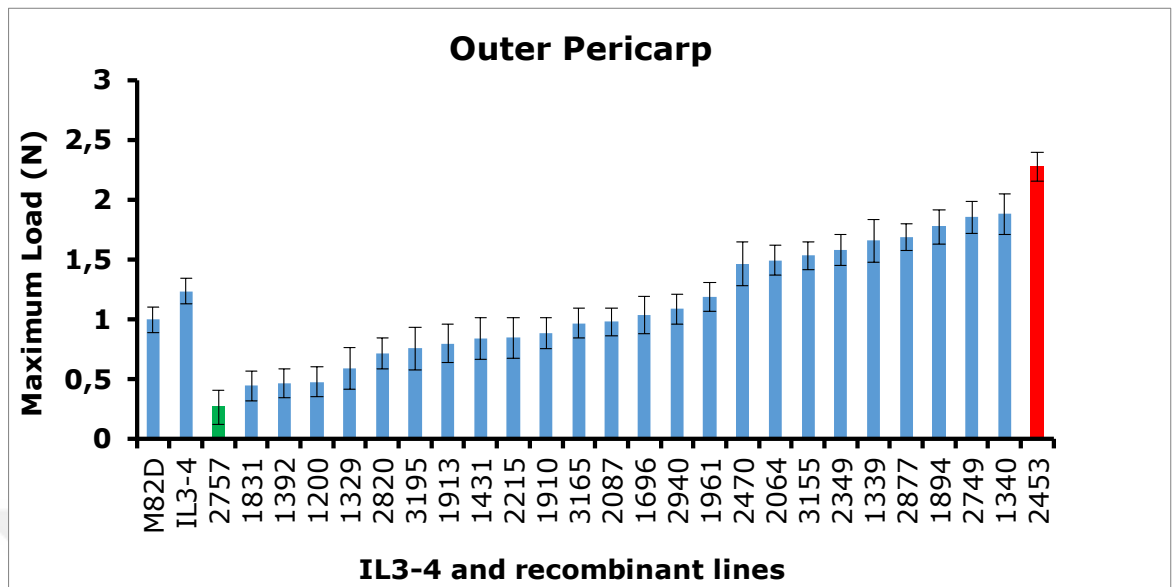


B

Parental Lines	Colour Index	Weight
M82	36.7 (a)	53.9 (b)
IL3-4	34.8 (a)	40.3 (b)

Figure 3.3 (A) Maximum load values for inner and outer pericarp of fruits from parental lines M82 and 3-4 and for the F₁, IL3-4 was significantly * ($P < 0.001$) firmer than M82 and heterozygous F₁. **(B)** (a) colour index, ($p = 0.0492$), and (b) fruit weight ($p = 0.132$) from each parental line were not significantly different SEM, $n = 10$ (biological replicates)

A



B

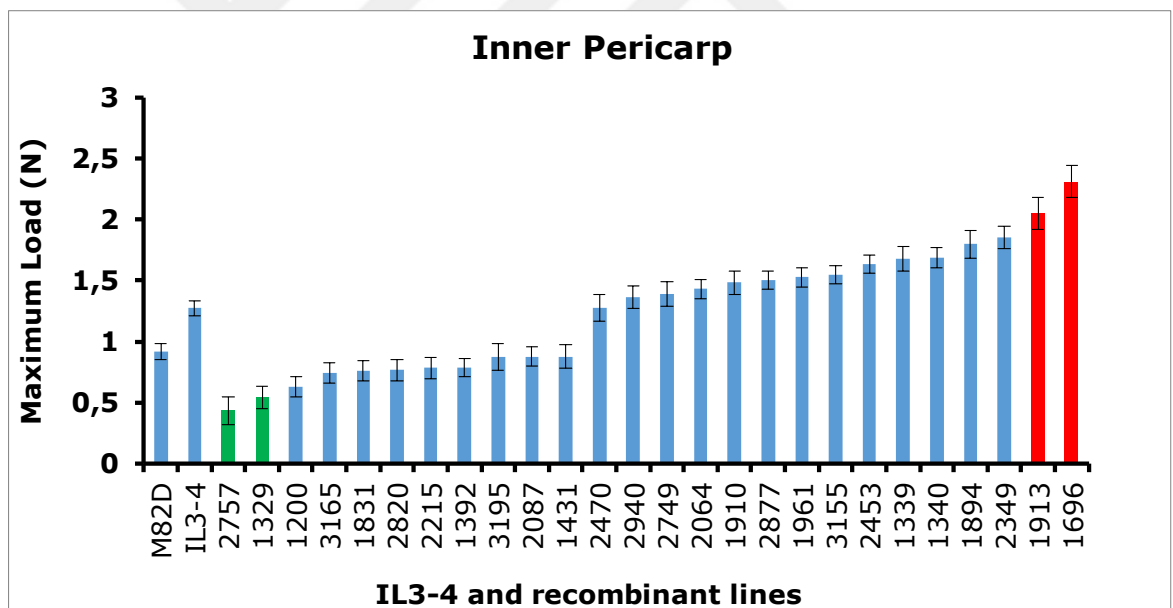


Figure 3.4 Maximum load (N) for pericarp of the recombinant individuals (A) outer and (B) inner pericarp. There was a significant difference ($P < 0.001$) in pericarp texture between M82 and IL3-4. It can also be seen that some recombinant lines were significantly softer (shown in green) than M82 parent and, some of those were significantly firmer than IL3-4 (shown in red) than IL3-4 parent. The difference between M82 and IL3-4 is less compare to figure 3.3A. There reason for this might be different time in a year. SEM, $n=10$

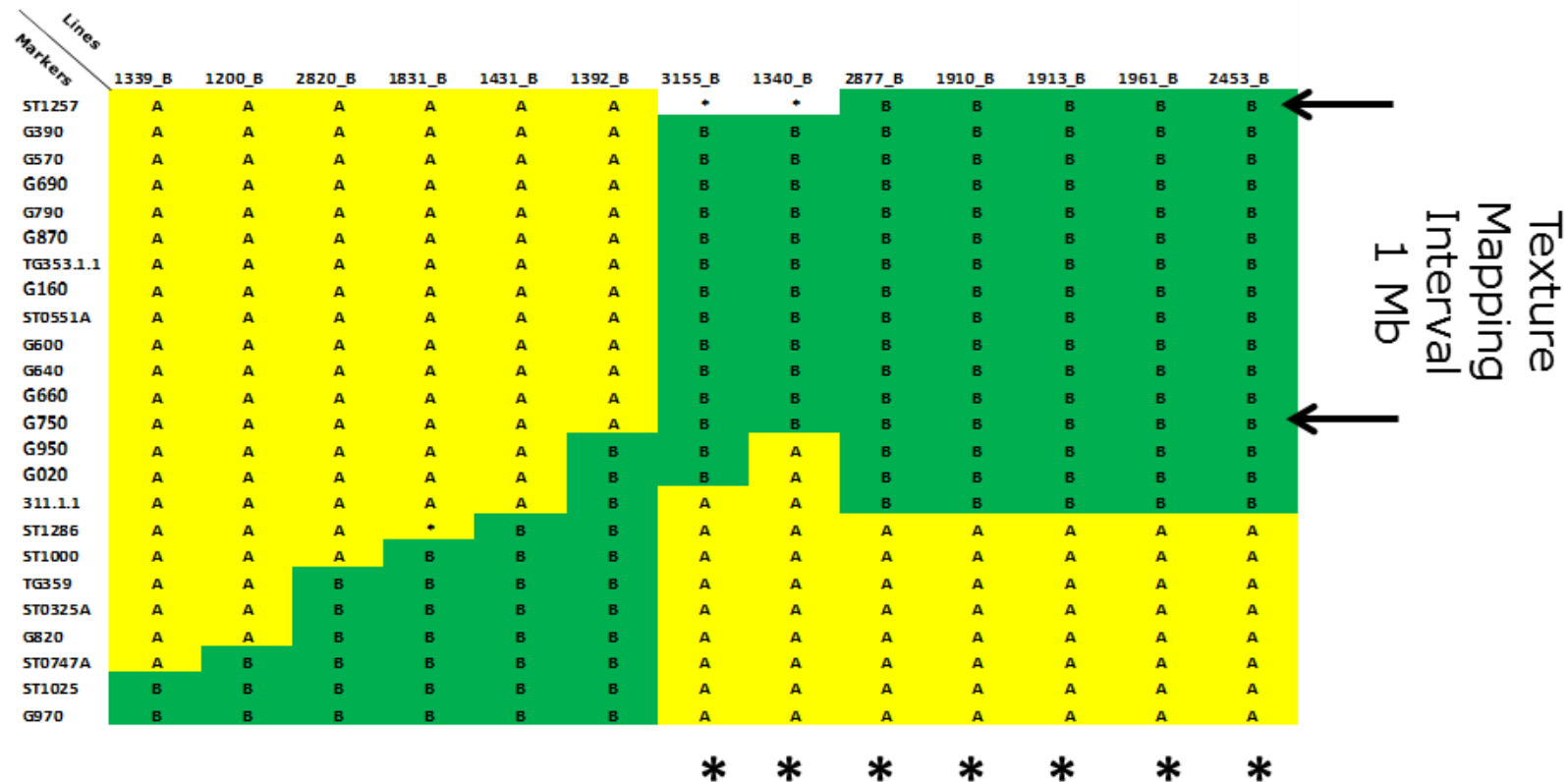


Figure 3.5 Example of genotype and phenotype data map. In total 30 QTL-NILs were genotyped with 16 markers and phenotyped for maximum load on the outer and inner pericarp. The QTL-NILs which were significantly firmer than M82 parent lines indicated by asteriks.

A M82 **B** *S.pennillii*

The mapping interval is relatively large, and contains 129 gene models based on the tomato genome sequence assembly SL2.40 (Appendix 9.12). One or more of these genes represents the QTL candidate. The QTL effect is on fruit softening and therefore a suitable candidate may be a gene involved in cell wall remodelling and it likely is expressed during the ripening process. This information can therefore be used to filter the large number of possible QTL candidate genes. The interval contained only one gene known to be expressed during fruit ripening (Tomato Genome Consortium, 2012; Supplementary Table 12) and that is known to be involved in cell wall degradation. This was a pectate lyase (*Solyc03g111690*) (Figure 3.6).

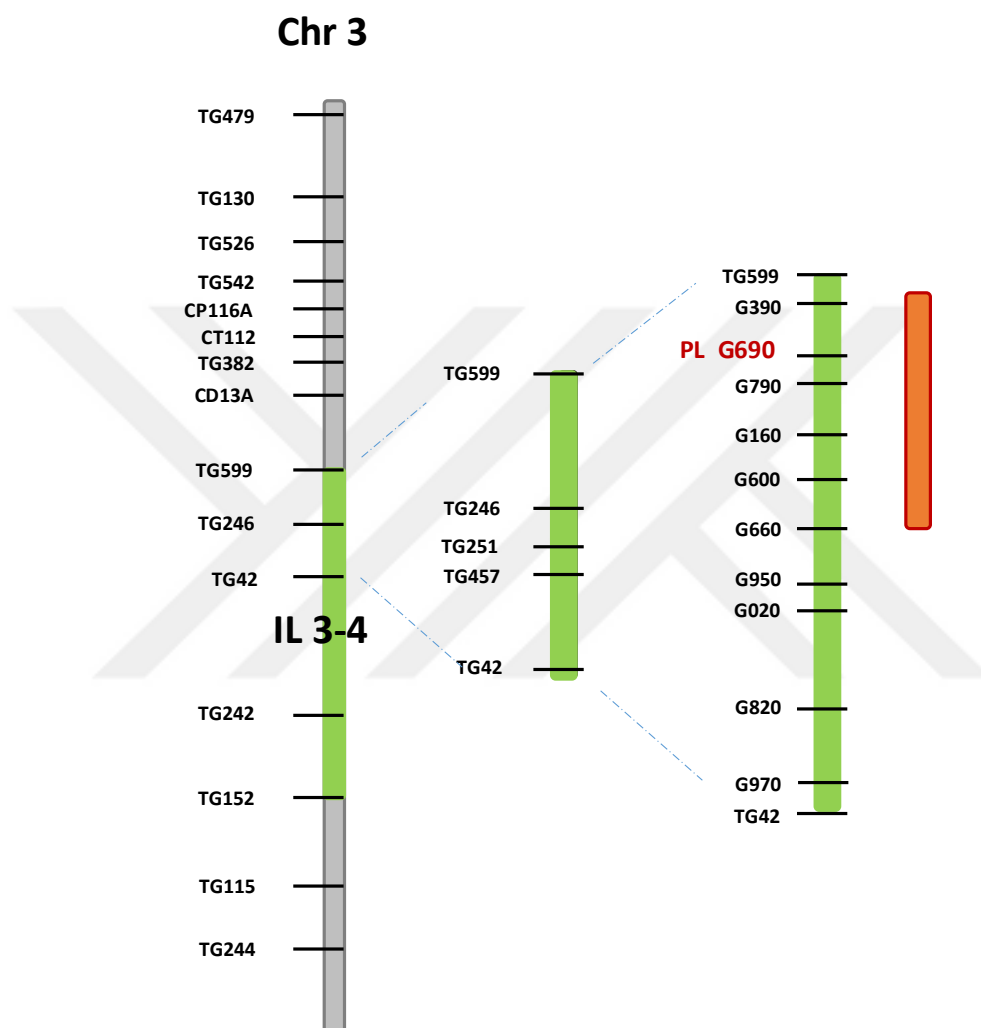


Figure 3.6 Fine mapping of IL3-4 texture QTL to a 1.05 Mb region delineated by markers G390 and G660. 1.05 Mb interval shown in brown. The location of the pectate lyase QTL candidate gene (Soly03g111690) is shown as PL opposite marker G690. The PL gene has also effect on IL3-3, however, IL3-3 is a very difficult line to grow (personal communication).

3.3.2 Investigating the expression of the QTL candidate gene

Prior to this study a tomato GeneChip experiment in the Seymour lab (unpublished) had revealed elevated expression of *Solyc03g111690* in M82 in comparison to IL3-4. In the present study qPCR confirmed these results and demonstrated that, there was a 5X difference in PL expression between the M82 and IL3-4 parents. While there were low levels of PL gene expression in IL3-4 at all developmental stages, in M82 fruits PL expression increased substantially during ripening (Figure 3.7).

The expression of the PL gene *Solyc03g111690* also was observed to be low in non-ripening mutants, RIN, NOR and CNR based on data from a microarray experiment in the Seymour laboratory (Figure 3.8).

The non-ripening mutants *rin*, *nor* and *Cnr* fail to soften or ripen normally and this is associated with abolition of ripening-related gene expression (Seymour *et al.*, 2013). While the absence of PL gene expression in these mutants does not prove that PL is involved in fruit softening the results are entirely consistent with this hypothesis.

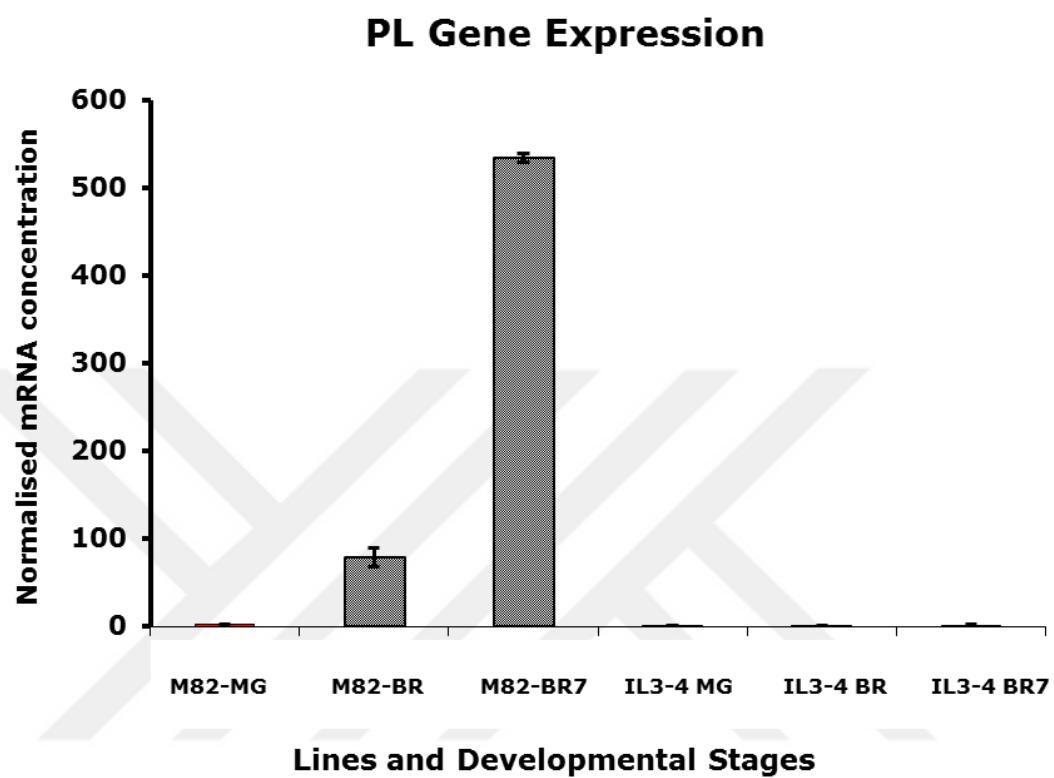


Figure 3.7 *Solyc03g111690.2* PL gene expression in M82 and IL 3-4 at different developmental stages. MG=mature green, BR=breaker, BR7= red ripe. SEM, n=3 (biological and technical replicates)

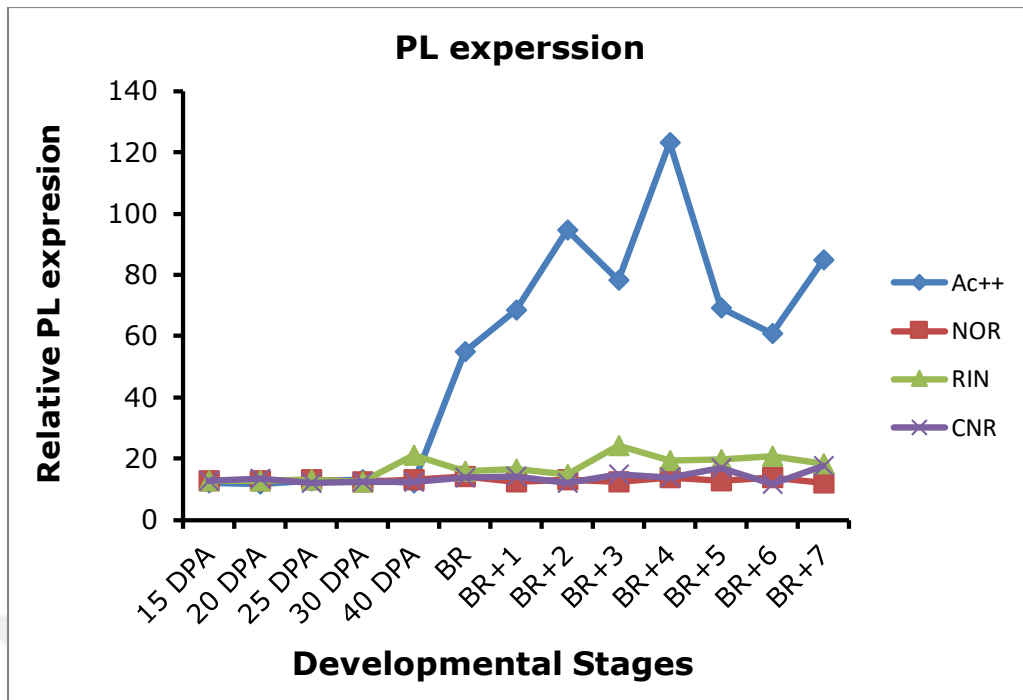


Figure 3.8 Relative expression of *Solyc03g111690* (PL) in different tomato ripening mutants and AC (ESB Data, Syngenta)

3.3.3 The texture QTL candidate gene is a pectate lyase (PL)

The genetic mapping experiments and the expression studies indicate that an excellent candidate for the firmness QTL candidate gene is PL. Cell wall catabolism involves the dismantling of polysaccharide networks by enzymes including those that degrade the pectin and cellulose networks (Sun *et al.*, 2011). Genes encoding a number of pectin degrading enzymes that are known to be expressed in ripening tomato fruits include PG, PME, β -gal and PL (The Tomato Genome Consortium 2012). PG, PE and β -gal have been the focus of many studies. All have been down-regulated in transgenic plants (Smith *et al.*, 1988; Smith *et al.*, 2002) and none of these experiments have resulted in significant changes in fruit firmness from silencing these genes. The activity of PL in tomato has not been investigated in detail.

The failure to investigate the role of PL has probably been due to difficulties in measuring the activity of these types of enzyme in crude plant extracts. Besford and Hobson (1972) were unable to detect PL activity in tomato fruit; high levels of PL gene expression in ripening tomato fruits indicate a role for PL gene products during ripening.

3.4 Conclusion

The fine mapping data indicated that a robust texture QTL was located in a c.1 Mb region of tomato chromosome 3 that also harboured a fruit expressed gene encoding a PL. This PL was lowly expressed in lines containing the *S.pennellii* allele marking it as a good candidate for our texture QTL gene.



Chapter 4

QTL Candidate Gene Validation– Generation of Transgenic PL Lines and Analysis of their Fruit Texture

4 QTL Candidate Gene Validation – Generation of Transgenic PL Lines and Analysis of their Fruit Texture

4.1 Introduction

As it has been mentioned in Chapter 3, the QTL region between markers G390 (619762256 bp) and G660 (63029600 bp) contains 120 gene models and we have nominated a PL, *Solyc03g111690* (Tomato Genome Consortium, 2012) as candidate gene based on function and differential expression between the two parents (Figure 3.7).

Alterations in the cell wall, which requires modifications of various cell wall components including pectin, makes a substantial contribution to plant growth and development. PLs are well known enzymes which are produced by *Erwinia chrysanthemi*, a major causal agent of soft-rot diseases that affect a wide range of plant species (Barras *et al.*, 1994). The action of the PL enzyme not only results in maceration of plant tissues but also can activate plant defence systems (Marin-Rodriguez *et al.*, 2002).

PL-like genes in plants were first reported from pollen (Wing *et al.*, 1989). They have also been found to show elevated expression in cortical cell separation during the emergence of lateral roots (Palusa *et al.*, 2007). Two PL-like genes were also highly expressed in mature tomato flowers, pollen and anthers. Moreover, a PL gene isolated (Medina-Escobar *et al.*, 1997) from ripe strawberry was proposed as a candidate for pectin degradation, contributing to the loss of fruit

firmness. In Jimenez Bermudez (2002) transgenic strawberry plants where a ripening-related PL was silenced using an antisense approach had firmer fruits supporting a role in fruit softening in strawberry.

Marin *et al.*, (2003) measured the activity of PL in banana fruit. Their assay was developed to measure the activity of the endogenous enzyme from banana pulp. Banana tissue showed a significant increase in calcium dependent PL activity during ripening. In banana, the climacteric peak corresponds with eating ripeness and the coincidence of PL activity peak with the climacteric peak is consistent with a role for PL in banana fruit softening. In contrast to PL, PG did not attain maximum values at the climacteric peak, but continued to increase in post-climacteric fruits, when bananas had attained full softening (Payasi and Sangwal, 2003).

PL is a good candidate for firmness QTL gene as it is the only known cell wall remodelling gene in the mapping region of QTL that is expressed in ripening tomato fruits. In tomato there are five PLs expressed in fruits, however the gene *Solyc03g111690* is the only one that is highly expressed in ripening stage (Reads Per Kilobase of transcript per Million, RPKM=1930.12) (Tomato Genome Consortium, 2012). This gene is also expressed highly in the fruits of the cultivar Ailsa Craig which is easily transformed where the fruits soften rapidly. It was decided to down-regulate the PL in this variety using RNAi to validate the function of the QTL candidate gene.

4.2 Materials and Methods

4.2.1 Gateway Cloning

The sequence of the PL (*Solyc03g111690*) candidate gene (Appendix 9.11.2) was obtained using the tomato genome browser at www.solgenomics.net. A 300 bp gene specific region of PL (Figure 4.2) was amplified from the 5' end of the sequence using the following forward and reverse primers with additional *att*B1 and *att*B2 sites attached to facilitate subsequent Gateway cloning (Figure 4.2).

Forward,

GGGGACAAGTTTGTACAAAAAAGCAGGCTACGCGTTTAATTGGACGTAG

Reverse

GGGGACCACTTTGTACAAGAAAGCTGGGTAAACAGAGGAAGTGCCCATG

with ***att*** sites in bold.

4.2.2 Amplification of PL PCR product and Gateway Cloning

The PL gene fragment was amplified using the following PCR conditions and the primers specified above. The programme involved a denaturing step of 95°C for 3 min, 30 cycles of 98°C for 10 seconds, 62°C for 30 seconds, and 72°C for 90 seconds, and a final extension step of 10 min at 72°C. The PCR product was purified from the gel according to QIAquick Gel Extraction Kit (Qiagen). Gel slice was weighed and 3 volumes of Buffer QG were added to 1 volume of gel. The mixture was incubated at 50 °C for 10 minutes to completely dissolve the gel. After the gel slice had dissolved, 1 gel volume of isopropanol was added and the solution thoroughly mixed. The sample was then placed to QIAquick spin column and centrifuged at 16168g

for 1 minute. The flow-through was discarded and 500 μL of Buffer QG was added to the same column. After 1 minute centrifugation at 16168g, 750 μL of Washing Buffer PE was added to wash the QIAquick which was then centrifuged at 16168g for 1 minute. In order to elute the DNA, the QIAquick column is placed to 1.5 mL microcentrifuge tube and 30 μL of Elution Buffer EB was added and the column was left incubate at room temperature for 3 minutes. It was then centrifuged at 16168g for 1 minute. The concentration of DNA was determined spectrophotometrically at 260-280 nm using a NanoDrop (LabTech). DNA at a concentration of 12.5 ng/mL, was used for the subsequent Gateway BP reaction.

BP Clonase Reaction This reaction was performed according to BP clonase mix kit protocol (Invitrogen) In a 1.5 mL tube; the following components were combined at room temperature.

The BP Clonase™ enzyme mix was thawed on ice for about 2 minutes and vortexed briefly. Then 1 μL of BP Clonase™ enzyme mix was added to reaction tube and mixed well by vortexing. The reaction tube was microcentrifuged briefly at 16168g. The reaction was then incubated at room temperature overnight and inactivated by the addition of 1 μL of Proteinase K, and incubation at 37 °C for 10 minutes. Resulting vectors were used to transform *E.coli* and plated on LB agar containing 100 mg/mL kanamycin 37 °C.

Cloning the entry vector in *E. coli* One Shot® TOP10 Chemically Competent *E.coli* cells were thawed and kept on ice for 2 minutes. Then 3 µL of BP reaction product was added to the cells. The sample was then incubated on ice for 30 minutes, and heat shocked 45 seconds at 42 °C and 2 minutes on ice, respectively. 200 µL of SOC media was then added to the sample and the cells placed in a shaking incubator at 37 °C for 1 hour. The transformed cells were then spread on LB agar plates (50 µg /mL kanamycin) and the plates incubated at 37 °C overnight. Next day, single colonies were picked and inoculated into LR media (2% (w/v) Bacto Tryptone, 0.5% (w/v) yeast extract, 0.4% (w/v) MgSO₄, 10 mM KCl, pH 7.5 with KOH), and incubated overnight in a shaking incubator (250 rpm, 37°C).

Plasmid Prep Purification Bacterial cell cultures were harvested by centrifugation at 5939g for 2 minutes. Plasmids were purified according to GeneJET Plasmid Miniprep Kit (Fermentas) protocol. All the supernatant was removed from the pellet which was resuspended by adding 250 µL of the resuspension solution. The culture was completely resuspended by vortexing and transferred to a microcentrifuge tube. 250 µL of lysis solution was then added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous. After that, neutralization solution was added and mixed immediately by inverting the 4-6 times. After 5 minutes centrifugation at 16168g, the supernatant was transferred to the GeneJET spin column. The samples was centrifuged for 1 minute at 16168g and transferred back to the column. 500 µL of the wash solution was added and centrifuged for 1

minute and this step was repeated. Finally, the GeneJET spin column was placed in a fresh 1.5 mL microcentrifuge tube, 30 μ L of the elution buffer was added and incubated 5 minutes. The sample was centrifuged at 16168g for 2 minutes and the last step was repeated. DNA concentration of the entry clone was determined spectrophotometrically by NanoDrop nucleic acid concentration at 260nm and purity using the 260/280 ratio. After DNA was isolated from positive transformants, it was sent for sequencing to Eurofins MWG/ Operon with M13 (-20 bp) primer to verify the correct orientation of the gene between *attL1* and *attL2* sites.

LR Clonase Reaction Entry vectors were then recombined into hairpin RNAi expression vector pK7GWIWG2(1) (Figure 4.1) (Karimi *et al.*, 2007) by LR reaction according to manufacturer's instructions (Invitrogen) to generate expression vector. In a 1.5 mL tube, 2 μ L of entry clone and 1 μ L of destination vector were added at room temperature.

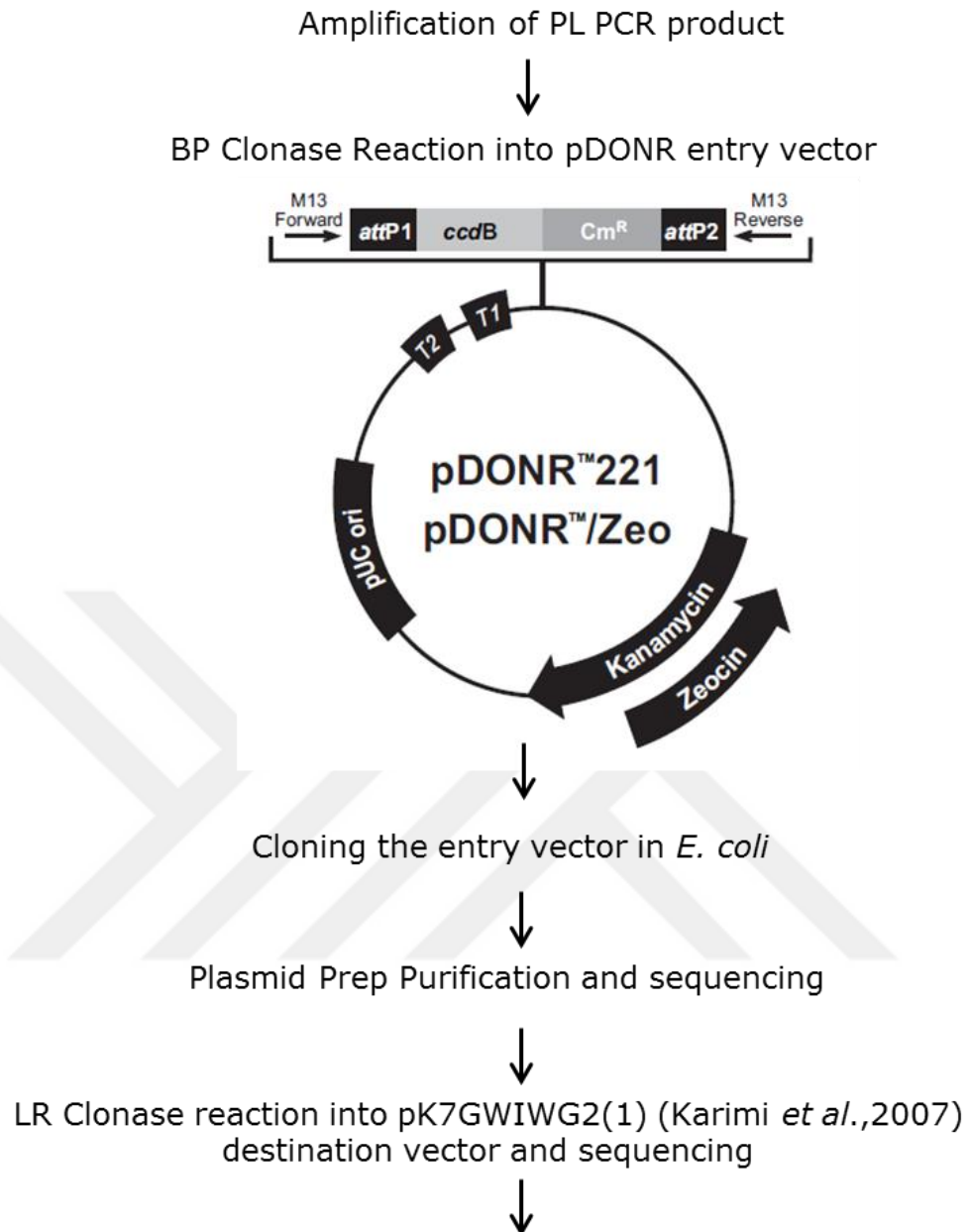
Next, TE buffer (pH 8.0) was added up to a final volume 8 μ L. The LR Clonase™ enzyme mix was thawed on ice for about 2 min and vortexed briefly twice, for 2 seconds each time. Then 2 μ L of LR Clonase™ enzyme mix was added to each reaction tube and mixed well by vortexing briefly twice. The reaction tube was microcentrifuged briefly at 16168g. The reaction was incubated at room temperature overnight and then 1 μ L of Proteinase K solution was added to each sample vortexing briefly and incubating at 37 °C for 10 minutes to terminate the reaction. Resulting

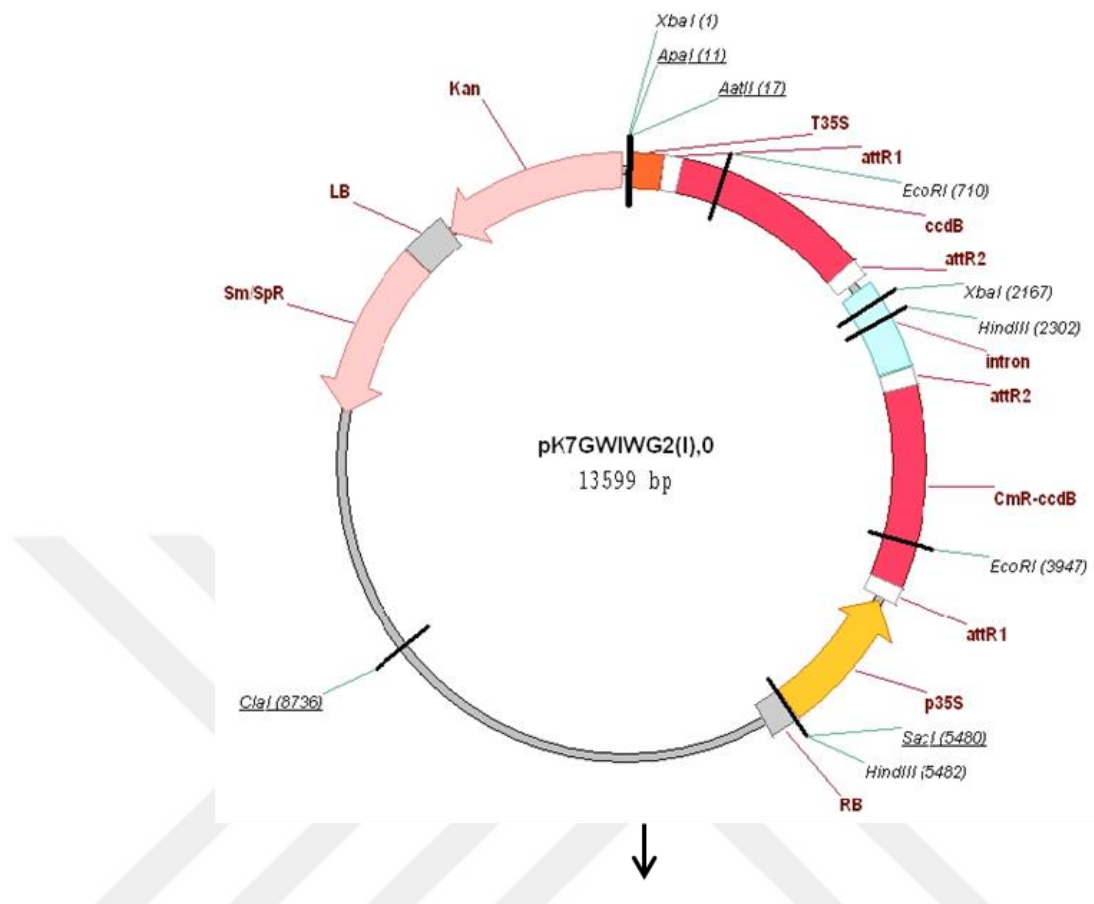
vectors were used to transform *E.coli* and plated on LB-Spectinomycin agar (50 µg/ml).

Transformation of the expression clone into *One Shot*[®] *Mach1*[™]-*T1R* *Chemically Competent E. coli* cells was as described in section (4.2.4).

PCR confirmation of destination vector the presence of the transgene in the destination vector was confirmed by PCR using primer combinations. 35S Terminator Forward GCAGGTCAGTGGATTTTGGTT-TTAGGAA with reverse primer of PL gene, and 35S Promoter Reverse CCCACTATCCTTCGCAAGACCCTTCCTC with reverse primer of PL gene. 10 µL of Megamix Blue PCR master Mix (Microzone), 0.5 µL forward/reverse primers, 2 µL of DNA (vector) and 7 µL of water was mixed, and PCR reaction (section 2) was used (Figure 4.3).

To confirm that the gene of interest had been inserted into the vector, the PCR product of 35S:PL that had been inserted into pK7GWIWG2 (1) (35S) was cut by *Hind*III. To do this, 14 µL of H₂O, 2 µL of 10X Buffer, 3 µL of Expression clone and 1 µL of *Hind*III were added and incubated at 37 °C for overnight. Reaction was deactivated at 65 °C for 20 minutes (Figure 4.4). The destination vector was then sent to the plant transformation service at Davis, University of California for generation of transgenic lines by agrobacterium infection (The Ralph M. Parsons Foundation, Plant Transformation Facility). The procedure is explained (Appendix 9.4).





The Ralph M. Parsons Foundation, Plant Transformation Facility

Figure 4.1 Flowchart describing steps involved in cloning of the PL gene fragment by Gateway cloning

4.2.3 Identification of transgenic lines

To determine the presence or absence of a T-DNA insertion in the transgenic plant lines, a set of PCR reactions was performed by using the PL gene specific reverse primer and the T-DNA 35S Terminator Forward (35S(F))(Appendix 9.2). In addition, DNA from wild-type AC⁺⁺ was used in the same way, as a positive control for the gene specific primer pair and as a negative control for T-DNA insertion (Figure 4.4). Sequence of transgene was also confirmed by sequencing (Appendix 9.11.4).

Water	7 µL
Forward Primer	0.5 µL (35 FW)
Reverse Primer	0.5 µL (2G111690.2R)
DNA	2 µL (20ng/ µL)

The conditions for the PCR were, denaturing step of 95°C for 3 min, 35 cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 1 min, and a final extension step of 10 min at 72°C.

4.2.4 Identification of single copy homozygous lines

The T₀ transformants obtained from the Davis Plant Transformation Centre in the USA were grown to fruiting and T₁ seeds were collected. Twelve seeds were sown per line. Plant DNA was extracted from leaf material using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (Section 2.2). Primers PDS and PL were amplified. The qPCR reaction contained,

2X Taq master mix	24 µL
Forward primer (35S-PL)	1.44 µL
Reverse Primer (35S-PL)	1.44 µL
PL probe	0.96 µL
H ₂ O	4.16 µL
DNA	(20 ng) 5 µL

Three replicates per DNA pool were used for PL and a known single copy phytoene desaturase (PDS) gene. Standard curves for each gene were run concurrently. TaqMan qPCR was run on a LightCycler480 System (Roche Applied Science), and PCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 50 s, and 72°C for 1 s, and a final cooling step of 40°C for 10 min. Standard curves were used to quantify DNA amplifications from crossing point values using absolute quantification with LightCycler480 software release 1.5 (Roche Applied Science) and normalized to the reference gene PDS (primer sequences in Appendix 9.2)(qPCR results were given in Appendix 9.5). The qPCR allowed us to

select a range of single copy homozygous and azygous lines for further study.

4.2.5 qPCR determination of PL gene expression

Quantification of PL gene expression in the PL knock out, azygous (internal control) and wild type Ailsa Craig (AC) lines was undertaken in validated using TaqMan quantitative RT-PCR (Section 2). Primers and dual-labeled fluorescent probes (5'FAM and 3'TAMRA) were designed using Primer3 (<http://frodo.wi.mit.edu/>).

PL (forward)	ACGCGTTTAATTGGACGTATG
PL (reverse)	AACAGAGGAAGTGCCCATG
PL (probe)	TTGAGTTTGAGTGCAAGGCCGTC

The PCR conditions were as mentioned in Chapter 2.

4.2.6 Determination of PL enzyme activity

20 grams of fresh breaker+7 tomato pericarp was homogenised with cold 80 % of acetone. The sample was washed with %100 acetone until colourless and left overnight to dry in air at room temperature. Then 40 mg of the acetone insoluble material was weighed and stirred for 2 hours in 1.9 mL of 50mM Tris-HCL, pH 8) at room temperature. The samples were then centrifuged for 30 minutes at 16168 g, and the absorbance of clear supernatant was measured at 235 nm (Figure 4.8). Enzyme activity was expressed as the difference in OD at 235nm after 30 minutes / mg cell material. Boiled samples were used as a control.

4.2.7 Analysis fruit texture and shelf life

The texture of transgenic fruits was determined by measuring the maximum load needed for a probe to penetrate the pericarp tissues according to methods described in Chapter 2. In order to determine the effect of antisense PL on shelf life, fruits which were collected at breaker+7 stages were left at 20°C for up to 4 weeks. Fruits were then visually inspected for integrity and the presence of mould.



4.3 Results and Discussion

The presence of the correct PL fragment in the plant transformation vector was validated at various stages during the cloning procedure. In (Figure 4.2) the 300 bp gene specific fragment is shown by an arrow. This fragment was used for Gateway cloning. The final step involved cloning the PL construct into the destination vector and this was verified by PCR amplification of a 300 kb fragment (Figure 4.3)(sequencing of clones and vectors in appendix 9.11). The destination vector was then sent to the plant transformation service at Davis, University of California for generation of transgenic lines by agrobacterium infection (The Ralph M. Parsons Foundation, Plant Transformation Facility). The procedure is explained in Appendix 9.4.

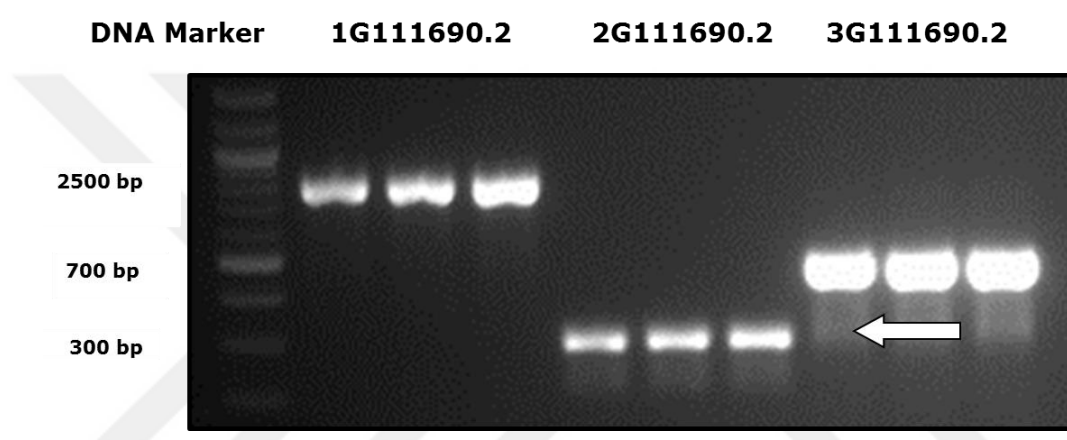


Figure 4.2 The gene specific PL PCR product (2G111690.2) showed by an arrow was chosen to be used for Gateway Cloning due to its appropriate size (300bp). Other PL gene products 1G111690.2 and 3G111690.2 were amplified from the same region with different product sizes (2500bp and 700bp). These primer products were not used for further experiments due to their big sizes for Gateway cloning.

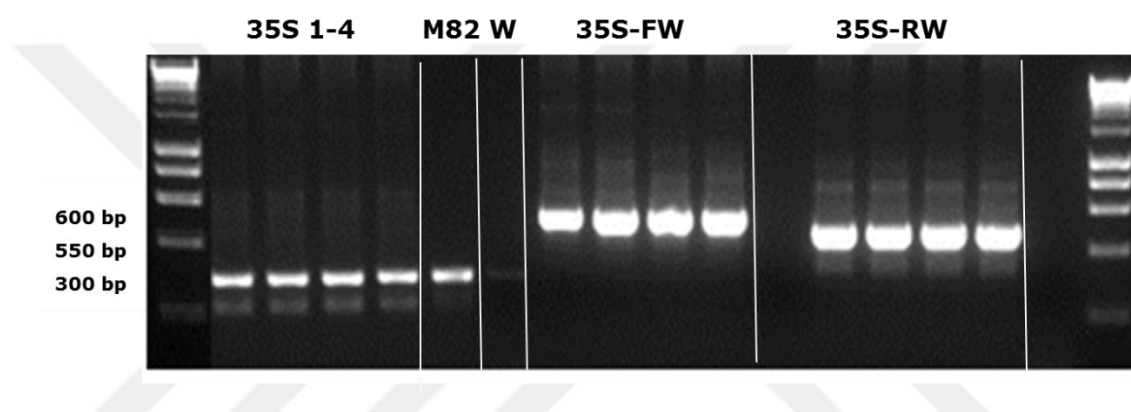


Figure 4.3 Cloning of PL gene fragment in to Gateway vector pK7GWIWG2(1), was validated samples prepared from four individual colonies were checked with each set of primers. 35S1-4 were amplified by 2G111690.2F/R. 35S-FW was amplified by 35T-FW and 2G111690.2R, 35S-RW was amplified by 35T-RW and 2G111690.2R. These regions are *attR* regions of the vector 35S= pK7GWIWG2(1), W= Water. (gene sequences is checked, given in appendix 9.11)

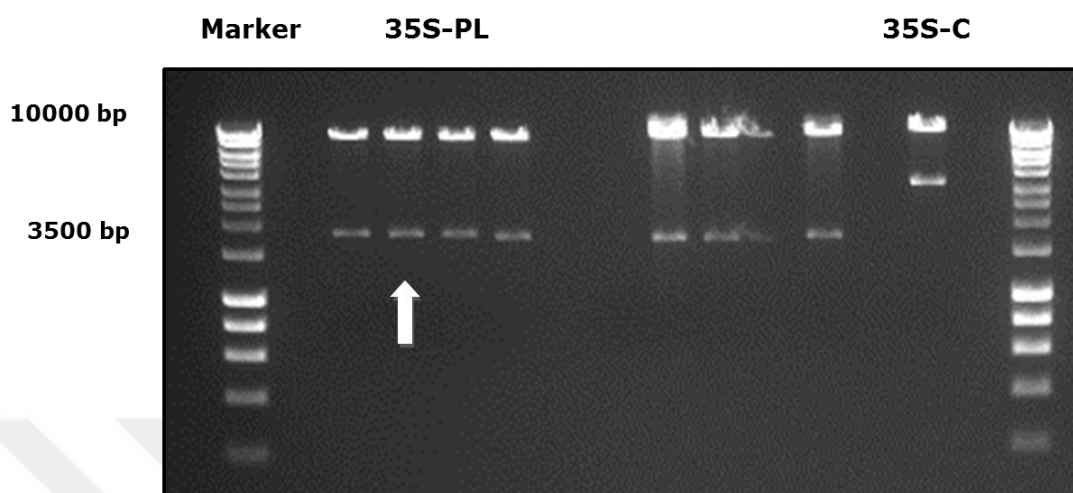


Figure 4.4 Gene construct has been inserted into 35S-pK7GWIWG2(1) destination vector to create expression clone. The plasmids were digested with *Hind*III. Two bands were seen on the gel as 10417 bp and 3167 bp, respectively. The insert was sent for sequencing to confirm the presence of the PL transgene in the vector (Appendix 9.11.4). Second expression clone shown by white arrow was sent to the plant transformation facility at Davis, CA, USA for generation of the transgenic plants. 35S-C= pK7GWIWG2(1) control vector.

4.3.1 Analysis of the PL transgenic lines

Thirteen transgenic Ailsa Craig lines were generated containing a 35S:RNAi constructs designed to down-regulate a putative ripening-related PL (*Solyc03g111690*). The presence of the T-DNA in all these plants was confirmed by PCR amplification (Figure 4.5).



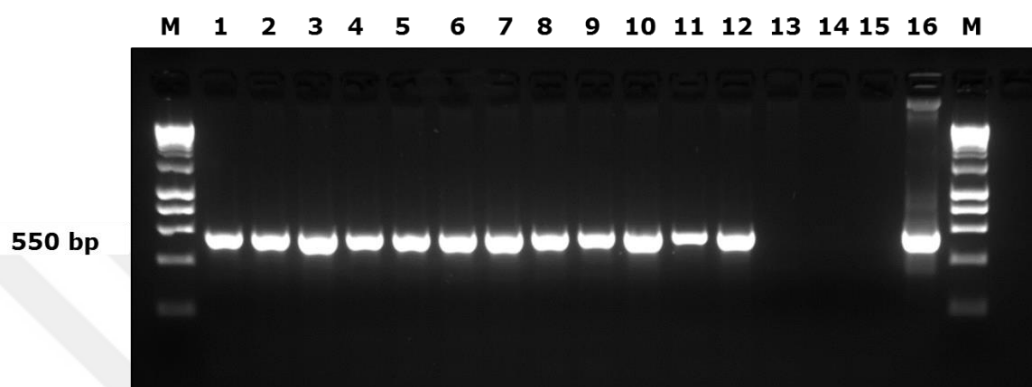


Figure 4.5 Lane 1 to 13: Transgenic lines containing the 35S::PL RNAi gene construct; **Lane 13** did not give product; **Lane 14:** Water Control; **Lane 15:** Ailsa Craig control; **Lane 16:** Plasmid control; **M:** Marker (hyperladder I, Bioline)

4.3.2 Identification of Homozygous lines

After the identification of the single gene copy insertions based on the Southern analysis (Appendix 9.5), putative homozygous lines were selected (T1, T5, T6 and T9). To confirm the presence of single insertions and to select homozygous lines qPCR tests were undertaken on T₁ progeny. Individual lines from T1, T5, T6, and T9 were selected based on the presence of two copies of transgene as determined by qPCR or as azygous controls (Appendix 9.6). qPCR analysis of the progeny of the T5 transgenic plants are shown as an example (Figure 4.6).



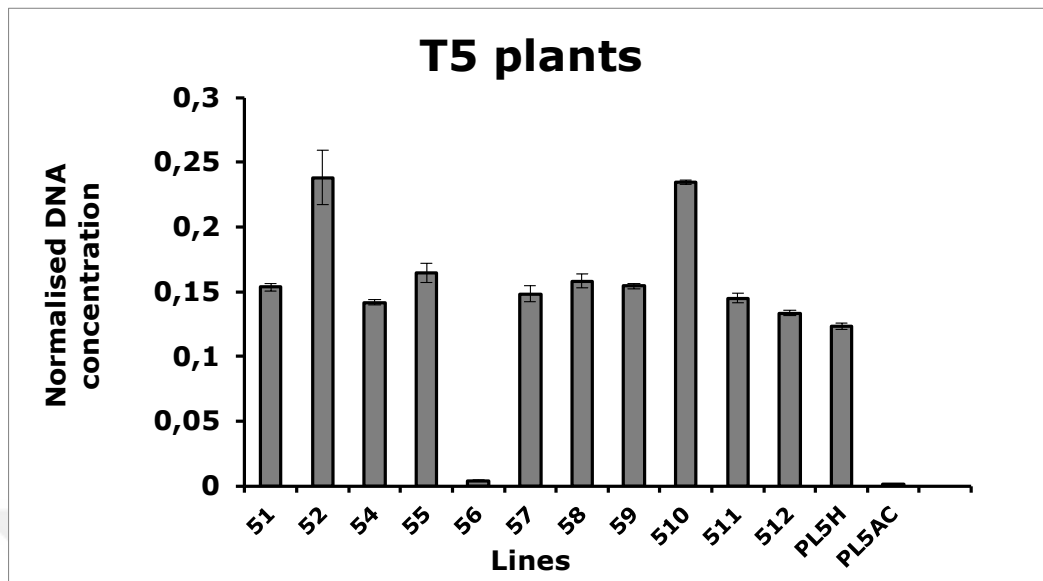


Figure 4.6 Twelve T_1 seeds from the T5 PL line were sown and seedlings used to identify of homozygous, heterozygous and azygous plants. PL5AC was Ailsa Craig wild type. Line T56 was azygous. DNA from the T_0 T5 line (PL5H) was used as heterozygous control. The T52, T55 and T510 plants were selected as homozygous individuals. SEM, $n=3$ (technical replicates)

4.3.3 PL gene expression

PL gene expression was determined at a range of stages of fruit development developmental and ripening (mature green 40 dpa,

breaker, breaker+4 (orange ripe) and breaker+7 (red ripe). For example the level of PL expression in azygous line (5-6) and Ailsa Craig wild type fruits was consistent with data reported by The Tomato Genome Consortium (2012) for *Solyc03g111690*. However, the expression level of PL in 5-5 transgenic fruits was almost completely abolished at all ripening stages (Figure 4.7A). Lines 6-4, 5-6 and 1-4 had been identified as azygous for the transgene and had high PL expression and lines 9-3, 5-5 and 1-2 were identified as homozygous transgenic lines by qPCR and had very low PL expression (Figure 4.7B).

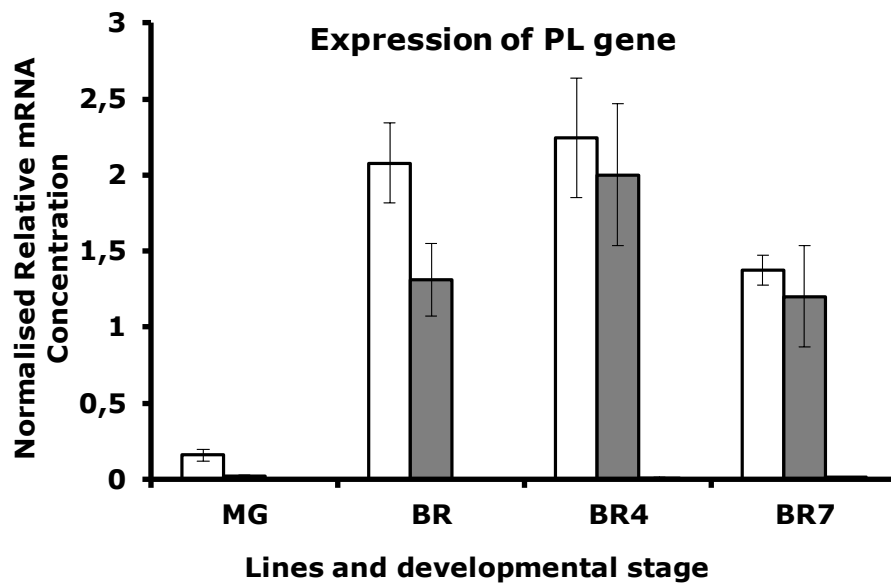
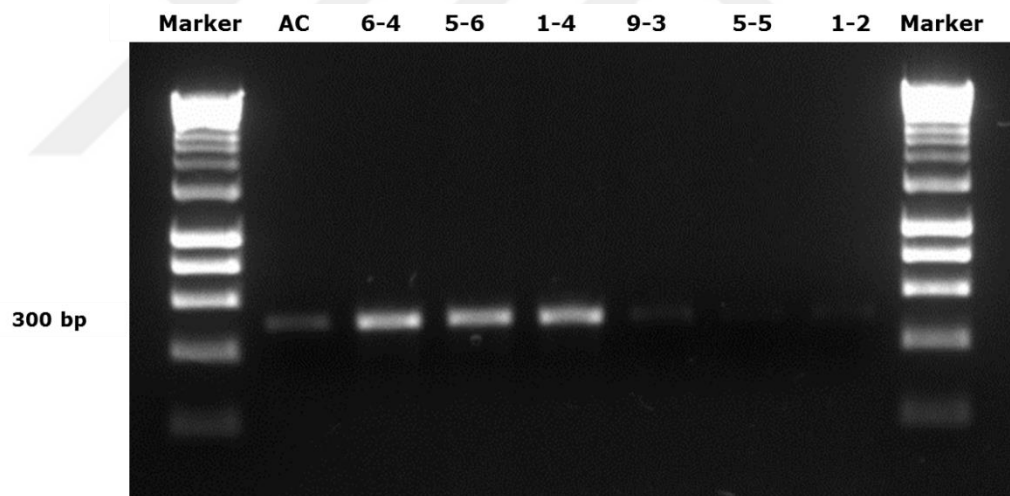
A**B**

Figure 4.7 (A) Comparison of PL gene expression during fruit development and ripening in Azygous (5-6)(grey bars), Ailsa Craig (AC)(white bars) wild type and PL:RNAi lines (5-5)(black bars which cannot be seen due to very low expression). MG (mature green), BR (breaker), BR4 (breaker+4), BR7 (breaker+7). **(B)** PL gene expression in fruit at Br+7 alone in further azygous (6-4, 5-6 and 1-4), AC and PL:RNAi lines (9-3, 5-5 and 1-2). SEM, n=3 (biological and technical replicates)

4.3.4 PL enzyme activity

Transgenic fruits showed severely reduced PL enzyme activity consistent with reduced PL gene expression. Various protocols to determine PL enzyme activity (Collmer *et al.*, 1988; Brooks *et al.*, 1990) were investigated including extracting cell wall proteins from fresh tissue with 1M NaCl (Tucker *et al.*, 1980). There was no detectable PL activity in any of the enzyme extracts. However, by preparing acetone powders from the tomato pericarp samples and incubating these in an alkaline buffer we were then able to detect activity that generated an increase in absorbance in the cleared enzyme extract at 235 nm. This was consistent with the generation of unsaturated bonds at the 4-5 position of the uronic acid residues of pectin during degradation by PL (Collmer *et al.*, 1988; Marin-Rodriguez *et al.*, 2003). PL enzyme activity

measured at 235 nm was significantly lower ($P < 0.001$) in PL antisense fruits compare to control fruits (Figure 4.8).



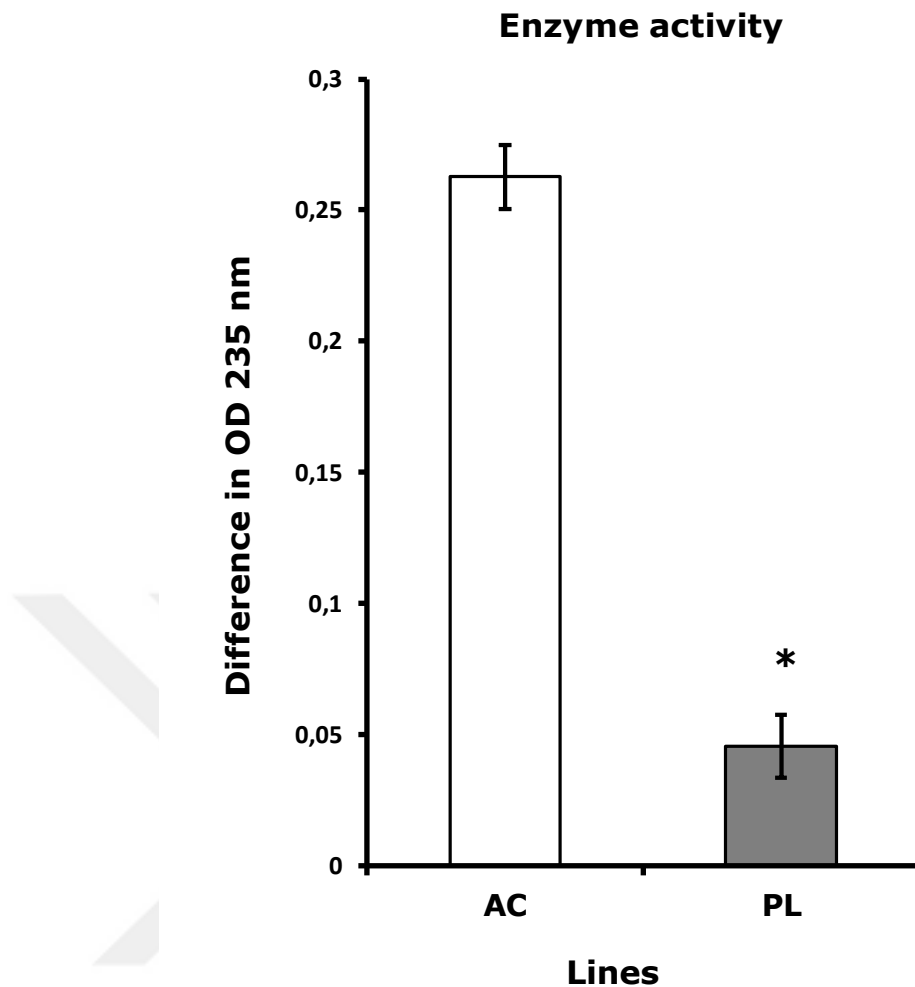


Figure 4.8 PL enzyme activity in acetone powders prepared from Br+7 red ripe fruit pericarp of Ailsa Craig and PL RNAi (5-5) line. 40 mg of acetone powder were incubated for 30 minutes. SEM, n=4 (biological replicates)

Previous workers have had little success in measuring PL activity in tomato (Besford and Hobson, 1972). In the current project attempts to extract the enzyme from the cell wall material were unsuccessful and only acetone powders gave measurable PL activity. It is possible that PL is either denatured during extraction or requires a cofactor that is bound

to the cell wall preparation. In conclusion the reduced PL activity in the RNAi line tested was entirely consistent with the lower levels of PL transcript measured in this line due to gene silencing.

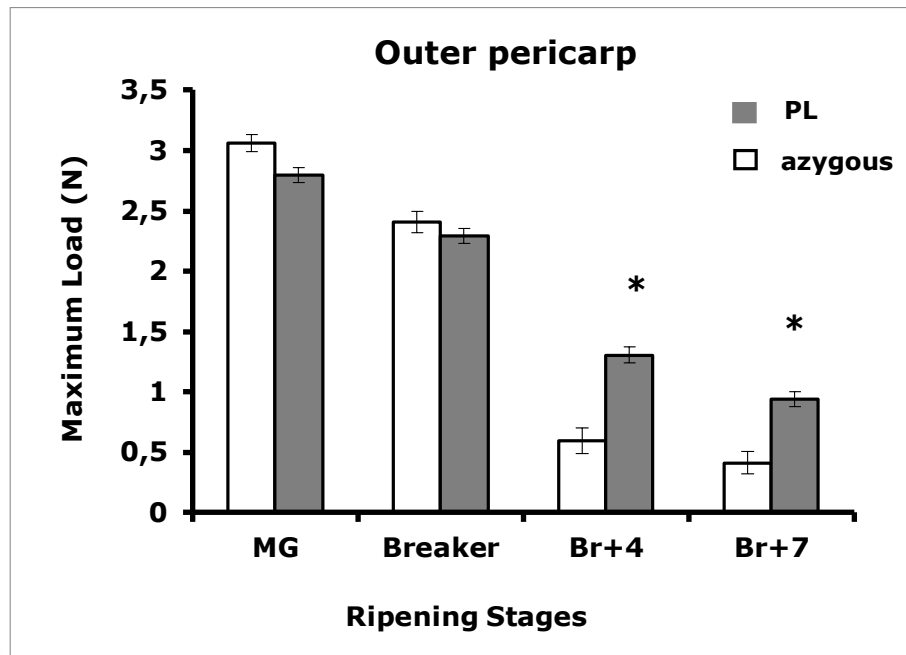
4.3.5 Texture Measurement on fruit from homozygous PL: RNAi lines

Texture of transgenic fruits was measured on T₀ and homozygous transgenic lines and appropriate controls grown on several occasions over a two year period. All transgenic lines gave plants that had a normal habit and produced ripening fruits that were visually indistinguishable from fruits from azygous control lines.

Several independent homozygous transgenic lines were grown and fruit texture was measured. The results for a representative line (5-5) are described and data for several other lines are shown in the Appendix 9.14. The firmness of the PL:RNAi fruit pericarp (Figure 4.9), was significantly enhanced ($P < 0.001$) in comparison to the azygous controls especially at the orange ripe (Breaker + 4) and red ripe stages (breaker +7) in both the outer and inner pericarp tissues. The fruit tissues of the PL:RNAi line were 2-3 fold firmer (Figure 4.9). There was a strong relationship therefore between reduced PL gene expression, enzyme activity and fruit firmness in tomato.



A



B

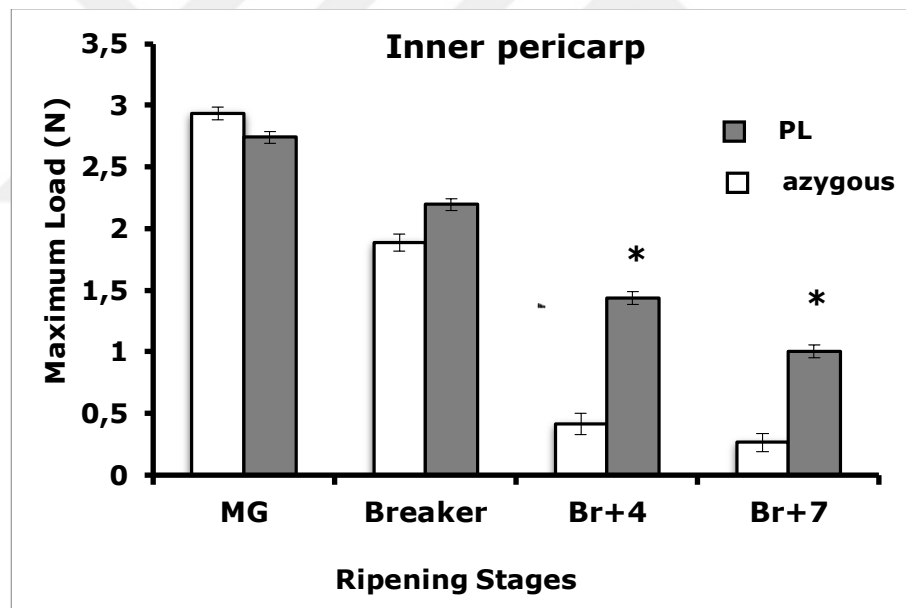


Figure 4.9 Changes in fruit pericarp firmness during ripening as a result of RNAi silencing of PL. **(A)** Outer pericarp and **(B)** inner pericarp firmness in PL:RNAi (5-5) and azygous lines (5-6), MG (Mature green), Br (breaker), Br+4 (orange ripe), Br+7 (red ripe). The firmness of the PL:RNAi lines was significantly enhanced * ($p < 0.001$) in comparison to the azygous controls at the breaker+4 and breaker+7 stages. SEM, $n=10$ (biological replicates)

The reduced rate of softening in the PL::RNAi fruits is substantial and much greater than reported for experiments where other cell wall remodelling proteins that have been silenced in tomato, e.g. polygalacturonase (PG), beta-galactosidase (β -gal), expansin (Exp1) (Smith *et al.*, 1988; Smith *et al.*, 2002). Indeed the silencing PG had no detectable effect on tomato fruit firmness (Smith *et al.*, 1990) and down-regulation of beta-galactosidase 4 (TBG4) only resulted in a 40% increase in texture above the control (Smith *et al.*, 2000). In addition, the PL RNAi fruits retained their integrity longer at 20°C in comparison with wild type indicating the potential for improved shelf life (Figure 4.10).

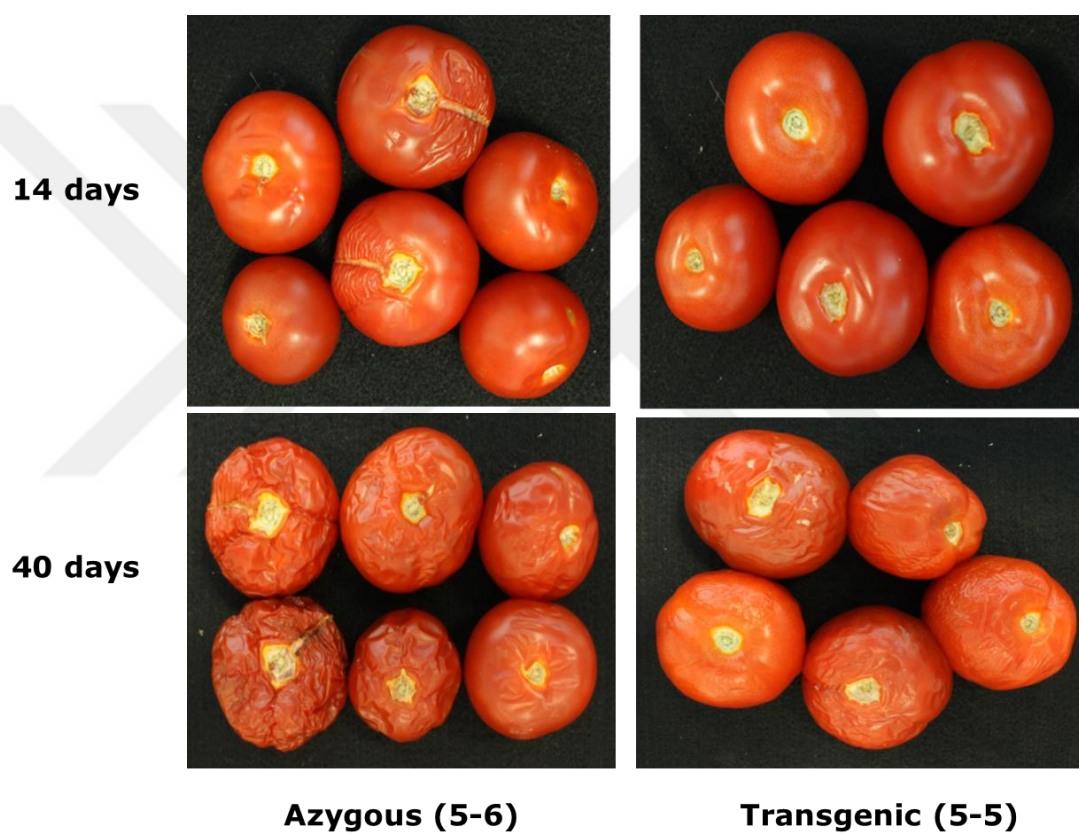


Figure 4.10 Azygous control (5-6) and PL:RNAi transgenic (5-5) fruits after storage at 20 °C 14 days (top) and 40 days (bottom).

4.4 Conclusion

The data from the analysis of the PL::RNAi transgenic plants indicates that PL is an excellent candidate for the gene underlying our firmness QTL on tomato chromosome 3. PL has a large effect on fruit softening during ripening as demonstrated by the effect of silencing the gene expression and the subsequent effects on PL enzyme activity and fruit texture. The results suggest that the *S.pennellii* PL allele may be useful in breeding programmes to slow the rate of fruit softening and therefore improve postharvest life. An important question that remains is whether silencing PL in tomato negatively affects tomato quality in relation to accumulation of pigmentation, sugars, acids and flavour volatiles.

Important next steps are to determine whether PL silencing affects other aspects of ripening beyond texture changes and also the mechanism of PL action.

Chapter 5

Effect of PL silencing on Fruit Metabolism and Ripening Related Gene Expression

5 Effect of PL Silencing on Fruit Metabolism and Ripening Related Gene Expression

5.1 Introduction

Fruit ripening involves a multitude of biochemical processes and is under strong genetic control (Seymour *et al.*, 2002). In addition to softening, fruit accumulate more sugars, acids and volatile compounds which are three major classes of chemicals responsible for flavour (Klee and Giovannoni, 2011). Modification of the levels of any of these compounds can lead to a loss of overall quality. Ethylene controls many of these physiological changes during ripening (Klee and Giovannoni, 2011).

In commercial tomato varieties ripening is often controlled by generating hybrids that contain one mutated copy of the *ripening-inhibitor* or *rin* gene (Kitagawa *et al.*, 2005). As described in Chapter 1, RIN is a master regulator of ripening. In the *rin* mutant normal ripening is completely abolished, but in *rin* hybrids the fruit ripen to a degree and shelf life is greatly extended although flavour can be impaired (Kitagawa *et al.*, 2005). An ideal situation would be to gain control over texture and shelf-life without negative effects on other aspects of ripening. The PL gene under the texture QTL on tomato chromosome 3 has the potential to control fruit texture, but its effects on other aspects of ripening need further investigation.

The aim of the work described in this chapter was to investigate how silencing PL influenced fruit metabolism and expression of other ripening related genes.



5.2 Materials and Methods

5.2.1 Extraction and analysis of carotenoids

All the metabolomics experiments were carried out with the help of Laura Perez and Paul Fraser (Royal Holloway, University of London). Carotenoids and tocopherols were extracted from freeze dried fruit. Extractions were made from sample powder (10 mg) in 1.5 mL centrifuge tubes. Metabolites were extracted by the addition of chloroform and methanol (2:1). Samples were stored for 20 min on ice. Subsequently, water (1 vol.) was added. Samples were centrifuged for 5 min at top speed (21100 g) in a Heraeus Pico21 centrifuge (Thermo Scientific). The organic phase, containing the pigment extract, was placed in a fresh centrifuge tube and the aqueous phase re-extracted with chloroform (x2 by volume). Organic phases were pooled and dried using the Genevac EZ.27. Dried samples were stored at -20°C and dissolved in ethyl acetate prior to chromatographic analysis.

Carotenoids were separated and identified by Ultra High Performance Liquid Chromatography with photo diode array detection (UPLC-PDA). An Acquity™ UPLC (Waters) was used with a BEH C18 column (2.1 x 100 mm, 1.7 µm) with a BEH C18 VanGuard pre-column (2.1 x 50 mm, 1.7µm). The mobile phase used was A: MeOH/H₂O (50/50) and B: ACN (acetonitrile)/ethyl acetate (75:25). All solvents used were HPLC grade and filtered prior to use through a 0.2 µm filter. The gradient was 30% A: 70% B for 0.5 min and then stepped to 0.1% A:99.9% B for 5.5 min and then to 30% A:70% B for the last 2 minutes. Column temperature was maintained at 30°C and the temperature of samples at 8°C. Online

scanning across the UV/Vis range was performed in a continuous manner from 250 to 600 nm, using an extended wavelength PDA (Waters). Carotenoids were quantified from dose-response curves. The HPLC separation, detection and quantification of carotenoids, tocopherols and chlorophylls have been described in detail previously (Fraser *et al.*, 2000).

5.2.2 Extraction and analysis of intermediary metabolites

Frozen tomato samples (Br+7) were freeze-dried (Christ Alpha 2-4 LSC Freeze Dryer) and ground to a fine powder by using a tissue lyser (Qiagen). 10 mg of powder was extracted with 1 mL of 50% methanol for 20 min at room temperature and shaking. 1 mL of chloroform was then added and centrifuged at top speed for 3 min to allow phase separation. 20 µL of the polar phase containing intermediary metabolites was transferred to a HPLC glass vial and spiked with 10 µL of the internal standard solution (1 mg/mL of ribitol in methanol). Samples were taken to dryness using a vacuum centrifuge Genevac EZ.27 and stored at -20°C until derivatisation and analysis.

Dried samples were derivatised to their methoxymated and silylated forms according to previous publication (Halket *et al.*, 2005). First, 30 µL of methoxyamine hydrochloride (20 mg/mL in pyridine anhydrous) was added to samples and incubated at 40° C for 1 hr. Following this reaction, samples were treated with 70 µL of MSTFA and heated at 40 °C for 2 hr. 1 µL of the final solution was splitless injected into a 7890B gas chromatograph on-line with a 5977A mass spectrometer (Agilent

Technologies, Palo Alto, California, US). Metabolites were separated in a DB-5MS 30 m x 250 μ m x 0.25 μ m column (J&W Scientific, Folsom, California, US) equipped with a 10 cm guard column and using a temperature gradient ranging from 70° to 320° C at 5° C/min. Helium was employed as the carrier gas and the flow rate was set at 1 mL /min. Inlet was heated to 280° C and the mass spectrometer transfer line to 250° C. A mixture of n-alkanes ranging from 8 to 32 carbons was used for retention index external calibration. Levels of metabolites analysed by GC/MS were quantified relative to the internal standard and corrected by dried weight of biomass. AMDIS version 2.69 software was used for peak deconvolution and identification of metabolites (Perez-Fons *et al.*, 2014).

5.2.3 Tomato Aroma Analysis by GC-MS

These experiments were conducted in the laboratory of Dr Ian Fisk at University of Nottingham, Sutton Bonington.

The protocol followed the methods developed by Buttery *et al* (1987). Tomato cut into four slices and placed into stomacher bag (Seward BA6141 Circulator 400, Fisher Scientific). This bag was placed into the Stomacher machine (Stomacher 400 Circulator machine, Seward) to be crushed at the standard speed for 2 min. The bag was left open for 3 min to allow some enzyme reaction to generate some key aroma compounds. Saturated calcium chloride (anhydrous granular, >93%, Sigma Aldrich, UK) were prepared and added at the same weight as

tomato into that bag, which was again placed into the Stomacher machine for mixing and homogenising at the standard speed for 2 min. The mixture was filtered by filter mesh (1mm) to remove the skin and large tissues. Each sample was prepared with 3 biological replicates by adding the tissue to a 30 mL glass vial. Then 10 ppm of 3-heptanone (internal standard) was added and the vial was then sealed. All samples were kept at -80 °C, and each sample was defrosted and allowed 30min to equilibrate at room temperature before GC-MS analysis. A vial of air was placed as blank sample to confirm the background signal is clear.

The Trace 1300 series Gas Chromatograph coupled with the Single-Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) was used. The SPME Fiber (50/30µm DVB/CAR/PDMS, Supelco, Sigma Aldrich, UK) was used to extract for 5 min then desorb for 5 min. Separation was carried out by a ZB-WAX Capillary GC Column (30 m, 0.25mm I.D., 1.00µm Film Thickness). The column temperature was held initially at 40 °C for 4 min till 150 °C. Full scan mode was chosen to measure volatile compounds (mass range from 20 to 300). A splitless mode was used, and the constant carrier pressure at 15 psi was applied. Volatiles were identified by comparison of each mass spectrum with spectra in reference collections (Microsoft Windows™ Version 2.0 of the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library). The quantification of volatiles collected from the headspace was calculated from GC peak areas, by comparison with the peak area of the internal standard (3-heptanone). Data were analysed by SPSS Statistics (version 22.0.0, IBM) and Excel 2010 (Microsoft). The important aroma

compounds in tomatoes are C6 compounds, especially some aldehydes and alcohols (Schwab *et al.*, 2008).

5.2.4 Ethylene Measurement

Fruits were harvested at breaker+4 and breaker+7 stages. Ethylene generation was measured by enclosing whole fruit in closed chambers (330 mL) for 2 hours then withdrawing 1 mL gas samples from the chamber and injecting it into a gas chromatograph (ATI-UNICAM 610 Series equipped with a dual capillary column and flame ionization detector).

After ethylene measurement, the gas samples were quantified according to formula;

C₂H₄ per fruit produced

$$\frac{((\text{ppm (sample)} - \text{background (air)}) \times (\text{volume (330 mL)} - \text{fruit weight (gram)}))}{(\text{time (2 hours)}) \times (\text{fruit weight (gram)})} = \text{nl g}^{-1} \text{h}^{-1}$$

5.2.5 RNA sequencing analysis

RNA extraction was carried out as described in Chapter 2, and the RNA samples were sent to Syngenta Biotechnology in North Carolina, USA for

generation of the RNASeq libraries and sequencing. Briefly the protocols were as follows. cDNA was prepared and cleaned using Ampure XP magnetic beads. It was fragmented to about 400bp using a Covaris S2 instrument and ligated to adapters using an Apollo 324 instrument and PrepX ILM DNA Library Kit. PCR amplification was performed using KAPA HiFi HotStart ReadyMix (2X). Six samples were pooled per lane and sequenced using an Illumina HiSeq to generate 100-nucleotide single end sequence reads.

After quality assessment by FastQC (Andrews 2010), untrimmed reads were aligned to the ITAG 2.3 tomato reference genome using Tophat version 2.0.12 (Trapnell et al 2009) with Bowtie2 version 2.2.3 (Langmead and Salzberg 2012), On average, 92% of the reads aligned per sample.

Reads aligned to annotated regions were counted using Syngenta counting algorithm. Gene counts were then loess-median, which applies intensity based corrections to account for biases in the data that may arise from non-linear relationships between two samples, normalized to correct for GC content bias using the R Bioconductor package EDASeq version 2.0.0 normalizing within lane (loess) and between lane (median) (Risso *et al.*, 2011).

Differentially expressed genes were determined by the R Bioconductor package edgeR version 3.8.4 (Robinson *et al.*, 2010). To control the family wise error rate, p-values were adjusted for multiple comparisons

using the Benjamini-Hochberg method producing an adjusted p-value or false discovery rate (FDR). An FDR < 0.05 was considered statistically significant. Genes were considered to be differentially expressed if they had an FDR < 0.05 and a $\log_2$ fold change greater than 1 or less than -1 (fold change of 2).

The gene ontology (GO) term enrichment process utilized GO terms extracted from the Gramene BioMart, <http://ensembl-gramene.org/biomart/martview>, as of November 7, 2014. The R Bioconductor package goseq version 1.18.0 (Young et al 2010) was used to perform GO enrichment analysis on differentially expressed genes from the downloaded terms correcting for gene length bias.

5.3 Results and Discussion

5.3.1 Metabolite analysis

The PL:RNAi fruits were indistinguishable from those of the azygous lines at all stages of development in terms of size and appearance. The colour of the PL::RNAi and azygous fruits was indistinguishable based on colour index (Figure 5.1). Consistent with these observations, metabolite analysis indicated no significant ($P>0.05$) differences in the content of carotenoid precursors such as phytoene or carotenoids between the PL:RNAi fruits and the azygous control at the red ripe stage (Figure 5.3). Suppression of PL levels also had no significant effect on tomato flavour which is the perception of many aroma and taste constituents and is very important quality factor. Sugars (mainly fructose and glucose) and acids (citric and malic) and their interactions are the most important factors responsible for sweetness, sourness and overall flavour intensity (Malundo *et al.*, 1995) and taste can be dramatically altered by the presence of flavour volatiles.

Analysis of the sugar, acid and volatiles in the ripe PL::RNAi and azygous fruits again revealed no significant ($P>0.05$) differences between treatments (Figure 5.3 and Table 5.1) which was consistent with measurement of soluble solids content and colour index (Figure 5.1).

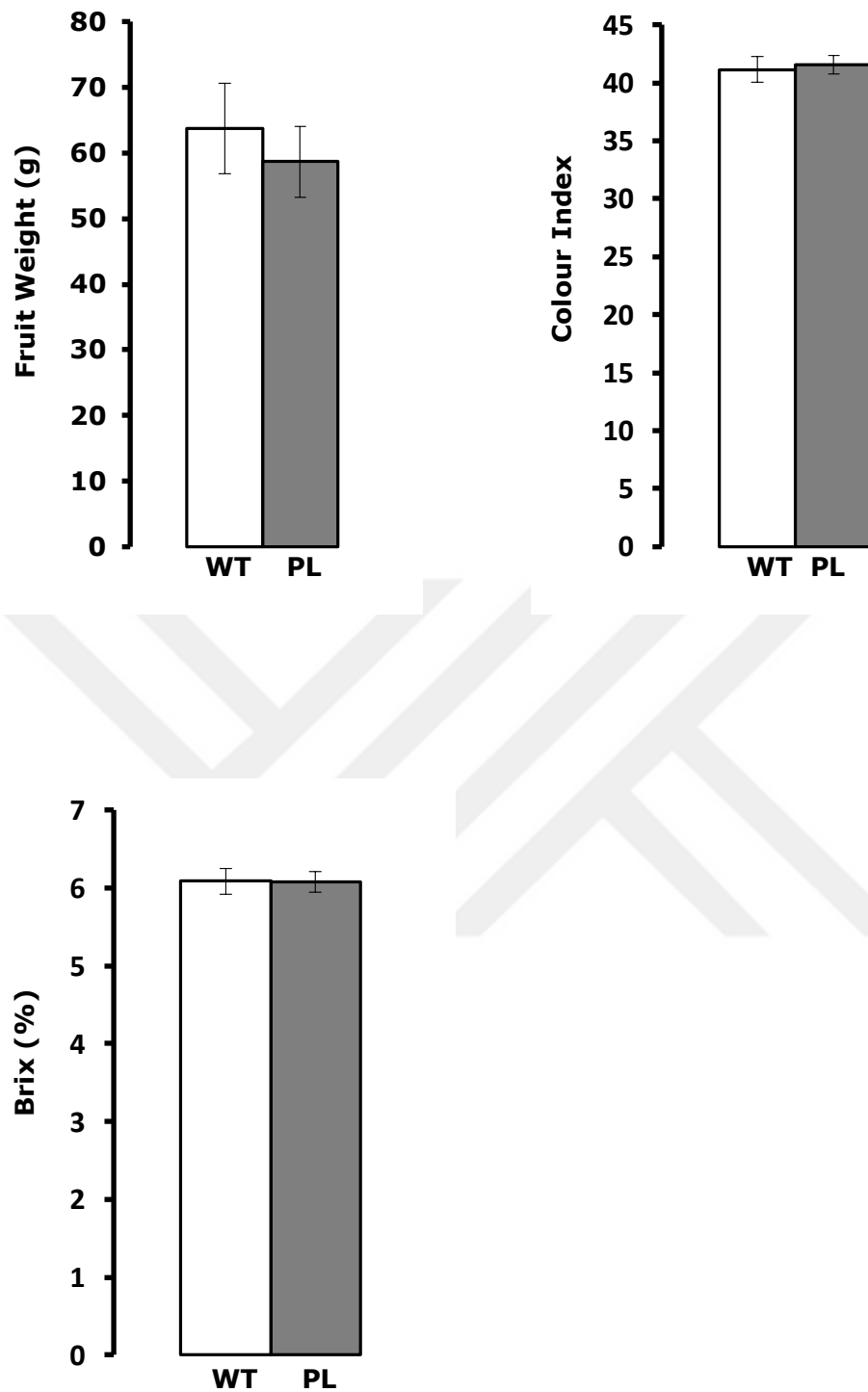


Figure 5.1 Fruit characteristics with respect to weight, colour index and brix measures in red ripe fruits from azygous (5-6) and PL:RNAi (5-5) lines. SEM, n=10 (biological replicates)

The aim of this study was to investigate whether PL suppression has affected carotenoids which play major role for flavour and colour of tomato. According to results, PL suppression has not dramatically changed any volatile compounds. There was only significantly difference for β -Carotene and lycopene. There was a big difference for the sucrose, however, it has been ignored due to the big error bar (Figure 5.3). All the detailed version of metabolomics (t-tests) is given in Appendix 9.7 and 9.8.



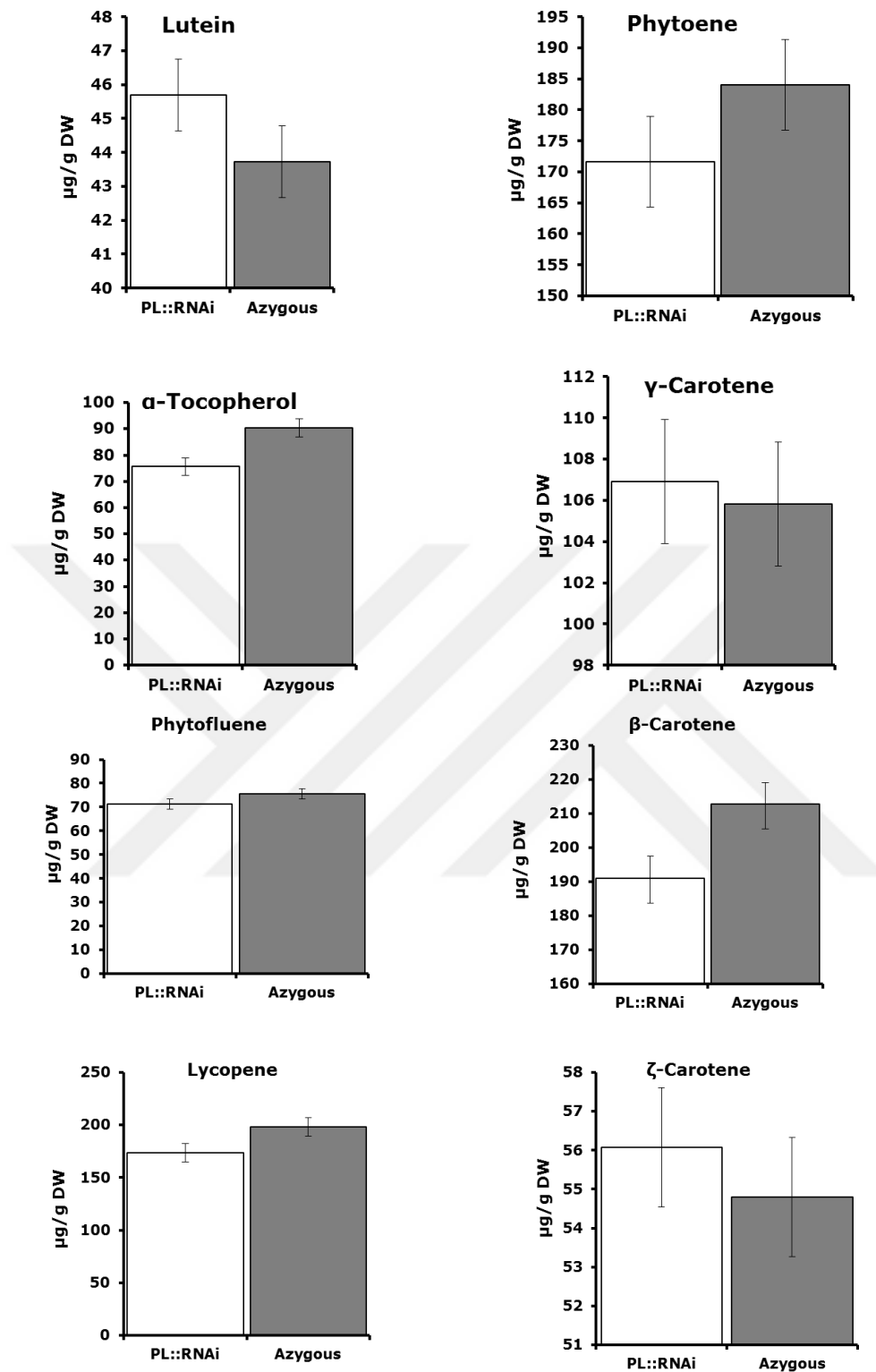


Figure 5.2 Analysis of carotenoid precursors, carotenoids and tocopherol composition of PL::RNAi and azygous fruits harvested at 7 days post breaker. SEM, n=3 (biological replicates) (all t-tests are in appendix 9.7)

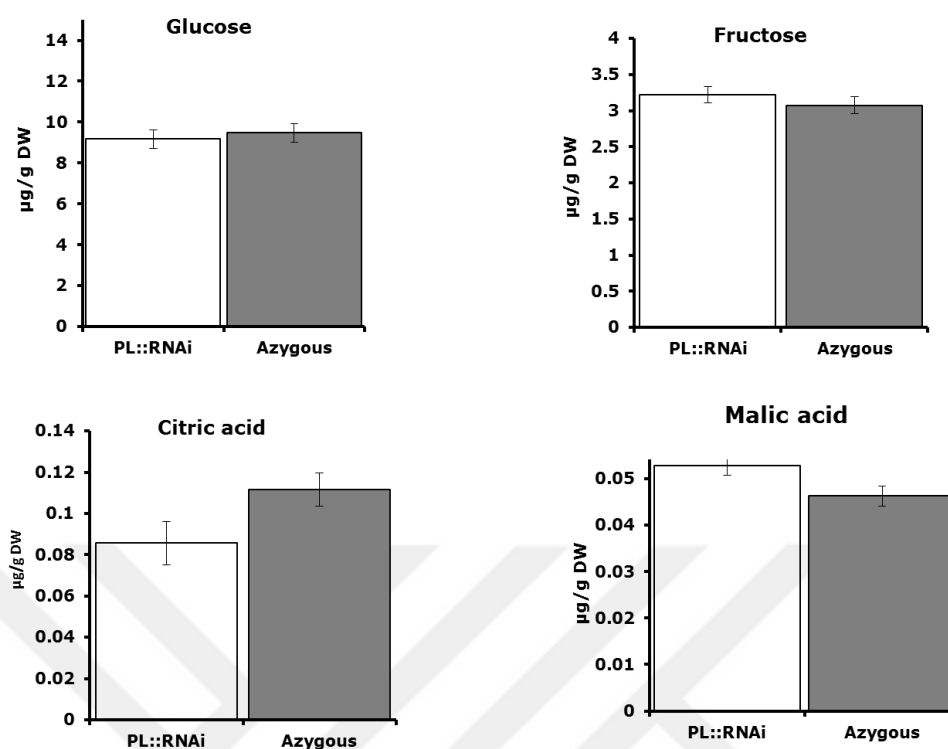


Figure 5.3 Analysis of sugar and organic acid composition of PL:RNAi and azygous fruits harvested at 7 days post breaker. SEM, n=3 (biological replicates) (all t-tests are in appendix 9.8)

Table 5.1 Six key compounds were selected from the identified compounds and listed in the table below with their reference odour and odour threshold adapted from Yilmaz (2001).

Chemical Group	Compound	RT (min)	Odour	Odour Threshold (ppm)
Aldehydes	hexanal	8.87	Floral, herbal	0.0045
Aldehydes	trans-2-hexenal	13.72	Green	0.017
Ketones	6-methyl-5-heptene-2-one	17.96	Floral, green	0.05
Alcohols	hexanol	18.38	Green, grass	0.5
Alcohols	cis-3-hexen-1-ol	19.60	green, floral	0.07

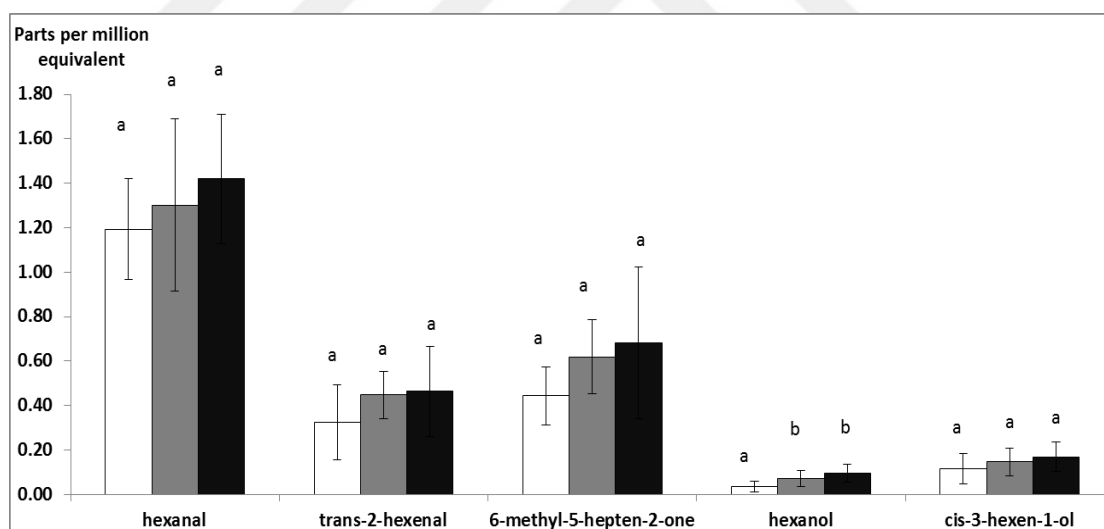


Figure 5.4 The concentration of each compound per sample was calculated in ppm (mg/kg). The average of three types: CT1 (AC⁺⁺) (white bar), CT2 (azygous, 5-6) (grey bar) and PL:RNAi (5-5) (black bar) was shown in the Figure above. The standard deviation was shown as the error bars, the letter a, and b indicated if there is a significant difference ($p < 0.05$). Apart from hexanol, there is no significant difference between these three types of tomato for most compounds including hexanal, trans-2-hexenal, 6-methyl-5-heptene-2-one and cis-3-hexen-1-ol.

Ethylene is known to play a critical role in fruit development. The level of it was measured to know if PL silencing was having an effect on ethylene biosynthesis. If so this could impact fruit softening by inhibiting the expression of other ethylene dependent cell wall remodelling genes. However there was no significant ($P>0.05$) effect on ethylene evolution of PL gene silencing ripening. ($P=0.478$ for Br+7, $P=0.109$ for Br+4) (Figure 5.5).



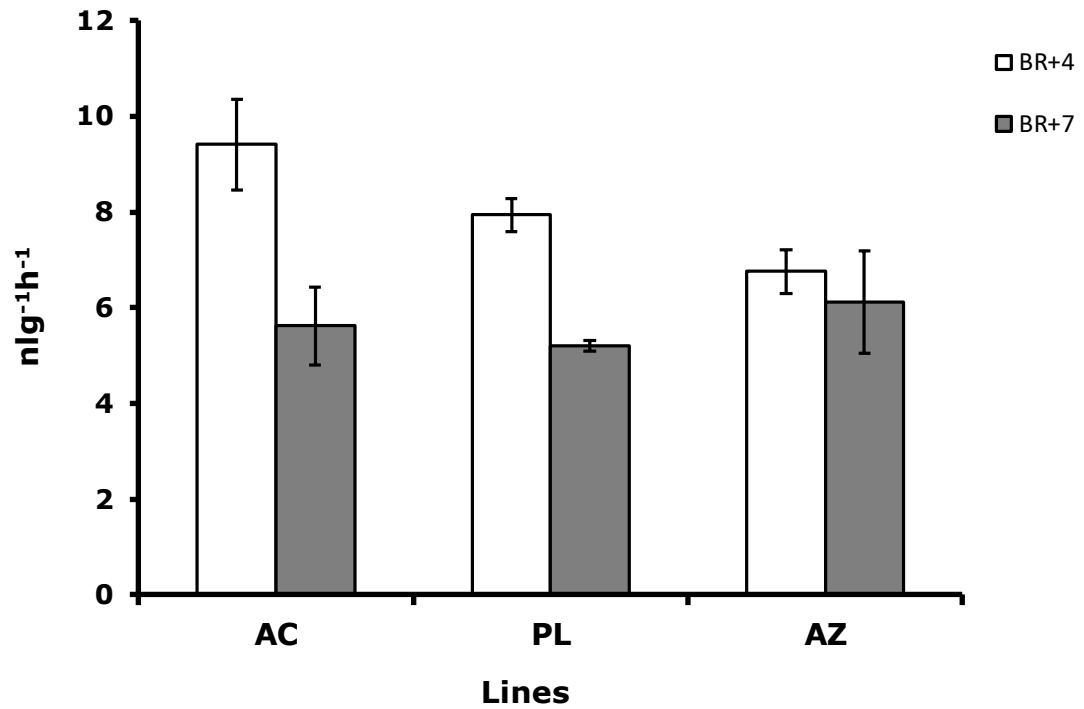
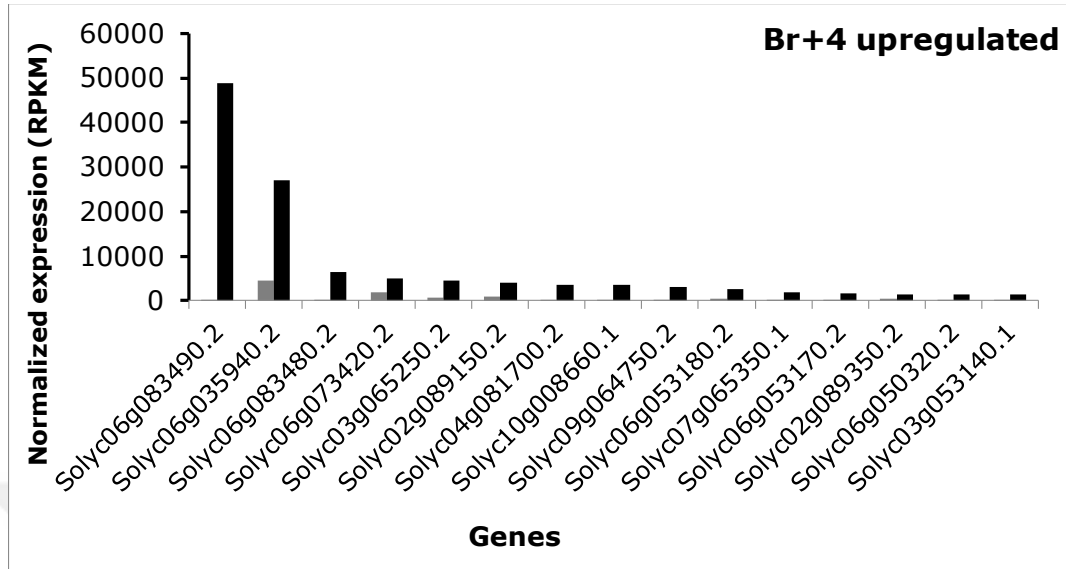


Figure 5.5 Ethylene evolution from fruit of 'Ailsa Craig', PL:RNAi (5-5) and azygous (5-6) lines at breaker+4, breaker+7 stages. There is no statistic different between transgenic and wild type lines. SEM, n=4 (biological replicates)

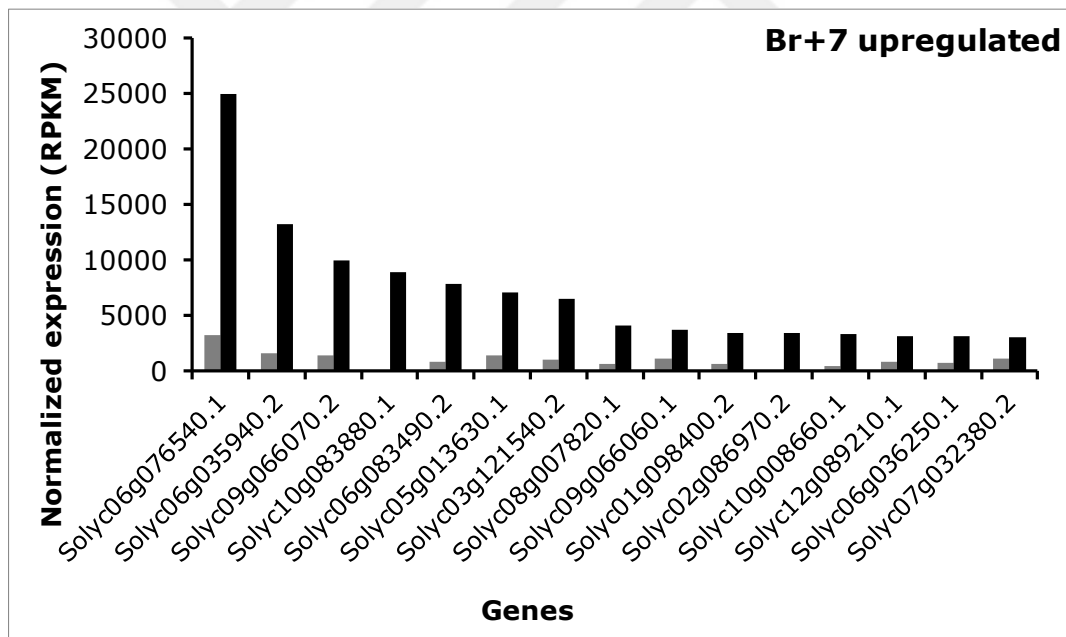
5.3.2 RNA-Seq analysis and specifically expression of cell wall Structure related genes

A comparison of the transcriptome of the PL:RNAi (5-5) and azygous control fruits (5-6) by RNASeq at orange and also red ripe stages ordered by GO terms indicated that the global expression profiles that were almost indistinguishable, with less than 60 genes out of some 15,000 genes expressed during ripening showing either elevated or reduced expression at breaker + 4 or breaker + 7 stages between the transgenic and wild type fruits.

A



B



■ PL:RNAi (5-5) ■ azygous control (5-6)

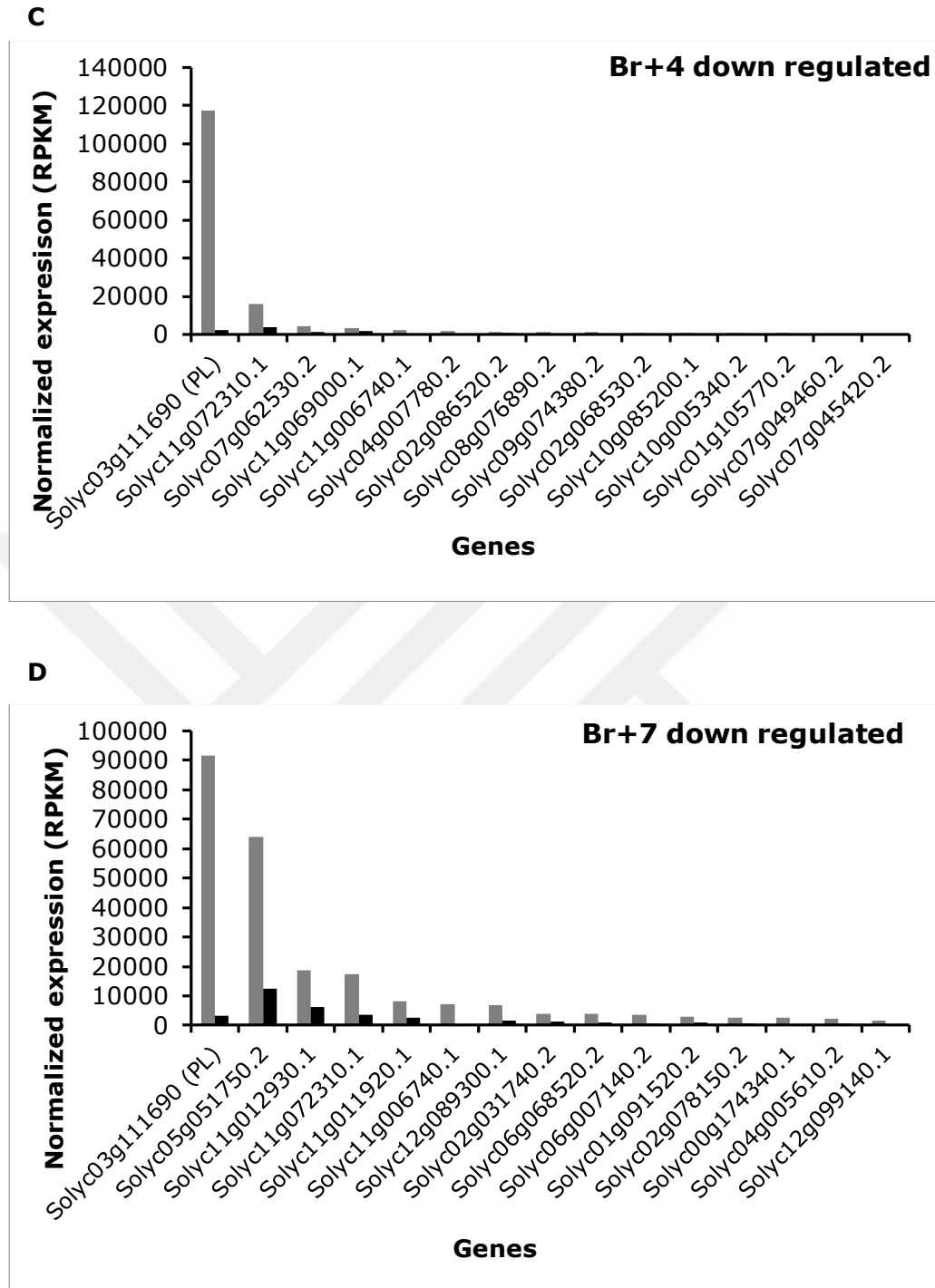


Figure 5.6 A comparison of the transcriptome of the PL:RNAi and control fruits by RNASeq at orange and also red ripe stages. 15 genes was shown for each developmental stage. *Solyc03g111690* (PL) gene expression efficiently down regulated by antisense PL in both ripening stages.

■ PL:RNAi (5-5) ■ azygous control (5-6)

In the RNAi lines, PL (Figure 5.6) expression was low in comparison to control fruits at breaker+4 (AZ: 117660.33 RPKM, PL: 2332) and breaker+7 (AZ: 91590.33, PL: 3185) and this was the only ripening-related cell wall structure-related gene (based on the Supplementary Table 12 in The Tomato Genome Consortium, 2012) that was altered at both these stages of development.

Three further cell wall structure related genes that are normally lowly expressed during the ripening phase and more normally associated with fruit development were down-regulated in the PL:RNAi lines. These were *Solyc01g091530*: fasciclin-like arabinogalactan protein, *Solyc03g115200*: β -1-3 glucanase and *Solyc05g005080*: endo-1 4- β -glucanase. The fasciclin-like gene encodes a protein similar to those responsible for modulating stem biomechanics and cell wall architecture in *Arabidopsis* (MacMillan *et al.*, 2010). The glucanase gene encodes a protein likely to, *Solyc03g115200*: β -1-3 glucanase, catalyzes endo-type hydrolytic cleavage of the 1,3- β -D-glucosidic linkages in β -1,3-glucans and the *Solyc05g005080*: endo-1 4- β -glucanase encodes a protein, involved in cell wall metabolism in higher plants (Buchanan *et al.*, 2012). However their expression was unchanged in the transgenic lines at breaker + 4, while at both 4 and 7 days post breaker stage substantial differences in texture were observed.

With respect to upregulated genes in RNA-seq data, the most prominent gene is *Solyc07g065350.1*: a putative pectinesterase, the gene product of which could act to strengthen cell to cell adhesion by facilitating the generation of calcium-pectate gels (Chapman *et al.*, 2012). However, the direct link to PL gene expression is unclear, although enhanced PE levels could contribute fruit firmness in PL lines (Phan *et al.*, 2007).

The function of the small number of other genes showing differences in expression in the PL:RNAi fruits requires further investigation. These include *PROTODERMAL FACTOR 2*-like and *CER1* (*Solyc06g035940* and *Solyc03g065250*) that were up-regulated at the orange and red ripe stages. They encode proteins likely involved in regulating epidermal development and cuticle properties which could have important effects on water loss and fruit shelf life (Martin and Rose, 2014) (Yeats and Rose, 2013) (Abe *et al.*, 2003). Previous transgenic experiments to modify cell wall degrading enzymes in tomato have also resulted in changes in cuticle properties. However measures of weight loss during ripening in PL:RNAi and azygous fruit (PL:RNAi; % 93.28 gram of water loss, azygous: % 93.72 gram of water loss, which are not significantly different) are not consistent with altered cuticle properties in the PL:RNAi lines.

5.4 Conclusion

Chemical and transcriptome analysis indicates that the PL:RNAi fruits develop and ripen fully with respect to weight, colour, ethylene evolution, sugar content and accumulation of metabolites. Ripening-related gene expression also proceeds as normal with the only major cell wall remodelling gene affected being the target PL. These data demonstrate that silencing PL can generate fruits that will soften more slowly without detrimental effects on other aspects of ripening, but what is the mechanism of PL action?



Chapter 6

Mechanism of Action of PL in Tomato Fruit Softening

6 Mechanism of Action of PL in Tomato Fruit Softening

6.1 Introduction

Cell walls are highly complex. Cell wall remodelling during fruit softening involves complex compositional and structural changes including changes in cell wall thickness, permeability of plasma membrane, hydration of the cell wall, decrease in the structural integrity and an increase in intracellular spaces (Redgwell *et al.*, 1997).

One of the most obvious changes in the cell walls of ripening tomato fruits is the degradation of the pectic polysaccharides. This involves an increase in their solubility and a reduction in their molecular weight (Seymour *et al.*, 1987). Pectins have been shown to be important in cell to cell adhesion and therefore their degradation is likely to contribute to cell separation and fruit softening (Ng *et al.*, 2013). In tomato the genes encoding the enzymes PG and PME are highly expressed during ripening and it was thought that these gene products were principally responsible for pectin degradation and fruit softening (Brummell *et al.*, 2006). However studies using antisense constructs to silence the PG and PE genes (Smith *et al.*, 1990; Hall *et al.*, 1993) failed to have significant impact on fruit softening. In the case of PG the decrease in pectin molecular weight during ripening was partially inhibited, but pectin solubilisation proceeded as normal (Smith *et al.*, 1990). There have been no reports in the literature on the effects of silencing PL in tomato and the role of this enzyme family in tomato fruit softening and cell wall degradation is unknown.

Cell wall pectins are highly complex with long chains of homogalacturonan 'smooth regions' interspersed with 'hairy regions' containing neutral sugar side chains (Voragen *et al.*, 2009). The middle lamella is known to be pectin-rich and may have a higher proportion of homogalacturonan where calcium junctions can form between adjacent pectin molecules. In the primary wall some more highly branched pectins appear to be tightly associated with the cellulose and hemicellulose fractions (Seymour *et al.*, 1990). The PL may act together with PG to degrade the pectin-rich middle lamella or perhaps these enzymes work mainly on 'smooth' or 'hairy' regions.

In this Chapter we investigate the mechanism of PL action on the tomato cell wall.

6.2 Material and Methods

6.2.1 Plant Material

PG and Expansin (Exp1) antisense lines were obtained from Dr Ann Powell University of California, Davis.

6.2.2 Alcohol Insoluble Solids Production (AIS) as crude cell wall material (CWM)

Fresh tomato pericarp (40 g) was skinned, cubed and boiled at 80 °C in 95% EtOH (100 mL) for 30 min. The sample was cooled to room temperature, homogenised using a coffee grinder and then filtered through Miracloth (Calbiochem Catalog No: 475855) and washed successively with hot 85% EtOH (200 mL), chloroform:methanol (1:1 v/v) (200 mL) and 100% acetone. The colourless AIS was air dried overnight at room temperature.

6.2.3 Fractionation of tomato CWM

All experiments were performed in 2 mL micro centrifuge tubes (Eppendorf). 7.5 mg of AIS weighed and put into tube with 1.5 mL of dH₂O. The sample was stirred 4 hours at room temperature, then centrifuged for 20 minutes at 10000g. The supernatant which contained the water soluble pectin was filtered through GF/A paper. The supernatant was made up in the tube to a known volume (1.5 mL) using dH₂O.

The same steps were repeated with the insoluble material replacing dH₂O with 50 mM of CDTA (pH 6.5), and with 50 mM Na₂CO₃+20mM NaBH₄, respectively. For the second solution (50 mM Na₂CO₃+20mM NaBH₄), samples were stirred for 18 hours at 4 °C, then moved to room

temperature for 2 hours stirring. After centrifugation, the 50 mM Na_2CO_3 +20mM NaBH_4 extracted material was adjusted to pH5.5 using acetic acid. All samples were kept at -20 °C for longer periods.

6.2.4 Uronic Acid Assay

Quantification of uronic acids in fruit serum was carried out spectrophotometrically according to the method of Carpita and Filisetti (1991). A 2% sodium chloride (NaCl) (Fisher Scientific), 3% boric acid (Sigma-Aldric) solution was prepared as 0.025 to 0.125 mg/L (WT/V) in water. 300 μL of each test sample and each standard was then added to a test tube in triplicate along with 300 μL of boric acid solution and 5 mL of 12M sulphuric acid (H_2SO_4) (Fisher Scientific). The samples were then vortexed to mix and incubated at 70 °C in a water bath for 40 minutes before being at room temperature. 0.2 mL of the dimethyl-phenol solution was then added to each sample and vortexed. Samples were left at room temperature for 5 minutes and the colour change read using a Multiscan FC (Thermo Scientific) microplate photometer at the wavelength 405 nm and 450 nm with the difference recorded. The absorbance reading at 405 nm was subtracted from that at 450 nm to correct for interference from hexoses. Averages of technical replicates were used for statistical analysis (Figure 6.1).

6.2.5 Determination of pectin molecular weights

Alcohol insoluble solids (AIS) which had been fractionated as in section (4.8.3) were used to determine molecular weights of pectin. The fractionated samples (H_2O , CDTA and $\text{Na}_2\text{CO}_3 + \text{NaBH}_4$) were dialysed (Thermo, 12-14000 kDa) overnight at 4 °C. The molecular weights of the samples (Ailsa Craig, azygous and transgenic PL::RNAi line) were then measured by ultracentrifugation. This work was undertaken in the laboratory of Professor S.E. Harding at Nottingham.

Polyuronide heterogeneity and molecular weights were determined as follows. The distribution of the sedimentation coefficients (heterogeneity) were determined from sedimentation velocity analytical ultracentrifugation (rotor speed of 400000 g, at 20.0°C in a Beckman (Palo Alto) Optima XL-I win interference optics) and a loading concentration of 0.3mg/mL to minimize any non-ideality effects. The data was analysed using the SEDFIT procedure of Dam & Schuck, (Dam and Schuck, 2004) and showed all pectin samples to be very polydisperse with material extending to at least 8S and as high as 17S in some cases. To obtain the (weight average) molecular weights sedimentation equilibrium experiments (on the same equipment) were then undertaken on samples from three separate fruits from each treatment at a rotor speed of 150000g, other conditions the same and data analysed using the SEDFIT-MSTAR procedure of Schuck, Harding and co-workers (Schuck et al, 2014) to estimate the weight average molar masses for pectic polysaccharides (Table 6.1).

6.2.6 Tissue Fixation, embedding and sectioning

Tomato fruit harvested Br+7 and 5 mm thick slices of pericarp tissue were cut. Tissue pieces were then fixed for 5 days at room temperature in Formalin-Acid-Alcohol (FAA) containing 50% absolute ethanol, 5% glacial acetic acid, 10% formalin and 35% dH₂O (all reagents Fisher Scientific).

Resin infiltration and embedding steps were carried out in 2 mL centrifuge tubes (Eppendorf). Tissue pieces were transferred to 70% ethanol before storage at 4 °C until required. The pieces were then dehydrated in two changes of 100% ethanol for 2h rotating at room temperature. LR white resin was prepared by mixing 65% LR white resin with 35% 2-Hydroxyethyl Metacrylate EM (HEMA) (TAAB Laboratories Equipment Ltd.) and dispelling any air by placing under vacuum for 10 minutes. Tissue was then infiltrated overnight with LR white resin by rotating at room temperature. The resin was changed a further two times over a nine hour period and then stored at 4 °C for three days to complete infiltration process. Tissue and resin were brought up to room temperature before opening the tubes to prevent absorption of atmospheric water. Single pieces of tissue were then placed into gelatine capsules (size 0, TAAB) filled with LR white resin before being polymerised in a 50 °C oven until hardened for 2-3 days. Sectioning of the tissue blocks and immunolabelling was carried out at Syngenta, Jealott's Hill by Dr. Judith Sheldon.

Sections were blocked in a 1/20 dilution of normal horse serum in buffer (tris buffered saline: 5mM TRIS; 14.5mM NaCl; 1mM CaCl; 2mM MgCl

at pH7.8) for 30 min. The blocking solution was taken off and sections incubated with a 1/10 dilution of LM20 monoclonal antibody (Verhertbruggen et al, 2009) in 1/20 normal horse serum in buffer for 1h at room temperature. Sections were washed in buffer prior to incubation for 1h with a Goat anti-Rat IgG (H+L) secondary antibody with Alexa Fluor® 568 conjugate, diluted 1/200 in buffer which again contained normal horse serum. After washing in buffer sections were counterstained with a 0.025% solution of calcofluor white, washed, and mounted in Citifluor anti-fade mountant. Sections were viewed using a Zeiss 780 confocal microscope (Figure 6.2).

6.3 Results and Discussion

6.3.1 Determination of solubility

The levels of water, CDTA and carbonate soluble pectins that could be extracted from the control and PL:RNAi cell wall material (CWM) are presented in (Figure 6.1). Water soluble pectin (WSP) consists of pectic polymers which are loosely bound to the cell wall through non-ionic and non-covalent bonds, while CDTA (chelator) (CSP) soluble pectin mainly contains ionically cross-linked pectin, and Na_2CO_3 (carbonate) soluble pectin is predominantly bound to cell wall polysaccharides through covalent ester bonds (Selvendran, O'Neill, 1987; Sila *et al.*, 2006).



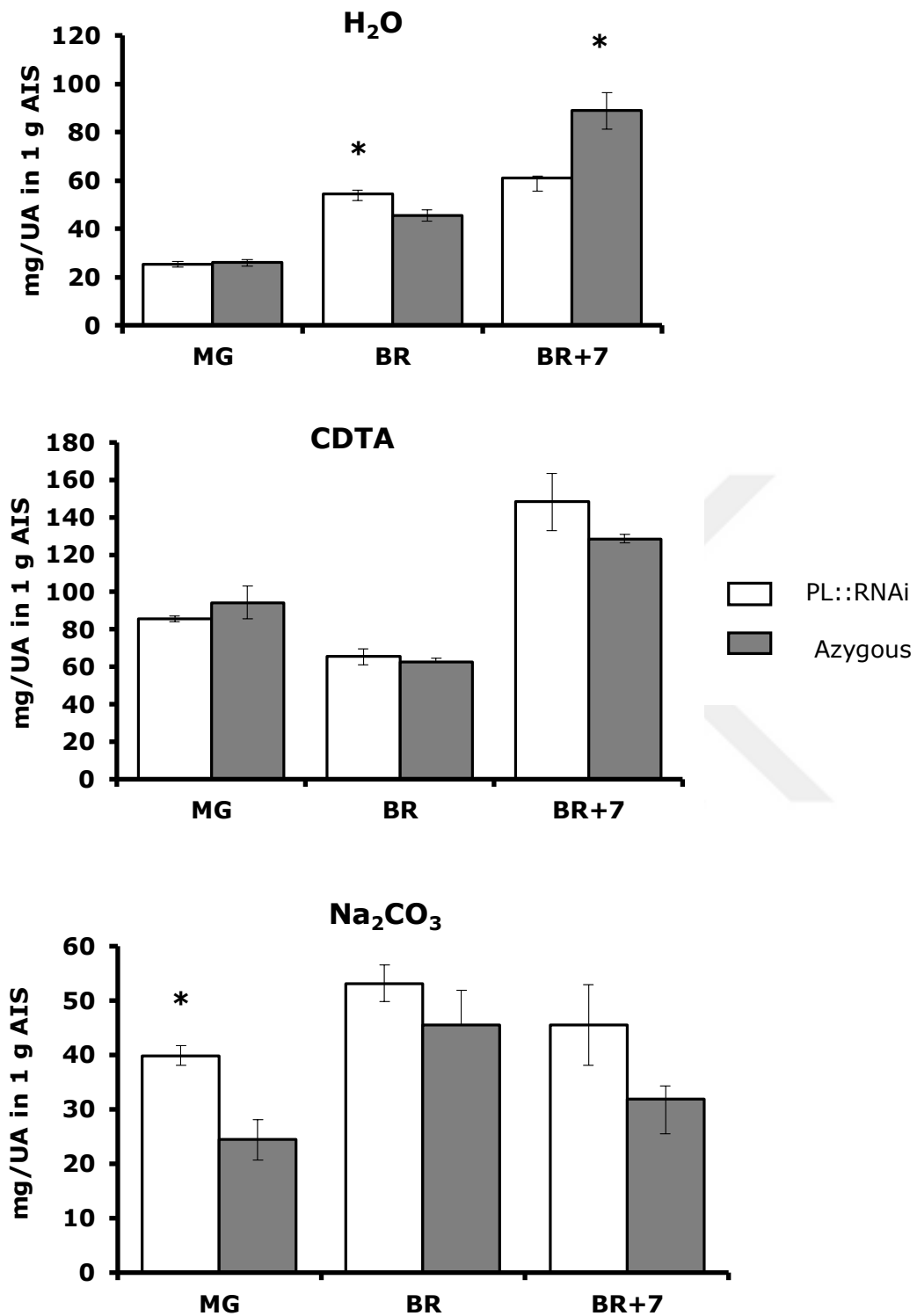


Figure 6.1 Fractionation of cell wall material from mature green (MG), breaker (Br) and breaker +7(Br+7) fruit of azygous (5-6) control and PL: RNAi (5-5) lines using water, the calcium chelator CDTA and sodium carbonate. Significant ^{*}(P=0.05) differences between the azygous extracts and those of the PL:RNAi treatment are marked by a asterics. SEM, n=3 (biological and technical replicates).

The fractionation of the CWM revealed a significant ($P < 0.05$) difference between the levels of WSP released by the azygous vs the PL:RNAi fruits at the breaker and breaker+7 stage, and Na_2CO_3 released by the same lines at the mature green stage. There were no other statistically significant effects of the RNAi construct detected in either the CSP or carbonate fractions, although there was evidence of lower levels of uronic acid that was extracted with carbonate from the azygous lines at breaker+7. Pectin solubilisation is commonly associated with ripening in tomato (Seymour *et al.*, 1987) and it was unaffected by silencing PG gene expression (Smith *et al.*, 1990). However the data indicated that PL plays a role controlling pectin solubility. It is possible that the trend toward lower levels of carbonate soluble pectins in the azygous lines reflects the source of some of the pectins that become water soluble.

6.3.2 Determination of MW of soluble pectin

In addition to reduced solubility, analytical ultracentrifugation experiments demonstrated that in Br+7 PL RNAi fruit the WSP had significantly ($P < 0.05$) higher weight average molecular weight (Table 6.1); c.158 KDa vs 89 KDa for the PL:RNAi. A weight average molecular weight of 158 KDa is similar to those previously reported from unripe tomato fruits (Seymour and Harding, 1987). This indicates that PL is necessary for normal pectin degradation. Also changes in weight average molecular weight in the carbonate fraction are much more pronounced in the azygous line consistent with the hypothesis that this may be one of the sources of soluble pectin normally released through PL action. Interestingly, in the RNAi PL fruits, solubilisation and

depolymerisation of WSP was inhibited even in the presence of normal levels of PG suggesting, PL action is a prerequisite for pectin depolymerisation by PG (Table 6.1).

Table 6.1 Weight average molecular weight of water, CDTA and carbonate soluble pectins extracted from cell wall material derived from azygous or PL::RNAi fruits at breaker +7. Where the values for the PL::RNAi line are significantly ($P < 0.05$) different from the control they are marked with a star. ($P = 0.011$ for H_2O ; $P = 0.078$ for CDTA; $P = 0.388$ for Na_2CO_3) SEM, $n = 3$

Sample	Apparent Weight Average Molar Mass (kDa)		
	Water	CDTA-S	Na_2CO_3
PL:RNAi	158.43(± 10) *	125.3 (± 10)	174.73 (± 64)
Azygous	89.6(± 12)	165.6 (± 14)	110 (± 13)

6.3.3 Changes in cell wall structure as determined by light Microscopy

Light microscopy imaging the pericarp cell walls with calcofluor white to visualise the cellulose domains and with a monoclonal antibody against pectin polysaccharides revealed greater cell-to-cell adhesion and more intense labelling with the pectin antibody probe especially at the cell junctions in PL:RNAi fruits (Figure 6.2). This is entirely consistent with the PL being involved in 'dissolving' pectin between cells and especially at cell junctions (Willats *et al.*, 2001), leading to cell separation and softening.

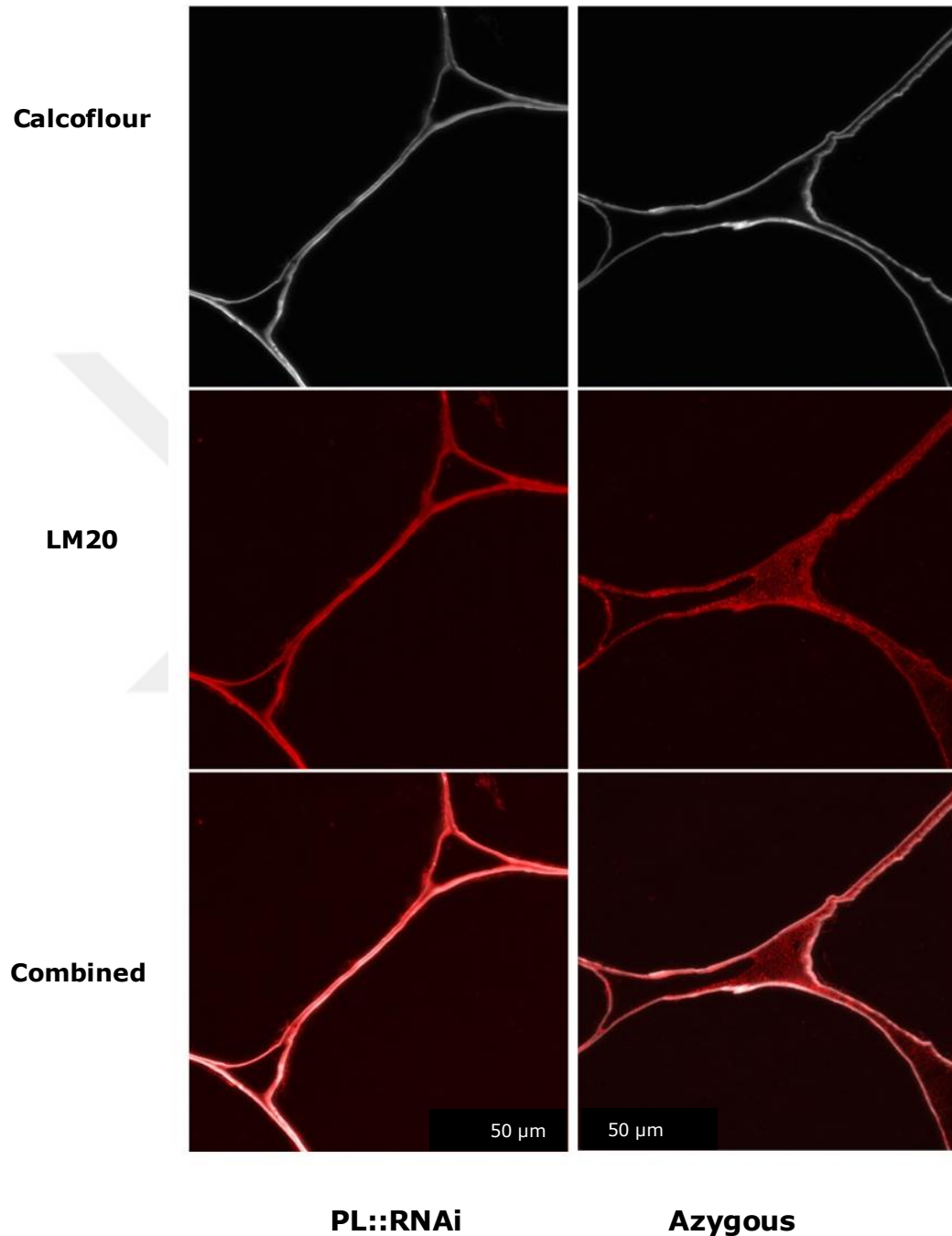


Figure 6.2 Pericarp cell walls from Azygous (5-6) and PL:RNAi (5-5) lines at Br+7 stage, labelled stained with calcofluor or labelled with LM20. This antibody recognises the homogalacturonan domain of pectic polysaccharide and requires methyl-esters for recognition of homogalacturonan, and does not bind to un-esterified homogalacturonan.

6.3.4 Crosses between PL:RNAi and a line where PG has been silenced using an antisense approach

To investigate synergistic or additive effects of combining PL silencing with inhibition of other cell wall degrading enzymes, crosses between were made with PL:RNAi lines and that expressing antisense transgenes to PG. The antisense lines were obtained from Dr. Ann Powell at University of California, Davis. We relied in these experiments on the dominant nature of the gene silencing effects. The texture of the F₁ fruits was analysed and a clear trend was apparent, in both outer and inner pericarp, and especially in the inner pericarp, the presence of the PL transgene and crosses with PL resulted in a significantly ($P < 0.05$) enhanced pericarp firmness (Figure 6.3).

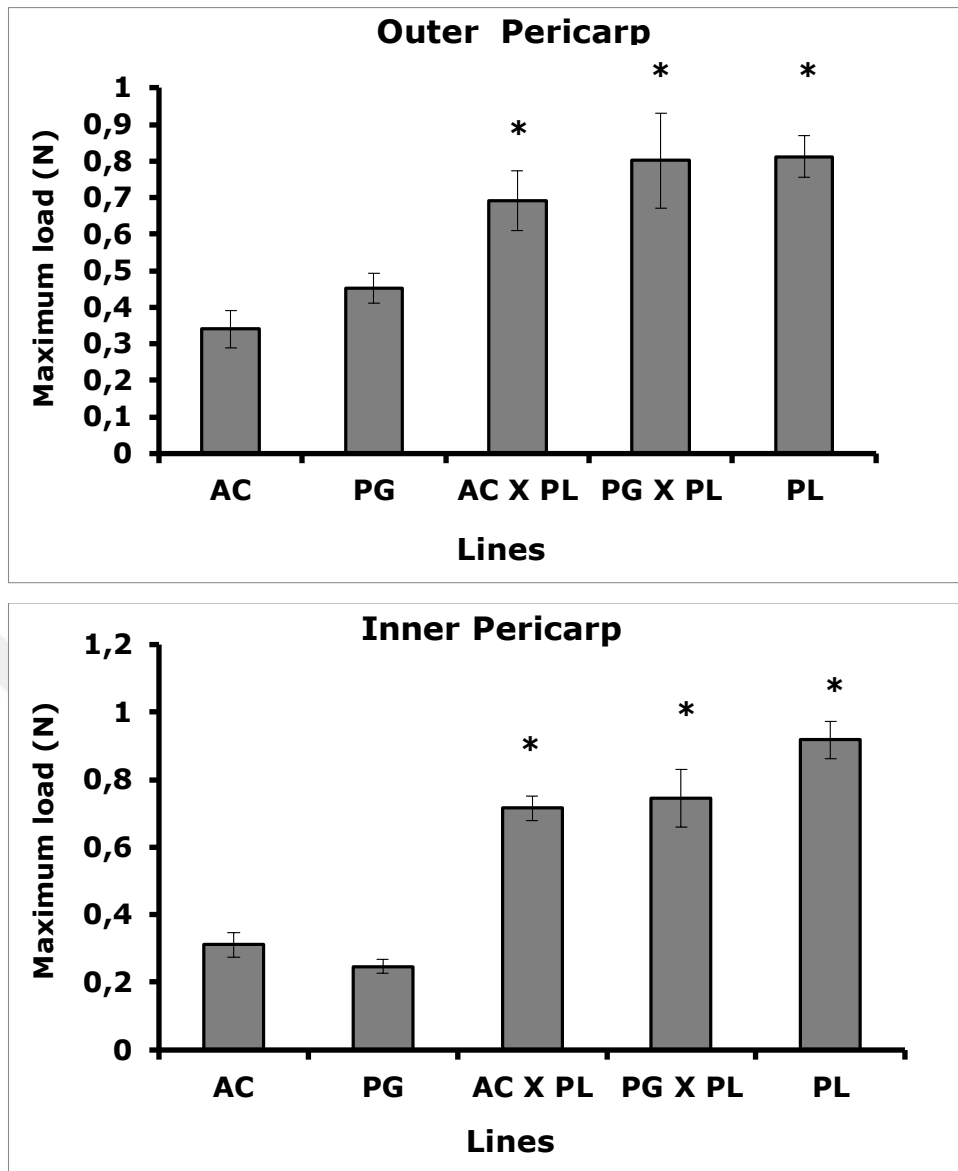
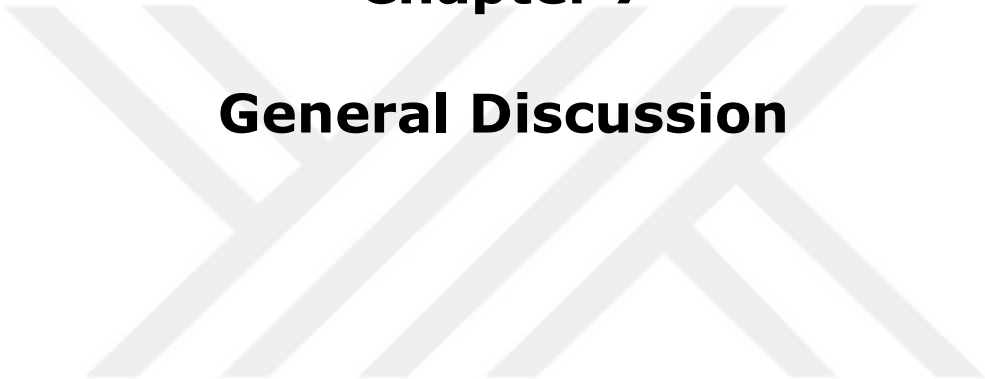


Figure 6.3 The effect of crossing a PL:RNAi line with a PG antisense on outer and inner pericarp firmness at breaker+7. AC (Ailsa Craig), PG (Antisense Polygalacturonase), ACxPL (ACxPL cross, PGxPL (PG-PL cross), PL (PL::RNAi). Stars show significant differences between lines and AC control. The presence of the PL transgene and crosses with PL resulted in a significantly $^{*}(P<0.05)$ enhanced pericarp firmness SEM, n=10 (biological replicates)

6.4 Conclusion

The mechanism of action of PL is on the pectic polysaccharide fraction and appears to be important for cell separation during ripening in tomato. Also PL is necessary for pectin depolymerisation and is important for solubilisation of pectins from the carbonate fraction which is thought to represent those pectic polymers that are more tightly bound to the cell wall. Combined silencing of PG with PL had no additional effects on fruit texture.





Chapter 7

General Discussion

7 General Discussion

Tissue softening is one of the most important changes that occur during the ripening of a fleshy fruit. It impacts organoleptic quality, shelf life and pathogen resistance. Moreover, the maturation of tomato fruit has received significant attention because of the fruits nutritional value and acceptance as a model system for studying fruit development and ripening (Giovannoni, 2004). However it has proved difficult to decipher the mechanistic basis of fruit texture changes in tomato. Silencing of highly expressed cell wall remodelling genes such as PG or PE has had little impact on texture phenotypes (Smith *et al.*, 1990; Hall *et al.*, 1993). The aim of this project was to investigate aspects of the genetic basis of fruit texture changes in tomato and specifically the gene underlying a major fruit texture QTL. This information should provide new approaches to breeding better tasting tomatoes that also have the extended shelf life needed for the modern supply chain.

The genomics revolution has provided the impetus for new approaches to understand the genetic basis of complex traits such as fruit texture. The trait has proved challenging even with tomato and all the genetic resources that are available (Seymour *et al.*, 2013). In this study, recently published tomato genome sequence (The Tomato Genome Consortium, 2012) was used to identify the gene under a texture QTL on chromosome 3. Then validate the function of that gene with RNAi technology.

Previous work has reported texture quantitative trait loci on tomato chromosomes, 2, 4 and 9 (Chaip *et al.*, 2007; Chapman *et al.*, 2012). The texture effects in all these cases are relatively small, however the publication of the tomato genome sequence (Tomato Genome Consortium, 2012) allows the genes underlying QTL to be identified and candidate genes nominated. On chromosome 2, candidate genes underlying a complex texture QTL were nominated as an ethylene response factor (ERF), and a PME (Chapman *et al.*, 2012). This QTL has now been introgressed into elite breeding lines and its market potential is being evaluated by an industrial partner.

Using the *S.pennelli* tomato wild species introgression lines (IL) we were able to demonstrate that a PL resides under a major quantitative trait locus (QTL) for firm fruit texture on tomato chromosome 3, IL3-4 and that the QTL is additive. Our texture QTL on chromosome 3 was mapped to 1 Mb region which contains 120 gene models with PL being the only cell wall remodelling gene in this region. Also in IL3-4 there was a substantial difference in PL expression when compared with M82 with very low PL expression in the introgression line associated with firmer fruits (Figure 4.9). For this reasons, PL was nominated as the QTL candidate gene. Therefore using the latest genetic and genomic information we have been able to identify a texture candidate gene under a QTL and our transgenic studied validated this gene as the genetic locus responsible for the fruit texture effects. The texture of red ripe fruits with low PL activity is up to 3 fold firmer than controls.

The sequence information from the *S.pennellii* genome assembly (<http://www.tomatogenome.net/> The Tomato Genome Consortium, 2012) was used to provide information on the nature of the likely genetic change responsible for the expression differences between *S. lycopersicum* and *S.pennellii*. Comparison of the *S. lycopersicum* and *S. pennellii* PL promoter region revealed several in/dels between M82 and IL3-4 and may reveal the cause of the low PL gene expression in IL3-4 (Table 7.1). These deletions/insertions may be in cis-regulatory elements and if so they could disrupt transcription factor binding to the PL promoter in *S.pennellii* reducing PL expression.

A number of ripening related transcription factors (TFs) have recently been identified of which *RIN* is necessary for normal ripening (Vrebalov *et al.*, 2002). Direct *RIN* target genes are involved in cell wall modification, ethylene signalling pathways and aroma-flavour formation (Fujisawa *et al.*, 2011). It has been revealed that *RIN* mostly binds to the cis-regulatory elements which contain the C-A/T-rich-G (CArG) box (Ito *et al.*, 2008). Table 7 indicates that similar motifs occur in the PL promoter region and show sequence differences between *S.lycopersicum* and *S.pennellii*. Moreover, a recent study comparing differential gene expression patterns between wild type and *rin* mutant identified in PL gene (*Solyc03g111690*) as being a likely *RIN* target, along with PG (Zhong *et al.*, 2013, Supplemental Data Table 9 of Zhong's paper).

Table 7.1 A comparison of 1.5 kb of the PL regulatory region between *S.lycopersicum* and *S.pennellii*

The bp distance on the chromosome	In/del bp with first sequence shown that of <i>S.lycopersicum</i>	Position from first ATG = -xxx bp
SL2.40ch03:56396588 ..56396591	deletion ACCC -> ACC	-43
SL2.40ch03:56396196 ..56396197	deletion TC -> T	-434
SL2.40ch03:56396146 ..56396149	deletion CAAA -> CAA	-484
SL2.40ch03:56396101 ..56396120	deletion TATTATAGCAAATAAATTAT -> TATTAT	-529
SL2.40ch03:56395995 ..56395995	insertion T -> TG	-635

The QTL mapping and transgenic experiments provided compelling evidence that PL gene expression has a large impact on tomato fruit softening. It is now important to understand the mechanism of PL action. To do this we must re-examine the action of PL in the context of what we already know about cell wall remodelling.

7.1 PL in transgenic fruits and its mechanism of action

The solubilisation and degradation of cell wall pectic polysaccharides are events common to a wide range of fleshy fruits (Seymour *et al.*, 2013). Pectins are a ubiquitous component of higher plant cell walls and are thought to play an important role in cell-to-cell adhesion (Willats *et al.*, 2001) (Figure 7.1). As cells expand, the corners of three-way junctions take much of the stress in keeping cells stuck firmly to their neighbours. Pectins are often preferentially deposited in the corners of three way junctions, as are some classes of hydroxyproline rich glycoproteins (Willats *et al.*, 2001). In tomato and other fruits, ripening results in cell separation in the pericarp tissues and it has long been thought that enzymes responsible for modifying pectin structure are responsible for these changes. However the exact mechanisms have proved somewhat elusive.

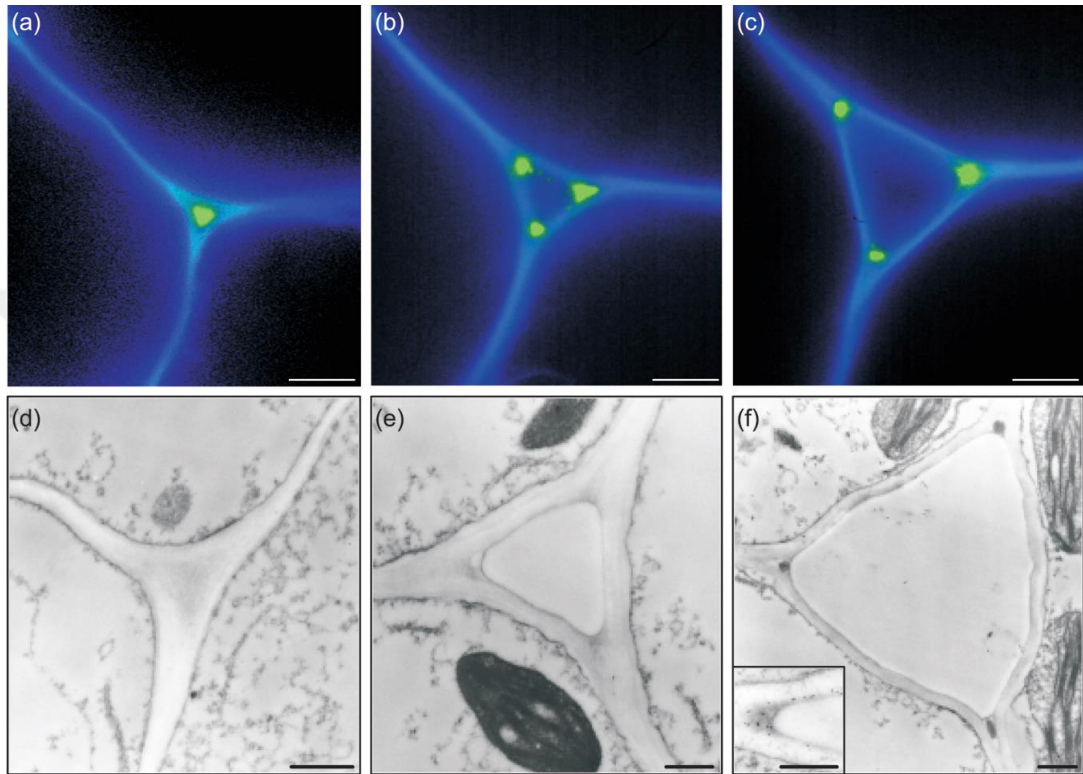


Figure 7.1 As cells expand, the corners of three-way junctions take much of the stress in keeping cells stuck firmly to their neighbours (a-c). Immunofluorescent labelling, with LM7 monoclonal antibody (green), shows homogalacturonan with low degree of random esterification and, the cellulose-binding fluorophor calcofluor (blue) cellulose in relation to intercellular spaces at different stages of formation. The junction shown in (d) consists entirely of cell wall material, whereas junctions at positions near the middle of the cortex are progressively separated resulting in the formation of intercellular spaces (e and f). Immunocytochemical results, using antibodies to a range of molecules that help to provide particularly strong adhesion between cells at their junctions, suggest that pectic polysaccharides are often preferentially deposited in the corners of three way junctions, as are some classes of hydroxyproline rich glycoproteins (adapted from Willats *et al.*, 2001)

Polygalacturonase was the first pectin degrading enzyme investigated in detail in ripening fruits. Its activity increases dramatically at the onset of normal ripening in tomato, while levels in non-ripening mutants remain low or undetectable (Hobson, 1964). In a ground breaking study the tomato PG gene was cloned and its expression inhibited by an antisense RNAi approach. (Smith *et al.*, 1988). Although PG activity was reduced to as low as 1% of normal levels, there was no significant effect on fruit softening. However pectin depolymerisation was significantly inhibited (Smith *et al.*, 1990). Pectin methylesterases (PME, EC 3.1.11), which demethylate pectins are highly active in developing and ripening tomato, but as with PG silencing of the major isoform PE2 had no effect on texture (Hall *et al.*, 1993). Indeed recent work (Phan *et al.*, 2007) has shown that silencing of *Pmeu1* gene leads to softer fruits.

The solubilisation of pectic polysaccharides in tomato has long been known to be associated with loss of galactosyl residues likely associated with side chains on these polymers (Seymour *et al.*, 1990). An exo- β -galactosidase enzyme was associated with these events (Pressey, 1981) and silencing of the gene, *TBG4*, encoding this enzyme resulted in red ripe fruit that were 40% firmer than controls (Smith *et al.*, 2002). The only other cell wall protein other than TBG4 that has been recorded to have an effect on fruit texture in tomato is the ripening-related expansin, Exp1. Silencing of the Exp1 gene resulted in a small enhancement of fruit firmness (Brummell *et al.*, 1999). Suppression of endo-1,4-beta-glucanase (cellulase, EC 3.2.1.4) *Cel2* (Brummell *et al.*, 1999) and *LeEXGT1* (Asada *et al.*, 1999), *LeXETB1* (de Silva *et al.*,

1994) which are 2 XET genes involved in hemicellulose modification failed to impact changes in fruit texture. We can contrast the limited effects of silencing PG, PME, β -gal and Exp with the very large impact of silencing PL. Antisense *Pmeu1* tomato fruits are significantly firmer between Br+3 and Br+8 ($p=0.019$) stages than wild type. PL also has proved important in ripening strawberry (Jimenez-Bermudez *et al.*, 2002). These results from previous studies are summarised in Figure 7.2



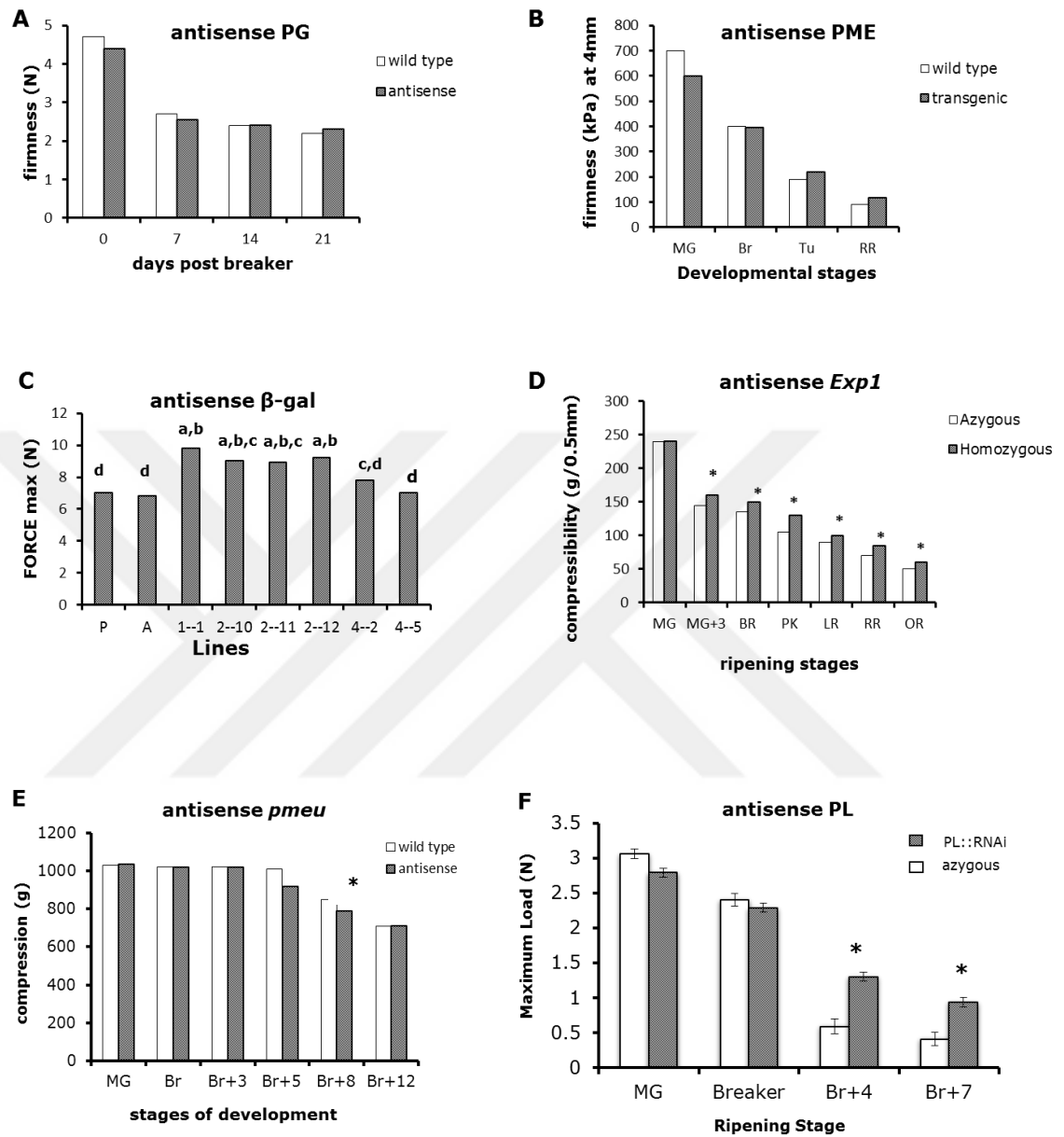


Figure 7.2 Down-regulation of cell wall related genes and their effects on fruit softening. **A:** Solyc10g080210 (*pg2a*) (Smith *et al.*, 1988) **B:** Solyc07g064170 (*PME*) (Tieman *et al.*, 1992) **C:** Solyc12g008840 (*beta-galactosidase4*) (Smith *et al.*, 2002), **D:** Solyc06g051800 (*exp1*) (Brummell *et al.*, 1999), **E:** Solyc03g123630 (*pectinesterase*, *pmeu*) (Phan *et al.*, 2007), **F:** solyc03g111690(PL). Significant (* $P < 0.05$) difference in fruit firmness between treatments are shown with asterisks where data was available.

Suppression of PG activity inhibited pectin depolymerisation; however these fruits showed normal pectin solubilisation and softening. It can be concluded that PG is necessary for a major portion of polyuronide degradation, but not for tomato softening (Smith *et al.*, 1990). Interestingly in our study the absence of PL lead to complete inhibition of depolymerisation indicating that PL is required for PG action leading to pectin depolymerisation.

In comparison with green unripe tomato fruits, at the red ripe stage normally ripening Ailsa Craig can show a reduction in fruit firmness of around 90%. In our RNAi PL lines the red ripe fruit retained about 40% of their 'green' texture as measured by determination of maximum load. This shows that PL accounts for around 30% of the softening in the pericarp tissues and therefore plays an important role in this process in tomato. The light and immunocytochemistry studies with the pectin antibody indicated that the middle lamella of the pericarp cells was normally highly degraded resulting in cell separation. This loss of cell adhesion failed to occur in the PL RNAi lines. These observations demonstrate that PL must be important in solubilizing pectins in the middle lamella region. The absence of pectin depolymerisation in the RNAi PL lines, even in the presence of normal PG activity indicates that PL is necessary for polyuronide degradation. However, what accounts for the softening effects that are not directly caused by PL action?

New insights into the relationship between fruit softening and cell wall remodelling that occurs during ripening must take into account the

structure of the cell wall itself. As it has been mentioned in Chapter 1, cellulose/hemicellulose contributes to the cell wall strength and tissue integrity along with the pectin polymer networks. Therefore, it is critical to consider the modification of polymer networks as a whole, rather than individual polymers and their degradation by cell wall enzymes/proteins. Evidence indicates that exo-galactanases (EGases) has an impact during early ripening, decreasing wall porosity to prevent other wall hydrolases to access wall components (Brummell and Harpster 2001). The task of expansin may be to promote conditions that favour the action of hydrolyses, such as EGases (*LeCel1* and *LeCel2*) or XTHs. For example, overexpression of *LeExp1* is correlated with cell wall hemicellulose depolymerisation, and occurs even in MG fruits (Brummell *et al.*, 1999a). In summary, cell wall modifying enzymes share their duties on direct degradation of polysaccharides and modification of polysaccharide side chains. So far, the overall gene manipulation studies suggest that there is cooperation between various enzymes in which the action of one enzyme is necessary for the action and access of other enzymes.

It is likely that efficient degradation of pectic polysaccharides requires cooperation of enzymes responsible for cleaving the set of different linkages in pectin chains. PG, PL and RGases might be needed to influence the action of PME or AE (acetylsterase) which remove backbone substituents. Silencing β -gal expression, which had a modest effect on fruit softening, may be important in releasing pectins for PL and PG action, although as the data suggest PLs is also involved in releasing tightly-bound carbonate soluble pectins from the cell wall. Rose and Bennett (1999)

suggested a potential cooperation between cellulose/hemicellulose and pectin degrading enzymes. For example, overexpression of PG in *rin* mutant which undergoes minimal softening caused increased depolymerisation of hemicellulose rather than the expected pectin depolymerisation. On the contrary, over expression of Exp which is responsible of cellulose/hemicellulose depolymerisation, showed increased pectin degradation. The previous studies on PG and β -gal along with current work on PL indicate that softening is likely the result of the synergistic actions of many cell wall enzymes (Figure 7.3).

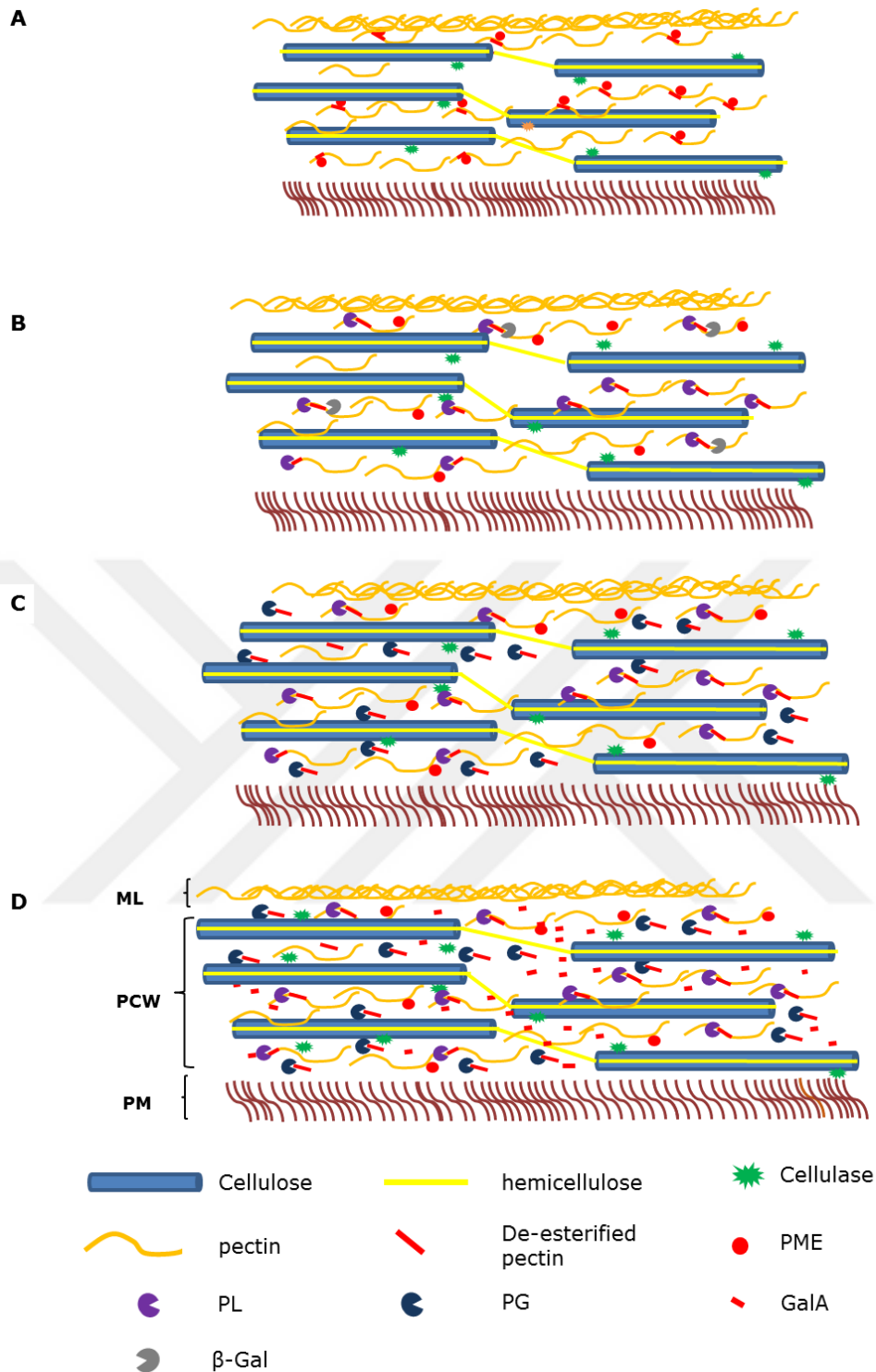


Figure 7.3 Cellulose microfibrils (blue bars) linked with hemicellulose (yellow lines) within a matrix of pectin (light brown). **A)** PME de-esterifies pectic homogalacturonan (HGA) backbone. **B)** Long chain solubilized from the wall by PL (purple) and β -Gal action. β -Gal might prepare pectin for PL action. **C and D)** In the final stage, soluble pectin molecules are degraded further by PG **ML**= middle lamella, **PCW**=primary cell wall, **PM**=plasma membrane

Different fruit types employ different combinations of enzymes for softening

Silencing of PG in strawberry provided reduced pectin solubilisation and increased firmness (Quesada *et al.*, 2009) but reducing PG levels in tomato had no effect on fruit softening (Smith *et al.*, 1990). In apple the effects of silencing PL gene expression have not been reported, but down-regulation of PG provided fruits higher intercellular adhesion than controls (Atkinson *et al.*, 2002; Atkinson *et al.*, 2012). This demonstrates fruits use different enzymic mechanisms to achieve changes in texture.

PME has two roles; removal of methyl groups that are necessary for PG action (Koch and Nevins, 1989), and formation of free carboxyl groups that facilitates calcium cross linking between adjacent pectic polysaccharides in cell walls (Fry, 1986). In tomato antisense transgenic PME1 fruits, an isoform of PME, softened faster than controls suggesting that this isoform acts to strengthen the cell wall through facilitating calcium cross linking (Phan, 2007). The role of PE in other fruits has been less extensively explored. Strawberry fruits with suppressed PL expression (Jimenez-Bermudez *et al.*, 2002) were significantly firmer than controls with lower degree of swelling, less pectin solubilisation and lower amount of ionically bound pectins representing a higher integrity of cell wall structure than controls. These data and those from the present study indicate that in both strawberry and tomato PL plays an important role in the softening process.

7.2 Conclusion

The present work is an important breakthrough because it is the first time substantial and specific control over tomato softening has been achieved. It also provides a starting point to re-evaluate the relationship between cell wall remodelling and texture changes. Modulating PL expression could have considerable advantages over the use of *rin* or delayed ethylene biosynthesis for enhancing product robustness because it selectively governs texture without impacting other ripening changes. The work opens up the possibility of breeding better tasting tomatoes with an extended shelf life to improve fruit quality and quantity in both developed and developing economies.

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9 Appendix

9.1 Speed DNA Extraction Buffer

1 M Tris HCL (pH 7.5)	200	mL
4 M NaCl	62.5	mL
0.5 M EDTA	50	mL
10% SDS	50	mL
(The recipe for 1 L)		



9.2 Primers used for genotype analysis, and transgenic plant

Sequences	Forward Primer 5'-3'	Reverse Primer 5'-3'
ST1257	TCTGTTTTACCTGGCGTTCTG	AATTGAGCTGCCAAATGAGG
G390	TTTCCTCTGGGGTTTCCTTT	GACTGTTGGGGCTTGAAATC
G570	GGGAGATGGATTTGCTTTCA	GGCCAGACTTTTGACCATGT
G690 (PL)	ACGCGTTTAATTGGACGTATG	AACAGAGGAAGTGCCCCATTG
G790	AAGCCAGAAAGCTTGCGTTA	GCTCAGCTTCAGTGCATTTG
G870	TTGAAAAGGTGCTGGGAACT	TGTACGCATCAAAGCTCGTC
G160	GCATGCACAATCAGTGAAGG	TCAAAGGGCTGAGGGTAGAA
ST0551A	AGCCTCACCGTATTCGTGTT	TGATCGAAGCACATCCAAAA

G600	ATTTGTTGATCGCTGGCTTC	CGACGATGGTCAGACCTTTT
G640	CAAACCTTTGCGATTCCATGA	TCTACTGGCAGGAAGGAGGA
G660	TGGTTGTTGACCATGAAAA	GGCCATTGAAGCGACATATT
G750	AGGTTGAATGGGAGTTGCAG	GCAGAAAACCGACTCAGAGG
G950	AACTTCAATGGGGCATATCG	TTTGCCACCCCTAATTGAAA
G020	TATATTTGCCCCAAGGCACT	ATCGGGACGGATAATGTCAC
ST1286	AAGGAATGGCAACTTTTGGA	ACCAAGGGTGATTCTGCAAC
ST0325A	ATCAATGGCGACTGCTTCTT	TGCCAATTGAAGAGGATGGT
G970	AAGCCAGAAAGCTTGCGTTA	GCTCAGCTTCAGTGCATTTG
qPL	GCGATCAGGAGTTAGAACTGG	AATCCCCCTTTTGCTTTGGTT
qPL probe	TTGAGTTTGAGTGCAAGGCCGTC	
attPL	GGGGACAAGTTTGTACAAAAAAGCAGGC TACGCGTTTAATTGGACGTATG	GGGGACCACTTTGTACAAGAAAGCTGGGTAA CAGAGGAAGTGCCCATTG
ELF-probe	TCGTTTTGCTGTGAGGGACATGAGGCA	
ELF-primer	ACCTTTGCTGAATACCCTCCATTG	CACAGTTCACCTCCCCTTCTTCTG
PDS-probe	TCTTTCCAAAAGTGCAGGTAACACTGC	
PDS-primer	CTAGGTTCTTGCTGCCTTGG	CCAACTTTTTGGCAATGCTT
35T(F)	GCAGGTCACTGGATTTTGTTTTAGGAA	
35P(R)	CCCACTATCCTTCGCAAGACCCTTCCTC	

9.3 PCR-DIG probe synthesis

Component	DIG labeled probe	Unlabeled DNA control	Labeled kit control	Final conc.
Water, PCR Grade	variable	variable	29.25 μ l	-
PCR buffer with $MgCl_2$ 10 $\times$ conc. (vial 3)	5 μ l	5 μ l	5 μ l	1 $\times$
PCR DIG Labeling Mix (vial 2)	5 μ l	-	5 μ l	200 μ M dNTP
dNTP stock solution (vial 4)	-	5 μ l	-	200 μ M dNTP
Upstream and downstream primer	5 μ l 5 μ l	5 μ l 5 μ l	5 μ l (vial 6)	0.1 – 1 μ M each
Enzyme mix (vial 1)	0.75 μ l	0.75 μ l	0.75 μ l	2.6 U
template DNA	variable	variable	5 μ l (vial 5)	1 – 50 ng gDNA 10 – 100 pg cDNA
Final volume	50 μl	50 μl	50 μl	

9.4 Tomato Transformation Protocol

9.4.1 Seed Germination

Seeds were surface sterilized in 50% Clorox + tween 20 for 30 minutes. Seeds then rinsed (3X) in 150-200 mL of sterile distilled water. Seeds were placed on agar solidified 1/2x Murashige and Skoog minimal organics medium (1/2x MSO) containing 15 g/liter sucrose, 10 gm Phytoagar (pH 5.6-5.8) and incubated for 7-8 days at 26 °C.

9.4.2 Agrobacterium Incubation

A glycerol freezer stock was prepared from a single bacterial colony isolated from a plate. 40-50 ul of the glycerol stock was used to inoculate 20 mL of MGL medium (pH 7.0) containing the appropriate antibiotics and incubated overnight at 28 °C in shaker at 250 rpm. The following day, 5 mL of the overnight growth was removed and transferred to 15 mL of TY medium (pH 5.5) containing the appropriate antibiotics plus 200uM acetosyringone. The culture was incubated overnight at 28 °C in shaker at 250 rpm. Following day, overnight culture is diluted by adding 1.5 mL of the bacterial culture to 20 mL of TY medium (pH 5.5), containing 200 uM acetosyringone.

9.4.3 Co-cultivation

Cotyledon pieces were cut and soaked in the Agrobacterium solution for 10 minutes. Cotyledon pieces were placed on co-cultivation medium consisting of Murashige and Skoog minimal organics medium (MSO) modified with 20 g/liter glucose, 1mM MES, 0.75mg/liter trans-zeatin, 1.0 mg/liter IAA, and 200 uM acetosyringone pH 5.6-5.8 and incubated at 23 °C.

9.4.4 Induction

After 3 days, the cotyledon pieces were transferred to induction medium consisting of MSO modified with 20 g/liter glucose, 1mM MES, 0.75mg/liter trans-zeatin, 1.0 mg/liter IAA, 400 mg/liter carbenicillin, 150 timentin, 100mg/liter kanamycin sulfate. After 10 days, the tissue was subcultured to MSO modified with 20 g/liter glucose, 1mM MES, 0.75mg/liter trans-zeatin, 0.0 mg/liter IAA, 400 mg/liter carbenicillin, 150 timentin, 100mg/liter kanamycin sulfate until shoots begin to form and incubated at 26 °C under a 16hr light 8 hours dark.

9.4.5 Elongation

Once shoots begin to form, developing shoots and callus were transferred to elongation medium consisting of MSO modified with 20 g/liter glucose, 1mM MES, 0.1mg/liter trans-zeatin, 400 mg/liter carbenicillin, 150 timentin, and 100mg/liter kanamycin sulphate and incubated at 26 °C under a 16hr light 8 hours dark.

9.4.6 Rooting

Once a shoot reaches 2-4 cm in height, the shoots were harvested and transferred to rooting medium consisting of MSO modified with 20 g/liter glucose, 1mM MES, 0.2mg/liter IBA, 400 mg/liter carbenicillin and 100 mg/ml kanamycin. Shoots gave roots in two weeks.

9.5 Southern Blotting

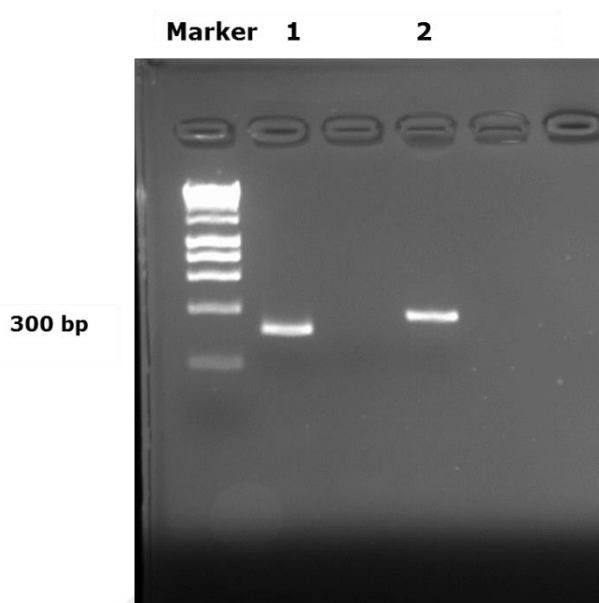


Figure 9.1 Lane 1: Plasmid control without DIG labelled. Lane 2: Genomic DNA DIG labelled. Genomic DNA DIG labelled product was slightly higher than plasmid control because of the DIG label.

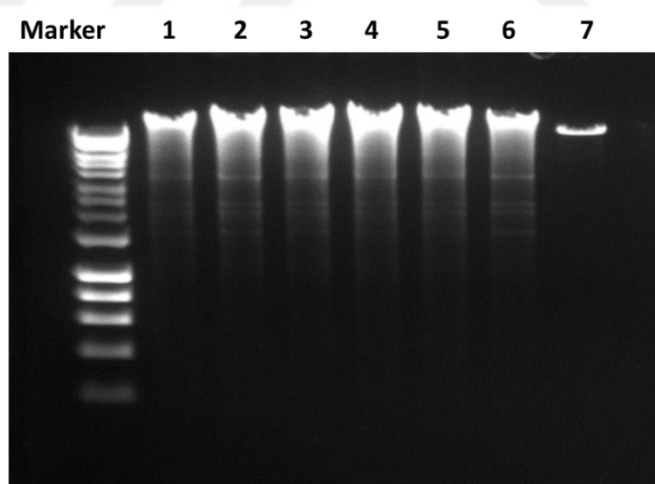


Figure 9.2 DNA was digested by SacI Lane 1: Transgenic line 2 (T2), Lane 2: T5, Lane 3: T6, Lane 4: T8, Lane 5 :T10, Lane 6: AC, Lane 7: Plasmid Control. M: Marker

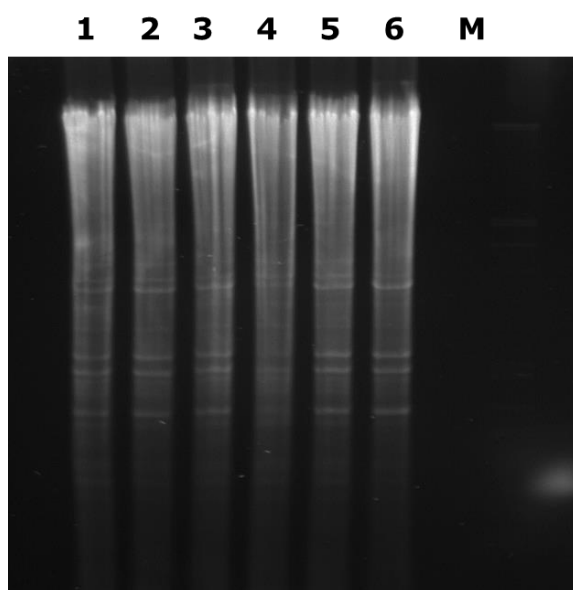


Figure 9.3 Separating DNA on gel **Lane 1:** Transgenic line 2 (T2), **Lane 2:** T5, **Lane 3:** T6, **Lane 4:** T8, **Lane 5:** T10, **Lane 6:** AC Control, **M:** Marker

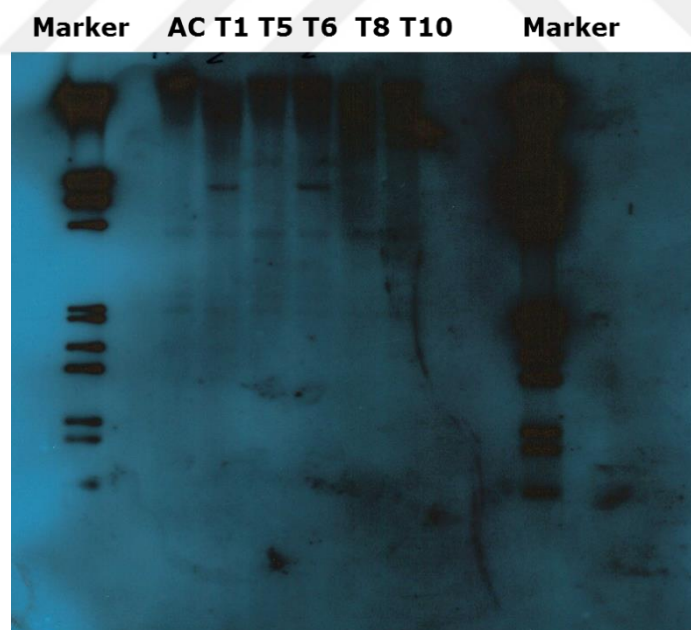
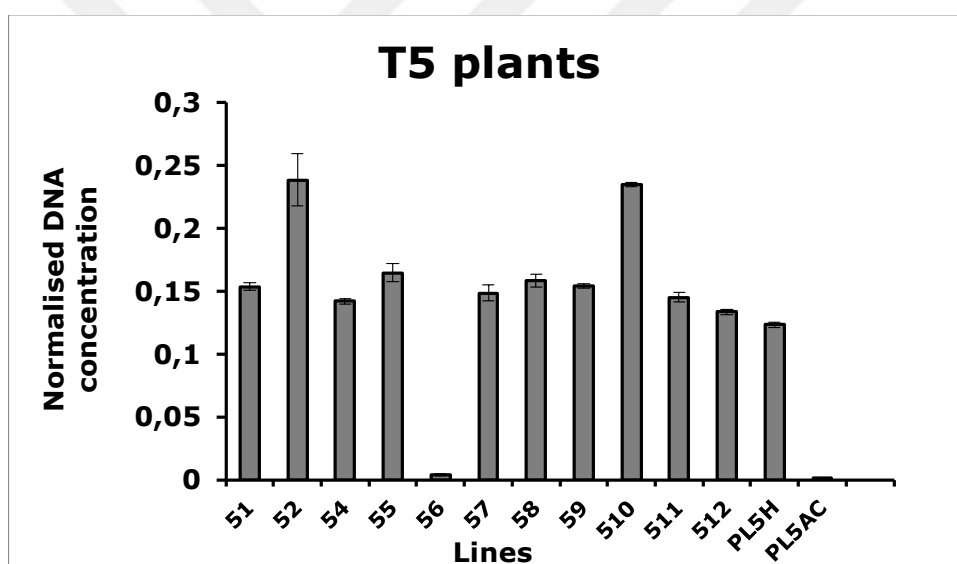
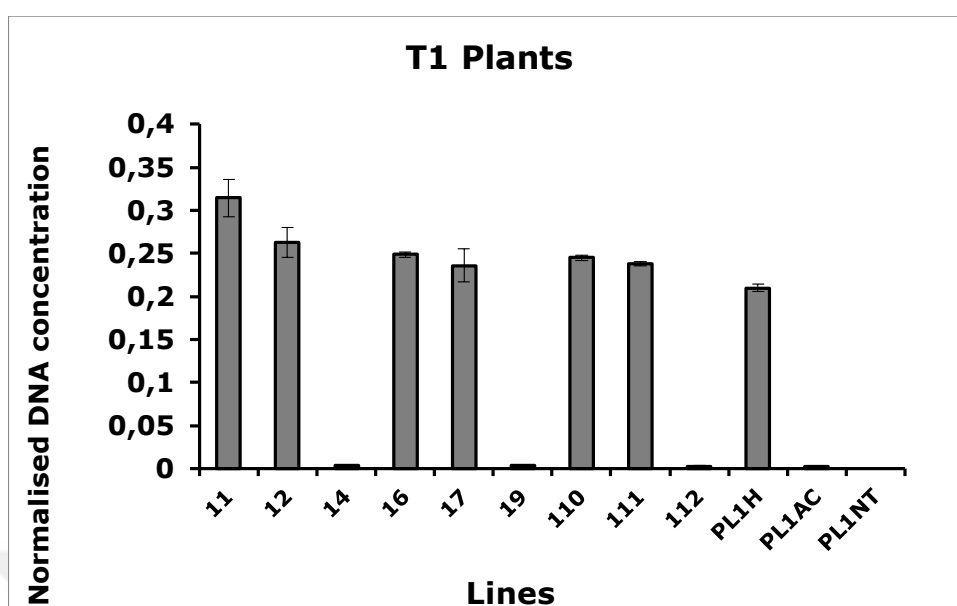
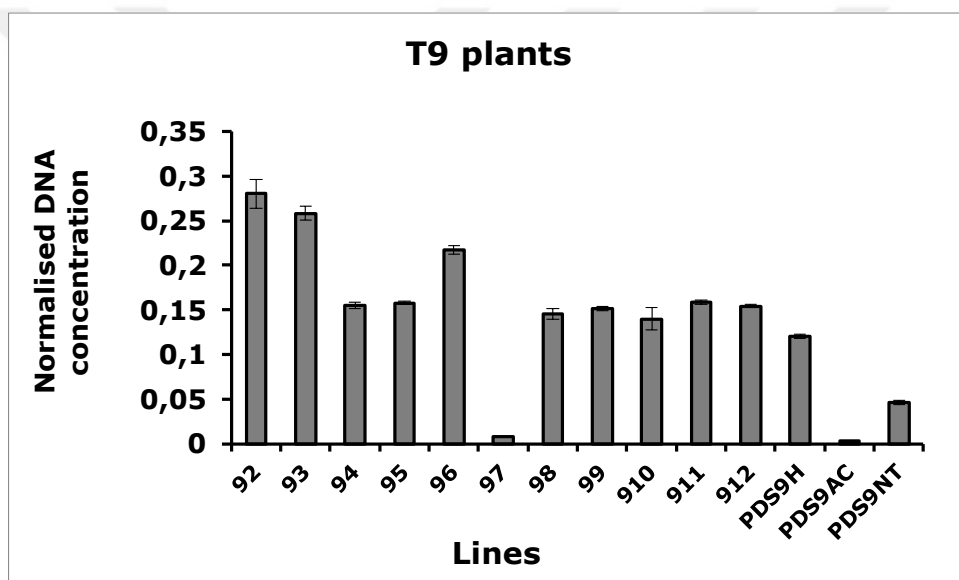
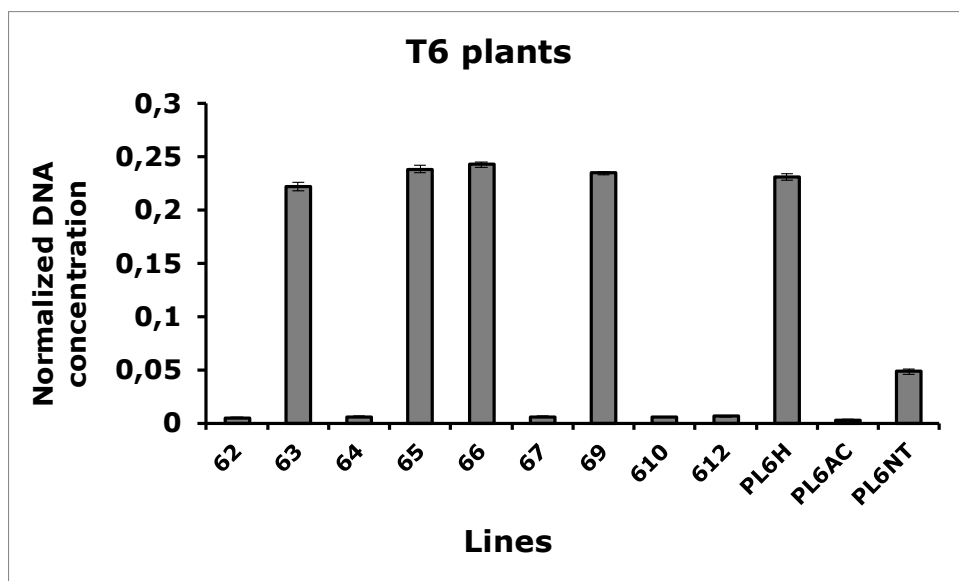


Figure 9.4 **Lane 1:** AC, **Lane 2:** Transgenic 1 (T1), **Lane 3:** T5, **Lane 4:** T6, **Lane 5:** T8, **Lane 6:** T10

9.6 Identification of homozygous lines





Homozygous lines identified by qPCR. High amplification levels compared to Heterozygous (H), and AC wild type, counted as homozygous line.

9.7 Carotenoids

Table Analyzed	Lutein	Lycopene	B-Carotene	Z-Carotene	G-Carotene	Phytofluene	Phytoene	a-Tocopherol
Column A	5-5	5-5	5-5	5-5	5-5	5-5	5-5	5-5
vs	vs	vs	vs	vs	vs	vs	vs	vs
Column B	T9-70	T9-70	T9-70	T9-70	T9-70	T9-70	T9-70	T9-70
Unpaired t test with Welch's correction								
P value	0.2293	0.0314	0.0334	0.6026	0.8175	0.2319	0.2589	0.001
P value summary	ns	*	*	ns	ns	ns	ns	**
Are means signif. different? (P < 0.05)	No	Yes	Yes	No	No	No	No	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
Welch-corrected t, df	t=1.230 df=27	t=2.275 df=26	t=2.241 df=27	t=0.5271 df=26	t=0.2330 df=27	t=1.224 df=26	t=1.153 df=27	t=3.803 df=21
How big is the difference?								
Mean ± SEM of column A	45.68 ± 1.178 N=15	173.6 ± 6.614 N=15	191.0 ± 7.317 N=15	56.08 ± 1.874 N=15	106.9 ± 3.492 N=15	71.27 ± 2.751 N=15	171.6 ± 7.861 N=15	75.60 ± 1.817 N=15
Mean ± SEM of column B	43.73 ± 1.059 N=15	198.4 ± 8.623 N=15	212.7 ± 6.370 N=15	54.80 ± 1.527 N=15	105.8 ± 3.008 N=15	75.49 ± 2.077 N=15	184.0 ± 7.328 N=15	90.31 ± 3.416 N=15
Difference between means	1.947 ± 1.583	-24.72 ± 10.87	-21.74 ± 9.701	1.274 ± 2.417	1.074 ± 4.609	-4.219 ± 3.447	-12.40 ± 10.75	-14.71 ± 3.869
95% confidence interval	-1.302 to 5.197	-47.06 to -2.376	-41.65 to -1.836	-3.695 to 6.243	-8.384 to 10.53	-11.30 to 2.868	-34.45 to 9.658	-22.76 to -6.667
R squared	0.05305	0.166	0.1569	0.01057	0.002006	0.05448	0.04695	0.4078
F test to compare variances								
F,DFn, Dfd	1.237, 14, 14	1.700, 14, 14	1.320, 14, 14	1.506, 14, 14	1.348, 14, 14	1.755, 14, 14	1.151, 14, 14	3.534, 14, 14
P value	0.6959	0.3323	0.6109	0.4532	0.5839	0.3045	0.7963	0.0244
P value summary	ns	ns	ns	ns	ns	ns	ns	*
Are variances significantly different?	No	No	No	No	No	No	No	Yes

PL and azygous carotenoids t-test

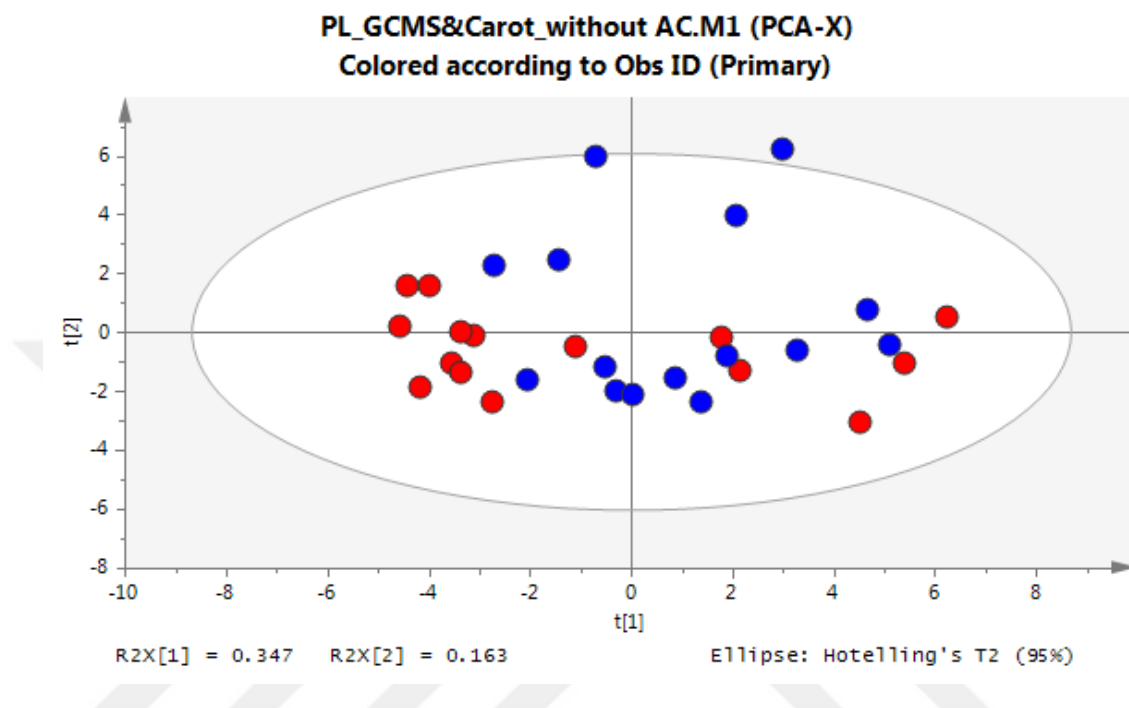
9.8 Sugars

Table Analyzed	Glucose	Fructose	Sucrose
Column A	5-5	5-5	5-5
vs	vs	vs	vs
Column B	T9-70	T9-70	T9-70
Unpaired t test with Welch's correction			
P value	0.745	0.4275	P<0.0001
P value summary	ns	ns	***
Are means signif. different? (P < 0.05)	No	No	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
Welch-corrected t, df	t=0.3294 df=22	t=0.8061 df=26	t=5.293 df=15
How big is the difference?			
Mean ± SEM of column A	9.172 ± 0.4521 N=15	3.219 ± 0.1133 N=15	0.02395 ± 0.002529 N=15
Mean ± SEM of column B	9.472 ± 0.7893 N=15	3.075 ± 0.1381 N=15	0.08849 ± 0.01193 N=15
Difference between means	-0.2996 ± 0.9096	0.1440 ± 0.1786	-0.06454 ± 0.01219
95% confidence interval	-2.186 to 1.587	-0.2233 to 0.5112	-0.09053 to -0.03855
R squared	0.004907	0.02438	0.6512
F test to compare variances			
F,DFn, Dfd	3.048, 14, 14	1.486, 14, 14	22.24, 14, 14
P value	0.0455	0.4684	P<0.0001
P value summary	*	ns	***
Are variances significantly different?	Yes	No	Yes

PL and azygous sugars t-test

9.9 PL-PCA analysis (GCMS&Carotenoids)

Score Plot

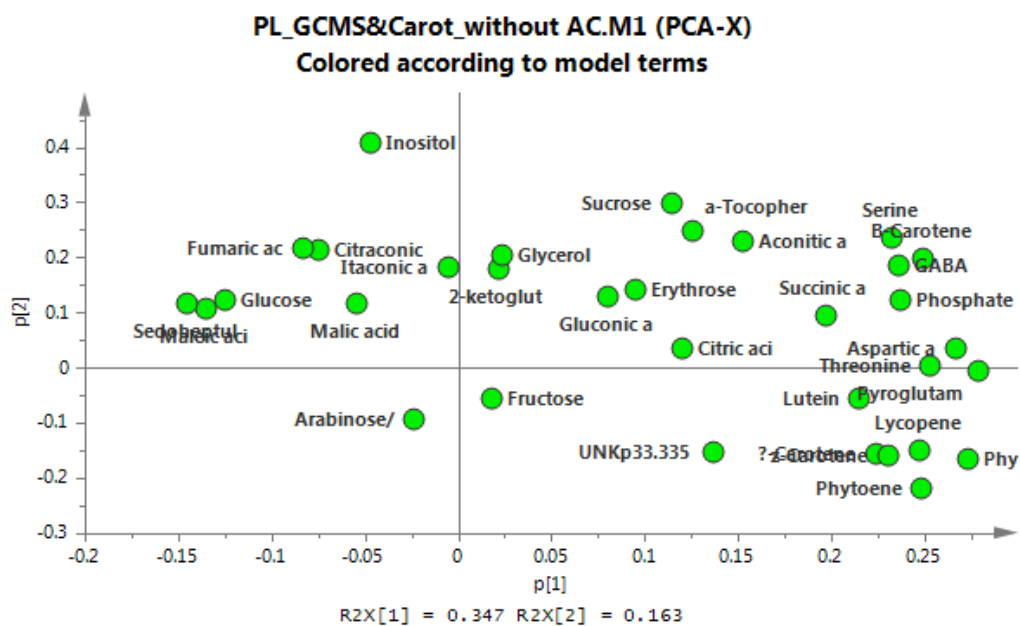


● 5-5

● T9-70

9.10 PL-PCA analysis (GCMS&Carotenoids)

Loadings Plot



9.11 Pectate lyase sequences

9.11.1 Protein sequence of PL

MGTSSVFLFLLSFLLLLPSLLASSNPQQVVDEVHRSINGSRRNLGYLSCGTGNPI
DDCWRCDPNWEKNRQRLADCAIGFGKNAIGGRDGKIYVVTDSGDDNAVTPKPG
TLRHAVIQTEPLWIIIFARDMVIQLKEELIMNSFKTIDGRGASVHIAGGPCITIYVT
NIIHGIHIHDCKQGGNAMVRSSPSHYGWRTVSDGDGVSIFGGSHVWVDHCSL
SNCKDGLIDAIMGSTAITISNNYMTHHDKVMLLGHSDTYTQDKNMQVTIAFNHF
GEGLVQRMPCRHRHGYFHVVNNDYTHWEMYAIGGSADPTINSQGNRFLAPDIRFS
KEVTKHEDAPESEWKNWNWRTDGDMLNGAFFTRSGVRTGSSSYAKASSLSAR
PSSLVANLVSSSGALNCKKGSRC

(<http://www.uniprot.org/uniprot/K4BK32>)



9.11.2 Gene sequence of PL

TATATAACTTAAACGCGTTTAATTGGACGTATGCTAGAGTAGTAATCATATGCTAACAAGAGATA
GATACTGTTACTTGAACAGACAAAACTACCGTAGTCTTCTTCTCTCTATCATCTAGGTTAGAGG
AATCTCCGTAAAACATGTAATCACGAAGGAACAAGGGACCCATCCCTTTTGTTTAATTACGCTAA
TTTCTCTACTTTTTCTTCTTTTATAAACTCCTCCATTTCCCTCTCTTTTCTTCATAACCCAAAAT
CCTCTGTTTTTTAAAGCTAATTTATAAAAACAAACAATGGGCACTTCCTCTGTTTTTCTACTATTCC
TTCTTTCTTTTCTTCTCCTTCTCCCGTCCCTCCTTGCCTCCTCTAATCCTCAACAAGTTGTCGAT
GAAGTACACAGGTACGTGCATTTTTTAATTTTTAATTTCAACATTGCATATTTGAAAAGGGAC
AGAGTCAGATAGGAACAAGGAGTTCATTTCGTATTAAAATAATTGCATATGGTAATAC TCATGTGT
ACATTATTATTATAATAATGCAGGAGTATAAATGGTTCAAGGAGGAATTTAGGGTATTTATCGTG
TGGTACCGGAAATCCAATCGACGATTGTTGGCGTTGTGATCCGAATTGGGAGAAAAACCGTCAGC
GGTTAGCTGACTGTGCAATTGGATTTGGGAAAAACGCTATTGGTGGTAGAGACGGTAAAACTAC
GTGGTGACCGATTTCGGGTGACGACAATGCCGTCACTCCGAAGCCGGGGACTCTCCGGCACGCAGT
GATTTCAGACTGAGCCACTGTGGATTATTTTCGCGAGGGATATGGTTATACAGTTGAAAAGAGGAAT
TAATTATGAATTCGTTTAAAACGATAGACGGAAGGGGCGCTAGCGTACACATAGCGGGTGGTCCG
TGTATAACAATACAGTACGTAACGAATATTATTATACACGGAATTCATATACATGACTGTAAACA
AGGTGGAATGCTATGGTGCGGAGCAGTCCATCGCATTACGGGTGGAGAAGTGTTCGGACGGTG
ATGGAGTGTCCATATTCGGTGGGAGCCATGTTTGGGTGGATCATTGTTCTTTGTCCAAGTGTAAAG
GACGGTCTGATTGATGCGATTATGGGGTCTACAGCAATAACCATTTCAAATAATTACATGACACA
TCATGATAAAGTTATGCTCTTGGGACATAGTGATACATATACTCAAGATAAAAACATGCAAGTAA
CTATAGCTTTTAAATCACTTTGGTGAAGGTCTTGTCCAAAGAATGCCAAGGTATGAGCCTGAGTTA
TTTAACTTTTATATACTGACAATTTTGACAATGTGAATTTTATGAATTATTATTTGGAATAAATG
CAGATGTAGACATGGTTACTTCCATGTGGTGAACAATGACTACACACATTGGGAAATGTATGCTA
TTGGTGGAAAGTGCTGATCCCACTATCAATAGTCAAGGGAACCGATTTCTTGCCCCTGATATCAGG
TTTAGCAAAGAGGTTAGTTAGTTATTTCACTTACATTTTTCTATCTAGTCTGTAAATGAGTTAAC
GGGTAATTTTATTATATTTATGTAGTTACAAAGCACGAGGATGCACCGGAGAGTGAATGGAAGA
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CTTGTGGTTTTTAGAGAGAGGCAAGCTAAGAAGTGTGATATATACATCAATATATATGTATTTA
TTTTTGA

- 5' UTR  CDS  3' UTR 

(http://59.163.192.91/tomato2/getGene.php?trans_fac_id=Solyc03g111690.2.1) (Tomato Genomic Resource Database)

9.11.3 PL into entry clone

ACATATGATTTTATTTTGACTGATAGTGACCTGTTTCGTTGCAACAAATTGATGA
GCAATGCTTTTTTATAATGCCAAC(TTTGTACAAAAAAGCAGGCTACGCGTTTA
ATTGGACGTATG)CTAGAGTAGTAATCATATGCTAACA**AGAGATAGATACTGT**
TACTTGAACAGACAAAACTACCGTAGTCTTCTTCTCTCTATCATCTAGG
TTAGAGGAATCTCCGTAAACATGTAATCACGAAGGAACAAGGGACCCA
TCCCTTTTGTTTAATTACGCTAATTTCTCTACTTTTTCTTCTTTATAAACT
CCTCCATTTCCCTCTCTTTTCTTCATAACCCAAAATCCTCTGTTTTTAAAG
CTAATTTATAAAACAAACAATGGGCACTTCCTCTGTTACCCAGCTTTCTTGT
ACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGG
TCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGATATCCCCTATAG
TGAGTCGTATTACATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCTCA
AAATCTCTGATGTTACATTGCACAAGATAAAAAATATATCATCATGAACAATAAAA
CTGTCTGCTTACATAAACAGTAATACAAGGGGTGTTATGAGCCATATTCAACGG
GAAACGTCGAGGCCGCGATTAAATCCAACATGGATGCTGATTTATATGGGTA
TAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGT
ATGGGAAGCCCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTT
GCCAATGATGTTACAGATGAGATGGTCAGACTAACTGGCTGACGGAATTTAT
GCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACT
CACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAGATA

Pectate lyase gene sequence analyzed from entry clone and shown in bold. The sequence performed with 2G111690.2F primer and the region delimited by brackets“()”. Remaining sequence belonging to entry vector.

9.11.4 PL into destination vector

TAGAAGTATTTTACAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACAC
ATGAGCGAAACCCTATAAGAACCCTAATTCCCTTATCTGGGAACTACTCACACA
TTATTCTGGAGAAAAATAGAGAGAGATAGATTTGTAGAGAGAGACTGGTGATT
TTTGCGGACTCTAGCATGGCCGCGGGATATCACAAG(TTTGTACAAAAAAGCA
GGCTACGCGTTTAATTGGACGTATG)CTAGAGTAGTAATCATATGCTAACAAG
AGATAGATACTGTTACTTGAACAGACAAAAACTACCGTAGTCTTCTTCTC
TCTATCATCTAGGTTAGAGGAATCTCCGTAAAACATGTAATCACGAAGG
AACAAGGGACCCATCCCTTTTGTTTAATTACGCTAATTTCTCTACTTTTTC
CTTCTTTATAAACTCCTCCATTTCCCTCTCTTTTCTTCATAACCCAAAATC
CTCTGTTTTTAAAGCTAATTTATAAAACAAACAATGGGCACTTCCTCTGTT
ACCCAGCTTTCTTGTACAAAGTGGTGATATCACTAGTGCG

Gene construct 35S::PL sequence was analysed from expression clone and shown in bold. The sequence performed with 35T-FW primer and the region delimited by brackets "()". Rest of the sequence belongs to destination vector pK7GWIWG2(1).

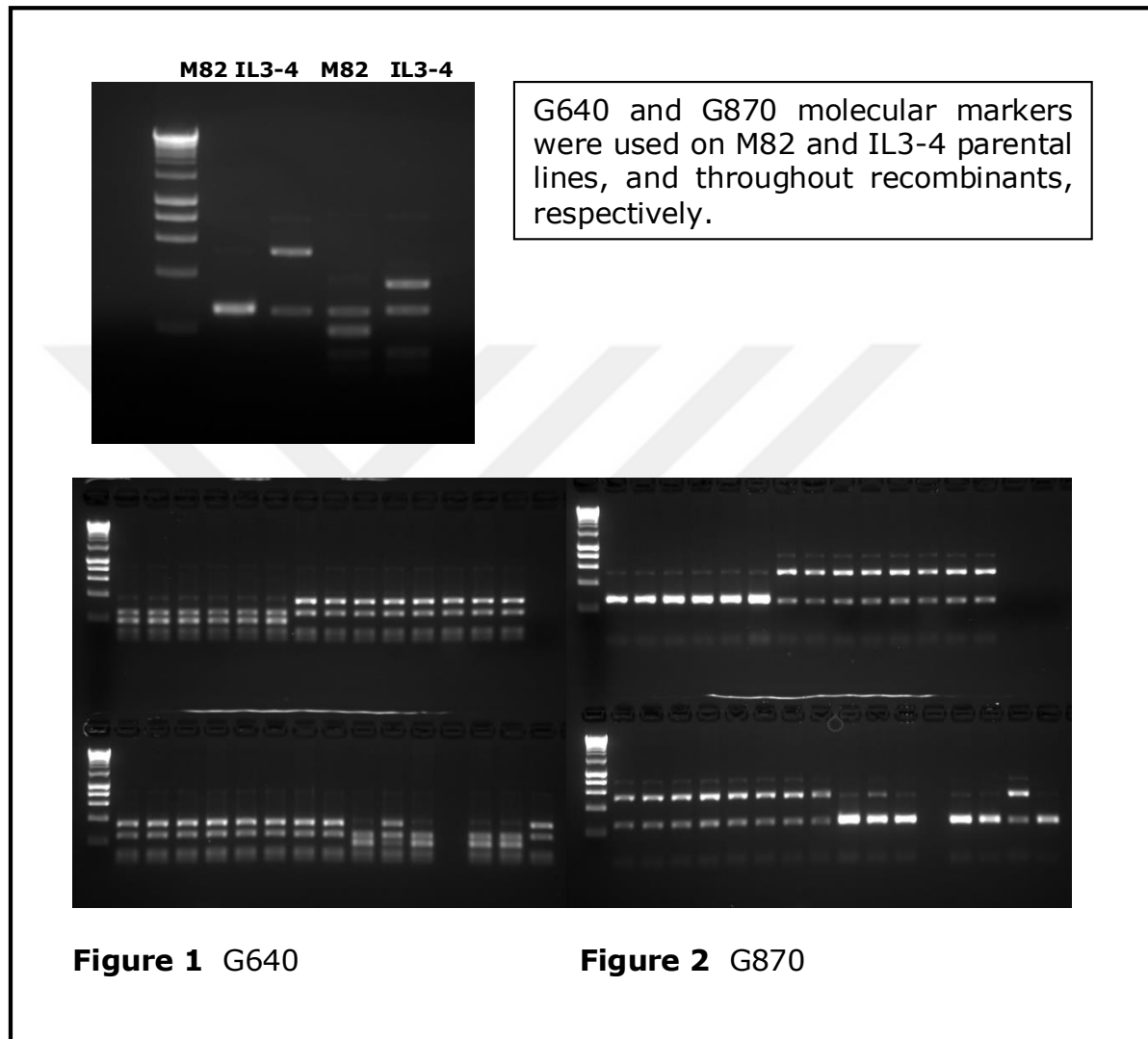
9.12 Gene models underlying 1 Mb region

Solyc03g111390	Kelch domain-containing protein 4
Solyc03g111400	Xanthine/uracil permease family protein
Solyc03g111410	B3 domain-containing protein
Solyc03g111420	DNAJ heat shock N-terminal domain-
Solyc03g111430	Unknown Protein (AHRD V1)
Solyc03g111440	DNAJ heat shock N-terminal domain-
Solyc03g111450	Nuclear transcription factor
Solyc03g111460	Nuclear transcription factor
Solyc03g111470	Nuclear transcription factor Y subunit C-2
Solyc03g111480	Mutator-like
Solyc03g111490	Ulp1 protease family C-terminal catalytic
Solyc03g111500	B3 domain-containing protein
Solyc03g111510	Zinc finger family protein
Solyc03g111520	Unknown Protein (AHRD V1);
Solyc03g111530	Cysteine-rich receptor-like protein kinase
Solyc03g111540	RLK, Receptor like protein,
Solyc03g111550	GDSL esterase/lipase
Solyc03g111560	Glucan synthase like 7
Solyc03g111570	Glucan synthase like 7
Solyc03g111580	Zinc finger CCCH
Solyc03g111590	Zinc finger protein-like
Solyc03g111600	Exodeoxyribonuclease III family protein
Solyc03g111610	HAD-superfamily hydrolase subfamily
Solyc03g111620	Os08g0119500 protein
Solyc03g111630	Programmed cell death 4a (AHRD V1
Solyc03g111640	Eukaryotic translation initiation factor
Solyc03g111650	Pentatricopeptide repeat-containing protein
Solyc03g111670	Receptor like kinase, RLK
Solyc03g111680	DNA polymerase III gamma/tau
Solyc03g111690	Pectate lyase
Solyc03g111700	Inactivated Zn-dependent hydrolase
Solyc03g111710	Speckle-type POZ protein
Solyc03g111720	Peptide methionine sulfoxide reductase
Solyc03g111730	Cathepsin B-like cysteine proteinase
Solyc03g111740	WD-40 repeat protein
Solyc03g111750	Genomic DNA chromosome 5
Solyc03g111760	Argonaute 4-like protein
Solyc03g111770	Abhydrolase domain-containing protein
Solyc03g111780	WD-40 repeat family protein
Solyc03g111790	Centromere protein X
Solyc03g111800	Receptor like kinase, RLK
Solyc03g111810	Sieve element-occluding protein 3
Solyc03g111820	Sieve element-occluding protein 3
Solyc03g111830	Protein-L-isoaspartate O-methyltransferase
Solyc03g111840	Polyadenylate-binding protein 1-like

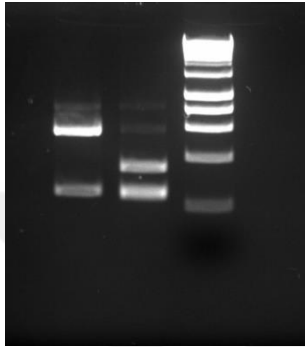
Solyc03g111850	Indole-3-glycerol phosphate synthase-like
Solyc03g111860	Glyoxal oxidase
Solyc03g111870	Copine-like protein
Solyc03g111880	Cytochrome P450
Solyc03g111920	Cytochrome P450
Solyc03g111930	Cytochrome P450
Solyc03g111940	Cytochrome P450
Solyc03g111950	Cytochrome P450
Solyc03g111970	Cytochrome P450
Solyc03g111990	Cytochrome P450 pseudogene
Solyc03g112010	Cytochrome P450
Solyc03g112030	Cytochrome P450
Solyc03g112040	Cytochrome P450
Solyc03g112050	Serine/threonine kinase
Solyc03g112060	Quinolinate synthase A
Solyc03g112070	Phosphoglycerate dehydrogenase
Solyc03g112080	Manganese-dependent ADP-ribose/CDP-alcohol diphosphatase
Solyc03g112090	Component of high affinity nitrate transporter
Solyc03g112100	Component of high affinity nitrate transporter
Solyc03g112110	Unknown Protein (AHRD V1)
Solyc03g112120	Unknown Protein (AHRD V1)
Solyc03g112130	AT5G28150-like protein
Solyc03g112140	Serine/threonine protein kinase
Solyc03g112150	Elongation factor Tu
Solyc03g112160	Protein DEHYDRATION
Solyc03g112170	Pectinesterase inhibitor
Solyc03g112180	Invertase inhibitor
Solyc03g112190	Pentatricopeptide repeat-containing protein
Solyc03g112200	Unknown Protein (AHRD V1)
Solyc03g112210	Unknown Protein (AHRD V1)
Solyc03g112220	Unknown Protein (AHRD V1)
Solyc03g112230	ZZ type zinc finger domain-containing protein
Solyc03g112240	Unknown Protein (AHRD V1)
Solyc03g112250	Multidrug and toxin extrusion protein 1
Solyc03g112260	Mate efflux family protein
Solyc03g112270	Lrr, resistance protein fragment
Solyc03g112280	C2 domain-containing protein
Solyc03g112290	Unknown Protein (AHRD V1)
Solyc03g112300	Os11g0444900 protein (Fragment)
Solyc03g112310	Receptor like kinase, RLK
Solyc03g112320	Ferric reductase oxidase
Solyc03g112330	U-box domain-containing protein 33
Solyc03g112340	Ring H2 finger protein
Solyc03g112350	ATP dependent RNA helicase
Solyc03g112360	60S ribosomal protein L27A
Solyc03g112370	Unknown Protein (AHRD V1)

Solyc03g112380	Armadillo/beta-catenin repeat family protein
Solyc03g112390	Myb transcription factor
Solyc03g112400	Unknown Protein (AHRD V1)
Solyc03g112410	Histone deacetylase
Solyc03g112420	Pentatricopeptide repeat-containing protein
Solyc03g112430	LOB domain protein 1
Solyc03g112440	Oleosin
Solyc03g112450	Signal recognition particle receptor beta subunit-like protein
Solyc03g112460	Alliinase (Fragment)
Solyc03g112480	Unknown Protein (AHRD V1)
Solyc03g112490	Unknown Protein (AHRD V1)
Solyc03g112500	Alpha-galactosidase
Solyc03g112510	RNA-binding protein 5-A
Solyc03g112520	Pre-mRNA-splicing regulator WTAP
Solyc03g112530	Guanine nucleotide-binding protein
Solyc03g112540	Retinol dehydrogenase 12
Solyc03g112550	Kinetochore protein Spc25
Solyc03g112560	Pentatricopeptide repeat-containing protein
Solyc03g112570	Unknown Protein (AHRD V1)
Solyc03g112580	Receptor like kinase, RLK
Solyc03g112590	Cell division protease ftsH homolog
Solyc03g112600	JmjC domain-containing protein
Solyc03g112610	Unknown Protein (AHRD V1)
Solyc03g112620	Carboxyl-terminal proteinase
Solyc03g112630	Fas-associated factor 1-like protein
Solyc03g112640	CRAL/TRIO domain containing protein
Solyc03g112650	Soul heme-binding family protein
Solyc03g112660	MOS1 (AHRD V1 *-*-D9IVG9_ARATH);

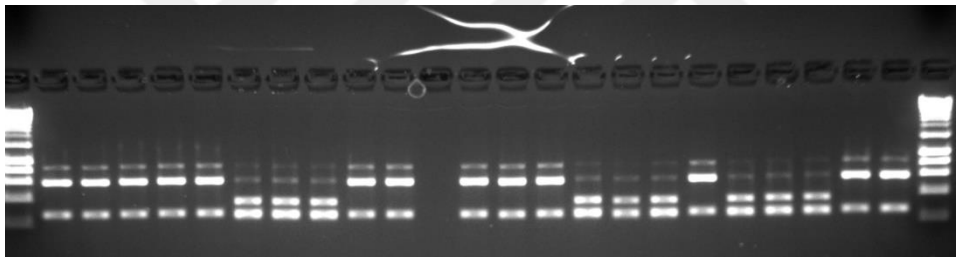
9.13 QTL Mapping markers

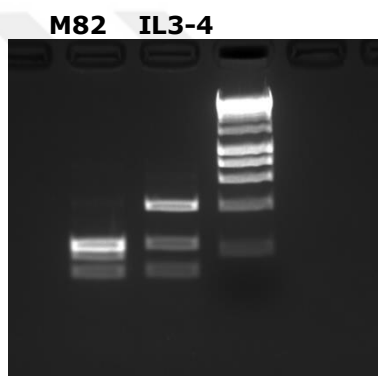


M82 IL3-4

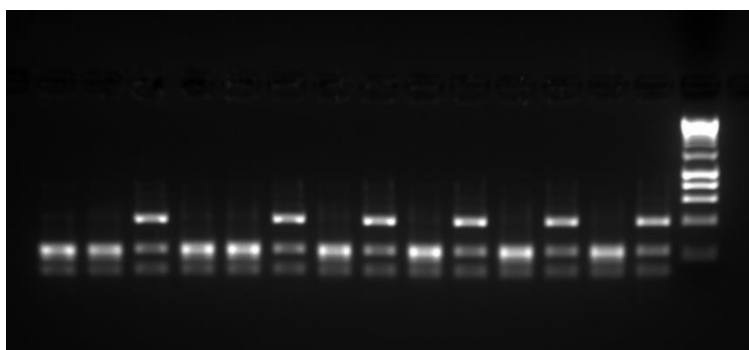


G690 molecular marker is used on M82, IL3-4 and throughout recombinant, respectively.

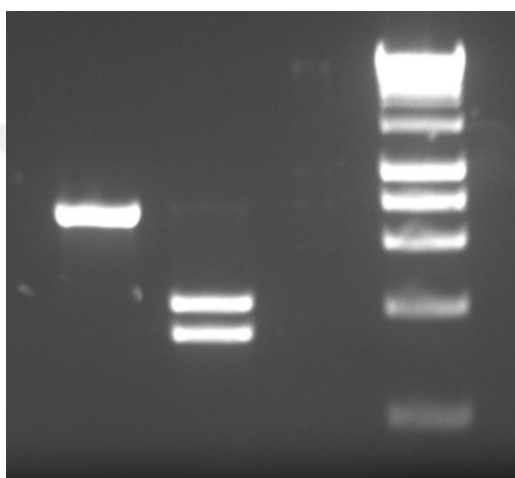




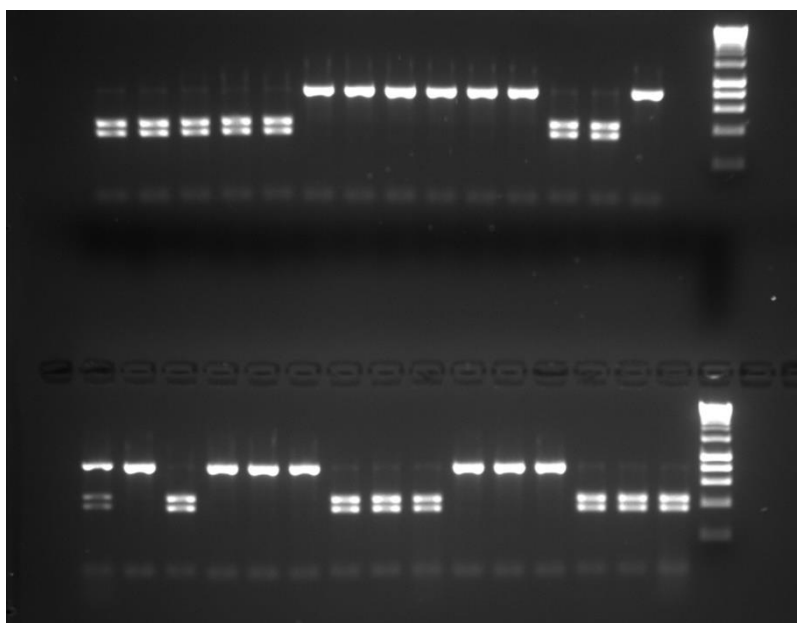
G570 molecular marker is used on M82, IL3-4 and throughout recombinant, respectively.

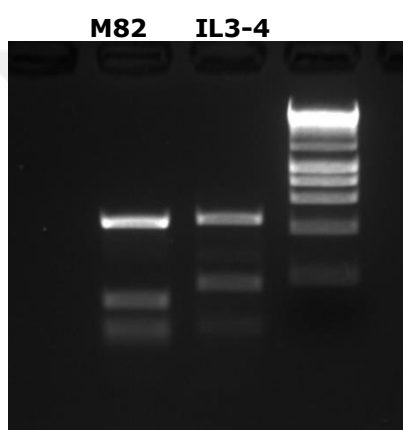


M82 IL3-4

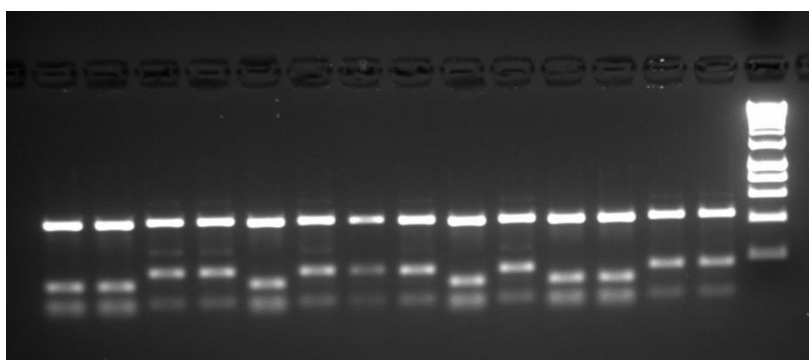


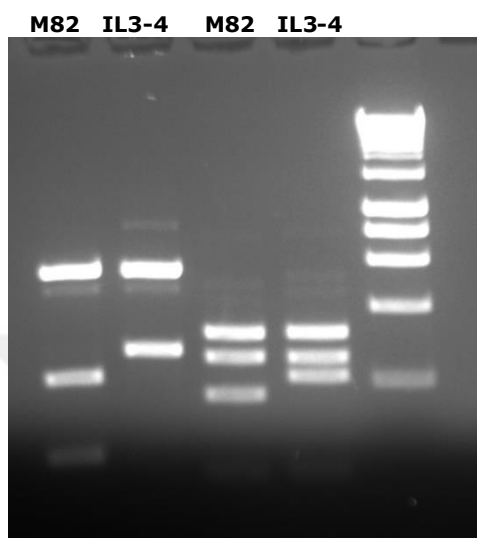
G970 molecular marker is used on M82, IL3-4 and throughout recombinant, respectively.





G390 molecular marker is used on M82, IL3-4 and throughout recombinant, respectively.





G750 and G020 molecular markers were used on M82 and IL3-4 parental lines, and throughout recombinants, respectively.

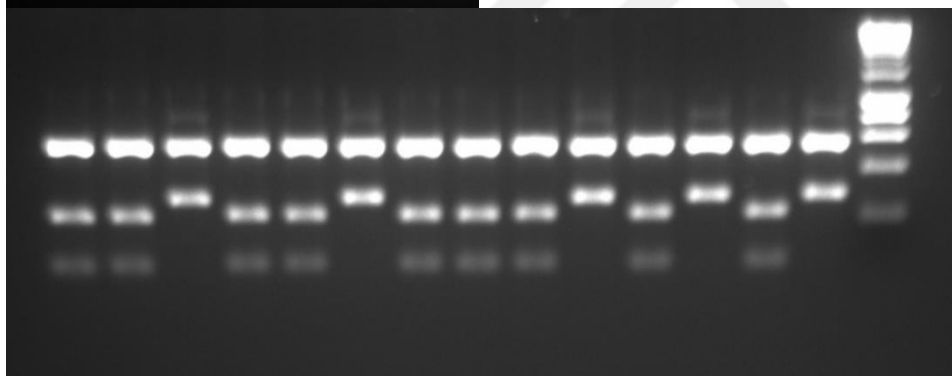


Figure 1 Marker G750

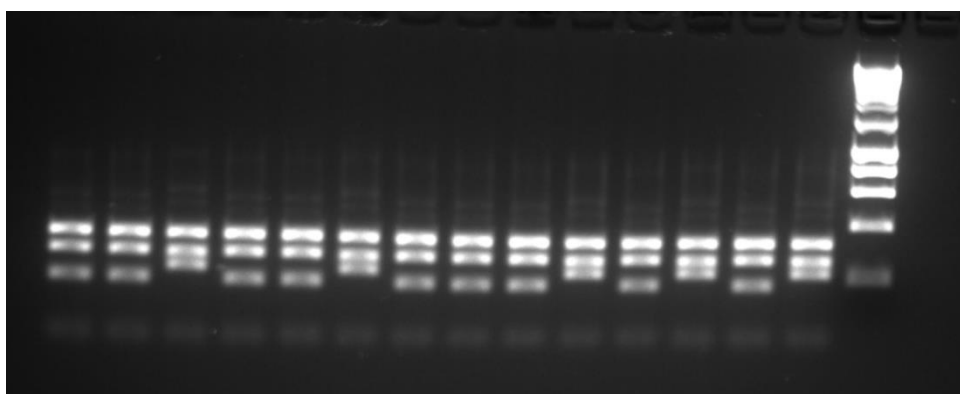


Figure 2 Marker G020

9.14 Texture Measurement of Transgenic Lines

