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Metabolic regulation and key genes of tomato secondary metabolism

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work unless otherwise stated. This work was carried out at the Max Planck Institute of Molecular Plant Physiology in Potsdam-Golm, Germany between March 2015 and September 2018. The material presented in this thesis has not been previously, in its entirety or in part, submitted at any university for any other degree.

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Sagar Sudam Jadhav

Potsdam, September 2018

Erklärung

Diese Dissertation ist das Ergebnis experimenteller Arbeit, welche vom March 2015 and September 2018 im Max-Planck-Institut für Molekulare Pflanzenphysiologie durchgeführt wurde. Ich erkläre, dass ich die vorliegende Arbeit an keiner anderen Hochschule eingereicht sowie selbständig und nur mit den angegebenen Mitteln angefertigt habe.

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Sagar Sudam Jadhav

Potsdam, September 2018

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Summary

Domestication syndrome has resulted in the large loss of genetic variation of crop plants. Because of such genetic loss, productivity of various beneficial secondary (specialized) metabolites that protect against abiotic/biotic stresses, has been narrowed in many domesticated crops. Many key regulators or structural genes of secondary metabolic pathways in the domesticated as well as wild tomatoes are still largely unknown. In recent studies, metabolic quantitative trait loci (mQTL) analysis using the population of introgression lines (ILs), each containing a single introgression from *Solanum pennellii* (wild tomato) in the genetic background of domesticated tomato (M82, *Solanum lycopersicum*), has been used for investigation of metabolic regulation and key genes involved in both primary and secondary metabolism. In this thesis, three research projects, i) understanding of metabolic linkage between branched chain amino acids (BCAAs) and secondary metabolism using antisense lines of BCAAs metabolic genes, ii) investigation of novel key genes involved in tomato secondary metabolism and fruit ripening, iii) mapping of drought stress responsive mQTLs in tomato, are presented and discussed. In the first part, metabolic linkage between leucine and secondary metabolism is investigated by analyzing antisense lines of four key genes (ketol-acid reductoisomerase, KARI; dihydroxy-acid dehydratase, DHAD; isopropylmalate dehydratase, IPMD and branched chain aminotransferases1, BCAT1) found previously in mQTL of leucine contents. Obtained results indicate that KARI might be a rate limiting enzyme for iC5 acyl-sucrose synthesis in young leaf but not in red ripe fruits. By integrating obtained results with previous reports, inductive metabolic linkage between BCAAs and other secondary metabolic pathways at DHAD transcriptional levels in fruit is proposed. In the second part, candidate genes that are involved in secondary metabolism and fruit ripening in tomato were found by the approach of expression quantitative trait loci (eQTL) analysis. To predict functions of those candidate genes, functional validation by virus induced gene silencing and transient overexpression were performed. Results obtained by analyzing T_0 overexpression and artificial miRNA lines for some of those candidates confirm their predicted functions, for example involved in fruit ripening (WD40, Solyc04g005020) and iC5 acyl-sucrose synthesis (P450, Solyc03g111940). In the third part, mapping of drought stress responsive mQTLs was performed using 57 *S. pennellii* ILs population. Evaluation of genetic architecture of mQTL analysis resulted in identifying drought responsive ILs (11-2, 8-3-1, 10-1-1 and 3-1). Location of well characterized regulators in these ILs helped to filter potential new key genes involved in drought stress tolerance. Obtained results suggests us our approaches could be viable for narrowing down potential candidates involved in creating interspecific variation in secondary metabolite content and at the level of fruit ripening.

Zusammenfassung

Das Domestikationssyndrom hat zu einem großen Verlust an genetischer Variation von Kulturpflanzen geführt. Aufgrund dieses genetischen Verlustes ist die Produktivität verschiedener nützlicher, sekundärer (spezialisierter) Metabolite, die gegen abiotische/biotische Belastungen schützen, in vielen domestizierten Nutzpflanzen eingeschränkt worden. Viele Schlüsselregulatoren oder Strukturgene des Sekundärstoffwechsels sind sowohl in den domestizierten als auch in den wilden Tomaten noch weitgehend unbekannt. In neueren Studien wurde die mQTL-Analyse (engl: metabolic quantitative trait loci) durchgeführt, unter Verwendung der Introgressionslinienpopulation (ILs), die jeweils eine einzelne Introgression von *Solanum pennellii* (Wildtomate) im genetischen Hintergrund von domestizierten Tomaten (M82, *Solanum lycopersicum*) enthalten, um Stoffwechselregulation und Schlüsselgene zu untersuchen, die sowohl am Primär- als auch am Sekundärstoffwechsel beteiligt sind. In dieser Doktorarbeit werden drei Forschungsprojekte vorgestellt und diskutiert, i) Verständnis der metabolischen Verbindung zwischen verzweigtkettigen Aminosäuren (BCAAs für engl: branched-chain amino acids) und dem Sekundärstoffwechsel unter Verwendung von Antisense-Linien von metabolischen Genen der BCAAs, ii) Untersuchung neuer Schlüsselgene im Tomaten-Sekundärstoffwechsel und Fruchtreifung, iii) Kartierung mQTLs in Tomaten, die auf Trockenstress ansprechen . Im ersten Teil wird die metabolische Verknüpfung zwischen Leucin und dem Sekundärmetabolismus untersucht, indem Antisense-Linien von vier Schlüsselgenen (Ketol-Säure-Reduktoisomerase, KARI; Dihydroxysäuredehydratase, DHAD; Isopropylmalatdehydratase, IPMD und verzweigtkettige Aminotransferasen1, BCAT1) untersucht werden, die in mQTLs für den Leucingehalt gefunden wurden. Die Ergebnisse weisen darauf hin, dass KARI ein geschwindigkeitslimitierendes Enzym für die iC5-Acyl-Saccharosesynthese in jungen Blättern, aber nicht in roten reifen Früchten sein könnte. Durch Integration der erhaltenen Ergebnisse mit früheren Berichten wird eine induktive metabolische Verbindung zwischen BCAAs und anderen sekundären Stoffwechselwegen auf DHAD-Transkriptionsebene in Früchten vorgeschlagen. Im zweiten Teil wurden Kandidatengene gefunden, die am sekundären Metabolismus und der Fruchtreife von Tomaten beteiligt sind, durch den Ansatz der eQTL-Analyse (engl.: expression quantitative trait loci). Um die Funktionen dieser Kandidatengene vorherzusagen, wurde eine funktionelle Validierung durch virusinduziertes Gen-Silencing und transiente Überexpression durchgeführt. Ergebnisse, die durch Analyse von T0-Überexpressions- und künstlichen miRNA-Linien für einige dieser Kandidatengene erhalten wurden, bestätigen ihre vorhergesagten Funktionen, z. B. Beteiligt an Fruchtreifung (WD40, Solyc04g005020) und iC5-Acylsaccharosesynthese (P450, Solyc03g111940). Im dritten Teil wurde die Kartierung von auf Trockenstress ansprechenden mQTLs unter Verwendung von 57 S. pennellii-IL-Populationen durchgeführt. Die Evaluierung der genetischen Architektur der mQTL-Analyse führte zur Identifizierung von ILs, die auf Trockenheit ansprechen (11-2, 8-3-1, 10-1-1 und 3-1). Die Position gut charakterisierter Regulatoren in diesen ILs half dabei, potenzielle neue Schlüsselregulatoren, die an der Toleranz gegenüber Trockenstress beteiligt sind, heraus zu filtern. Die erhaltenen Ergebnisse legen nahe, dass unsere Ansätze geeignet sein könnten, potentielle Kandidatengene einzugrenzen, die interspezifische Unterschiede im Sekundärmetabolitengehalt und auf der Ebene der Fruchtreife verursachen.

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List of Abbreviations

α-KIC	α-ketoisocaproic acid
ALS	acetolactate synthase
amiRNA	artificial microRNA
AP2a	APETALA2a
BCAAs	Branched chain amino acids
BCAT1	branched-chain aminotransferase1
BCFAs	Branched-chain fatty acids
BCKAs	Branched chain keto acids
BILs	backcross inbred lines
bp	base pairs
CBB cycle	Calvin-Benson-Bassham cycle
CNR	COLORLESS NON-RIPENING
Del/Ros1	Delila/Rosea1
DHAD	dihydroxyacid dehydratase
eQTL	expression quantitative trait loci
FAMEs	fatty acid methyl esters mixture
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
GABA	gamma-aminobutyric acid
iC4	2-methylpropanoic acid
iC5	3-methylbutanoic acid
ILs	introgression lines
IPCC	Intergovernmental Panel on Climate Change
IPMD	isopropylmalate dehydratase
KARI	ketolacid reductoisomerase
LC-MS	Liquid Chromatography-Mass Spectrometry
MADS	MCM1, AGAMOUS, DEFICIENS, SRF TF-family
mg/l	miligram per litre
mGWAS	metabolome-based Genome-Wide Association Studies
mM	milimolar
mQTL	metabolite Quantitative Trait Loci

MYB	myeloblast TF-Family
NAC	no apical meristem (NAM), ATAF1-2, and CUC2 (cup-shaped cotyledon) - TF family
NADPH	reduced form of Nicotinamide Adenine Dinucleotide Phosphate
PPP	pentose phosphate pathway
qRT-PCR	quantitative real-time Polymerase chain reaction
QTL	quantitative trait loci
RILs	recombinant inbred lines
RIN	RIPENING-INHIBITOR
RNA-seq	RNA sequencing
rpm	rotation per minute
RT	retention time
SBP	SQUAMOSA promoter binding proteins
SGAs	steroidal glycoalkaloids
SGTs	Secreting glandular trichomes
SlADH	*S. lycopersicum* alcohol dehydrogenase
SlAREB1	*S. lycopersicum* ABA response element binding protein 1
SNP	single nucleotide polymorphism
SPME-GC-MS	solid phase microextraction-gas chromatography mass spectrometry
TAGL1	AGAMOUS-LIKE1
TCA	tricarboxylic acid
TCP	teosinte branched1 (tb1), CYCLOIDEA (CYC) and PROLIFERATING CELL FACTORS - TF family
TD	threonine dehydratase
TFs	transcription factors
µl	microlitre
VIGS	virus induced gene silencing
WT	wild type

Chapter 1. General Introduction

1.1 Tomato is the most important vegetable crop worldwide

Tomato, *Solanum lycopersicum* (*S. lycopersicum*), is a member of the Solanaceae family. The species is originated in South America but domesticated in Central America. It is the most important crop in the world in terms of global net production value according to FAOSTAT, 2012 with a net value of >US$55 billion (Vincent et al. 2013). It is called as the luxury crop since it is the main component of food basket in the developed countries. Tomato belongs to one of the most important angiospermic family named Solanaceae. With 90 genera and over 3000 species, Solanaceae is hyperdiverse in terms of habit, habitat and morphology (Knapp et al., 2004). According to statistics published by United Nations Food and Agriculture Organization (faostat.fao.org), with Asia being the top producer, total production of tomato is 177 million tonnes worldwide. Healthy diet provides macro and micronutrients, adequate calories and also vitamins. World Health Organization (WHO) recommends a minimum 400g of fruit and vegetables per day (excluding potatoes and other starchy tubers), mainly for balancing nutrients in the body and also for prevention of chronic diseases. Among the fruits and vegetables, tomatoes are rich source of not only valuable phytochemicals such as the red pigmented lycopene, beta-carotene, phytoene, phytofluen, polyphenols, glycoalkaloids and flavonols for example quercetin and kaempferol; but also minerals (potassium) and fiber (Perez-Fons et al., 2014; Martinez-Valverde et al., 2002; Tohge et al., 2014). This has led to immense interest in increasing the levels of these secondary metabolites through transgenic approach (Verhoeyen et al., 2002; Bovy et. al., 2002; van der Rest et al., 2006; Colliver et al., 2002; Schofield et. al., 2005; Itkin et. al., 2011; Seymour and Granell 2014).

Availability of more than 75,000 accessions of tomato (Larry and Joanne 2007; Minoia et. al., 2010; Okabe et. al., 2011; Saito et. al., 2011), several mutants affected in fruit size, shape, development and ripening (Liu et. al., 2002; Tanksley, 2004; Xiao et. al., 2009; Rodríguez et. al., 2011), introgression lines, sequenced genomes of various species of tomato (Tomato Genome Consortium, 2012; Bolger et al., 2014; Schmidt et al., 2017; Lin et al., 2014; The 100 Tomato Genome Sequencing Consortium, 2014), short generation time, easy transformation technology and

publically available RNA transcriptome data makes tomato a model system for ``Fruit Omics´´ (Pesaresi et. al., 2014; Gascuel et al., 2017).

1.2 Untapped genetic potential of tomato wild species

Earlier studies suggest that tomato might have domesticated in two phases as first in the Andes and more recently in Mexico (Peralta and Spooner 2007, Blanca et. al., 2012). There are 13 distinct wild species of tomato worldwide (Peralta et al., 2006). *Solanum chmielewskii* (*S. chmielewskii*), *Solanum neorickii* (*S. neorickii*) and *Solanum pennellii* (*S. pennellii*) are all green-fruited species native to Peru. *S. pimpinellifolium* is the only red-fruited wild species and the only one to have exhibited a natural introgression with *S. lycopersicum* (Schauer et al., 2005). The cultivated tomato varieties contains only 5% of the genetic diversity present in the wild relatives and it has been found that self-incompatible wild species such as *S. pennellii* can have tremendous amount of untapped genetic potential (Miller and Tanksley 1990; Bai and Lindhout 2007). Qualitative traits such as flower colour, stem colour and pod number per peduncle etc. are under monogenic or oligogenic control while quantitative traits such as yield, height and weight etc. are under polygenic control means under multiple chromosomal loci. Such quantitative trait loci (QTL) associated with transcript levels, protein modifications or metabolite levels are known as expression (eQTL), protein (pQTL) or metabolite (mQTL) QTL respectively. Such QTL studies are helpful in deciphering evolutionary perspectives of gene function and can be carried out by using bi-parental segregating populations such as introgression lines (ILs), backcross inbred lines (BILs) and recombinant inbred lines (RILs). But individual locus characterization is difficult in F2 or recombinant inbred lines (RILs) because of epistatic interactions (Lippman et al., 2007; Chapman et al., 2012). Therefore, scientists have developed exotic libraries of inbred or introgression lines for different wild species by overcoming the difficulties of reduced recombination and/or linkage drag. For example, *S. pennellii* 716 ILs (Eshed and Zamir, 1994), *S. lycopersicoides* ILs (Canady et al., 2005), *S. sitiens* ILs (Pertuze et al., 2003), *S. chmielewskii* ILs (Frary et al., 2003), *S. habrochaites* ILs (Monforte and Tanksley, 2000), *S. neorickii* LA2133 BILs (Fulton et al., 2000), and the *S. cheesmaniae* RILs (Paran et al., 1995) as well as *S. cheesmaniae* ILs (Robert et al., 2001, Peleman and van der Voort 2003) and *S. pennellii* BILs (Ofner et al., 2016).

Development of ILs has major impact on this field of tomato metabolomics. ILs have enabled many scientists worldwide to fish out many promising candidate genes involved in different tomato metabolic pathways and have also increased our understanding of the genetic, metabolic and hormonal network underlying tomato fruit ripening (Lee et. al., 2012; Rambala et al., 2017; Barrantes et al., 2016; Alseekh et al., 2015; Schauer et al., 2006, 2008).

Recent advances in sequencing and metabolomics techniques are making it possible to filter the promising candidate genes by eQTL (expression Quantitative Trait Loci) and mQTL approaches, association mapping, multi-parent advanced generation inter-cross (MAGIC), nested association mapping as well as by using mutant derived populations (Beló et. al., 2008; Kover et. al., 2009; McMullen et. al., 2009; Druka et. al., 2010; Rambla et al., 2015).

1.3 Genome sequencing and postgenomics in tomato research

International groups of scientists have decoded genomes of *S. pimpinellifolium* accession LA1589, two crop varieties of *S. lycopersicum* named Heinz 1706 and M82 (The Tomato Genome Consortium 2012) and *S. pennellii* accession 716 a distant relative of the cultivated tomato (Bolger et al., 2014). RNA-Seq data of *S. lycopersicon* cv. "Heinz1706" supports 30,855 out of predicted 34,727 protein-coding genes. Wild species *S. pennellii* 716 have unique adaptations in terms of morphology, mating system, chemistry; and biotic and abiotic stress resistance. In line with this, study of *S. pennellii* 716 genome sequence has deciphered many drought and salt stress responsive candidate genes (Bolger et al., 2014).

Population of *S. pennellii* ILs have been developed through multiple rounds of self- and back-crossing *S. pennellii* 716 with *S. lycopersicum* cv. M82 (Eshed and Zamir, 1994). These ILs are composed of marker-defined genomic regions of the wild species *S. pennellii* (accession code LA716), substituting for the homologous intervals of the cultivated variety M82 (Fridman et al., 2004). Hence, they represent whole genome coverage of *S. pennellii* in overlapping segments in the genetic background of M82. Development of ILs has facilitated the robust assessment of mean phenotypic values of QTL-containing chromosome segments and hence easier identification of genes controlling quantitative traits.

1.4 QTL approach using *S. pennellii* ILs

Development of *S. pennellii* ILs has enabled to perform several approaches towards crop improvement. For example, since fruit quality improvement has been a fundamental human pursuit (Causse et al., 2001; Lecomte et al., 2004), fruit trait related QTL analysis have been perfommed. Usage of *S. pennellii* ILs population has resulted in identification of QTLs as well as candidate genes for primary metabolites, cell wall invertase, fruit yield, extreme heterosis, vitamin content in fruit, drought and salt tolerance; and beneficial secondary metabolites such as volatile organic compounds, acyl-sugars, carotenoids, tocopherols, cell wall components and sesquiterpenes (Schauer et al., 2006, 2008; Fridman et al., 2004; Stevens et al., 2007; Bolger et al., 2014; Liu et al., 2003; Tieman et al., 2006; Fraser et al., 2007; Perez-Fons et al., 2014; Schilmiller et al., 2010; de Godoy et al., 2013; Fernandez-Moreno et al., 2017; Rambla et al., 2017). Some of the important QTL studies have been discussed below.

1.4.1 QTL analysis for better fruit yield

Domestication resulted from human selection for a relatively small number of desirable traits with subsequent breeding and improvement, emphasizing adaptations to local conditions, tolerance to pathogens and increased yield (Giovannoni 2018). Though little is known about actual evolutionary pathway of fruit bearing species, the most likely scenario could be that early humans selected for mutations associated with larger fruit, and gradually, enough ‘‘large-fruited’’ mutations accumulated to give rise to our present-day cultivars (Tanksley, 2004). Domestication of tomato also has resulted in dramatic shape variation. Fruits from wild species of tomato are almost invariably round whereas tomatoes from domesticated species are round, oblate, pear shaped, torpedo-shaped, and bell pepper–shaped (Bai and Lindhout 2007). Four loci named fw1.1, fw2.2, fw3.1, and fw4.1 were first identified as QTLs in crosses between small-fruited wild tomatoes and their large-fruited cultivated counterparts (Grandillo et al., 1999). Except fw3.1, other three loci affect fruit size and mass but not shape (van der Knaap et al., 2002; van der Knaap and Tanksley, 2003). Moreover, it is established that natural genetic variation at locus fw2.2 alone can change the size of fruit by up to 30%; with ORFX (open reading frame X) being single gene responsible for this effect (Frary et al., 2000). The threading program LOOPP assigned ORFX as encoding human

oncogene RAS protein (Meller and Elber 2001). Frary et al., 2000 have also found that ORFX is expressed early in floral development and controls carpel cell number.

By using *S. pennellii* ILs, 138 loci putatively involved in pathways related to fruit size and composition (organic acids and sugars), and of 81 QTLs controlling the variation of the corresponding traits were mapped (Causse et al., 2004). Another study by Gur and Amir (2004) have proved that pyramiding three independent yield-promoting genomic regions introduced from *S. pennellii* can increase yield by more than 50% higher than that of a control market leader variety under both wet and dry field conditions that received 10% of the irrigation water.

1.4.2 Improvement of sugar contents for BRIX

Brix is a measure of the Total Soluble Solid (TSS) content in the tomato or tomato product. The TSS in tomatoes is mainly sugars (fructose). QTL analysis on *S. pennellii* ILs has helped in mapping Brix9-2-5 QTL within a flower- and fruit-specific invertase (LIN5) (Fridman et al., 2000). This QTL increases sugar yield of tomatoes. Upon pursuing this finding in depth by QTL analysis of five different tomato species delimited the functional polymorphism of Brix9-2-5 to an amino acid near the catalytic site of the invertase (Fridman et al., 2004). This amino acid was affecting enzyme kinetics and fruit sink strength. Increase in brix is due to increased apoplastic invertase activity that increases sucrose and glucose in ripe fruit pericarp and at earlier time points in columella tissue. These results highlight the power of *S. pennellii* ILs for high-resolution perspectives on complex phenotypes.

1.4.3 mQTL by global metabolomic analysis of primary metabolites

Comprehensive metabolic profiling in parallel with whole-plant phenotype characterization of *S. pennellii* ILs has enabled to identify 889 fruit mQTLs and 326 loci that modify yield-associated traits (Schauer et al., 2006). These 889 fruit mQTLs include most plant amino and organic acids, sugars, sugar alcohols, fatty acids, vitamins C (ascorbate) and E (a-tocopherol). Same group went further to evaluate heritability of these traits (Schauer et al., 2008). For this purpose, the *S. pennellii* ILs were grown alongside lines heterozygous for the introgression (ILHs) allowing evaluation of both heritability and the mode of inheritance. These studies revealed that most of the metabolic QTL (174 of 332) were dominantly inherited, with relatively high proportions of additively (61 of 332) or recessively (80 of 332) inherited QTL

and a negligible number displaying the characteristics of overdominant inheritance. This work also finds that mean heritability of the mQTL was generally relatively low; and a handful of the traits displayed reasonable heritability.

1.5 Gene regulatory network underlying tomato fruit ripening

Tomato is a climacteric fruit and genetically programmed tomato fruit ripening process is accompanied by dramatic changes in colour, texture, aroma and metabolite profile (Alexander and Grierson, 2002; Carrari and Fernie, 2006; Tohge and Fernie 2015). Ethylene is a major endogenous cue underlying this process. Additionally, environmental stimuli such as temperature and light also affect ripening. Tomato is a perishable good and therefore understanding genetic, metabolic and signalling cascade underlying ripening process is important in order to avoid post-harvest losses (Seymour et al., 2013). Plant developmental processes including ripening, are tightly regulated by transcription factors (TFs). Screening of mutants and ILs as well as transcriptome analysis have helped scientists to identify various QTLs and candidate genes that are involved directly or indirectly in tomato fruit ripening (Fortes et al., 2017; Lee et. al., 2012; Karlova et. al., 2011; Nguyen et. al., 2014; Shima et. al., 2014; Fujisawa et. al., 2013; Fernandez-Moreno et al., 2016). For example, by using *S. habrochaites* near isogenic line population (Monforte and Tanksley 2000), Dal Cin et al., (2009) identified QTL on chromosome 6 that caused over a threefold increase in fruit ethylene. Moreover, an ethylene response factor (ERF2.2) (Solyc02g077840.1) was identified under Firs.p. 362 QTL2.2 (Chapman et al., 2012). It has been shown that TFs such as EIN3 (Ethylene insensitive 3), NOR (Non-ripening), MADS box protein RIPENING-INHIBITOR (RIN) and COLORLESS NON-RIPENING (CNR), a SQUAMOSA promoter binding protein (SBP) functions in ethylene dependent manner (Chao et al., 1997; Solano et al., 1998; Vrebalov et al., 2002; Wilkinson et al., 1995; Manning et al., 2006). Contrary, TFs FUL1 (FRUITFULL1) and FUL2 influence ripening in an ethylene-independent manner (Bemer et. al., 2012). RIN and AGAMOUS-LIKE1 (TAGL1) regulate ripening through the regulation of 1-aminocyclopropane-1-carboxylate (ACC) synthase 2 (ACS2) while TF HB1 (HD-Zip homeobox protein 1) functions via regulation of ACC oxidase 1 (ACO1) expression (Itkin et al., 2009; Vrebalov et al., 2009; Lin et al., 2008). It has also been found that TF APETALA2a (AP2a) regulates fruit ripening and carotenoid accumulation via regulation of ethylene biosynthesis and signalling (Karlova et. al.,

2011). Recently, SlZFP2 (*Solanum lycopersicum* zinc finger protein) a transcriptional suppressor of CNR was found (Weng et. al., 2015). Surprisingly, recent finding implicate even long non coding RNAs named lncRNA1459 and lncRNA1840 in tomato fruit ripening (Zhu et. al., 2014).

Besides these reports, effect of epigenetic regulation of SBP (SQUAMOSA promoter binding protein like) box promoter, SlDML2 (*Solanum lycopersicum* DNA methylase 2*)* and RIN on tomato fruit ripening has also been documented (Manning et al., 2006; Liu et al., 2015; Zong et al., 2013).

1.6 Genetic regulation of tomato secondary metabolism

Secondary metabolism changes dynamically during tomato fruit ripening process. For example, chlorophyll and α-tomatine content decrease as breaker stage approaches while flavonoids, lycopene, esculeosides and flavour aroma volatiles increase post breaker stage (Tohge et. al., 2014; Wang and Seymour 2017). Many candidate genes and regulators in the pathways of some of these secondary metabolites have been identified and very well characterised either through transient or permanent transformations. Earlier candidate gene characterization studies were based on the research in bacteria and yeast, use of mutants and ILs, transgenic approach and coexpression correlation analysis (Fernie and Willmitzer 2004; Heidel et al., 2006; Hobbs et al., 2004; Schauer et al., 2004; Ozaki et al., 2010). One of those approaches genetically manipulated expression of hydroxycinnamoyl CoA quinate transferase (HQT) (Niggeweg et al., 2004; Clé et. al., 2008) and cinnamoyl-CoA reductase (CCR) (van der Rest et al., 2006) in tomato leaves and showed not only increased chlorogenic acid (CGA) levels but also altered rutin content.

Developments in biotechnology have speed up these attempts. Genetic loci for important metabolic traits have been recently mapped in tomato and arabidopsis by using metabolome-based genome-wide association studies (mGWAS) (Sauvage et al., 2014; Tieman et al., 2017; Wu et al., 2018). There are handfuls of eQTL and mQTL studies in grapevine and arabidopsis (Sonderby et. al., 2007; Huang et al., 2013). One of the recent study in barley identified eQTL for genes involved in drought stress in the juvenile phase and early leaf senescence based on a genome wide association study (Wehner et al., 2016). Similar study by Bolger et. al. (2014), by using panel of 76 *S. pennellii* ILs identified QTLs that are positively related to drought and salt stress. However, very few studies of eQTLs and mQTLs

approaches have been conducted in tomato. A large-scale mQTL analysis of *S. pennellii* ILs has detected total 679 mQTLs of secondary metabolism (Alseekh et al., 2015). These 679 mQTLs corresponded to 147 permissive (67 stringent) hydroxycinnamates, 75 permissive (30 stringent) flavonols, 151 permissive (84 stringent) glycoalkaloids, 13 permissive (5 stringent) acyl-sugars, 111 permissive (67 stringent) N-containing compounds, 80 permissive (38 stringent) phenolics, and 102 permissive (49 stringent) unclassified compounds. In this study, heritability analysis had revealed that mQTLs of secondary metabolism were less affected by environment than mQTLs of primary metabolism. With the help of this mQTLs data, they have also narrowed down and characterized two candidate genes for glycoalkaloid mQTLs. In the second study, integration of the genome, transcriptome, and metabolome data on a population of between 399 and 610 diverse tomato accessions led to identification of 3,526 mGWAS signals, 2,566 cis-eQTL, 93,587 trans-eQTL and 232,934 expression metabolite correlations (Zhu et al., 2018). In this study, one mGWAS signal (03:67080052) of the SGA hydroxytomatidenol (SlFM0964) was also supported by the eQTL of Solyc03g118100, an oxidoreductase gene that was previously reported to play an important role in SGAs biosynthesis (Umemoto et al., 2016). In addition to these studies, understanding epigenome underlying secondary metabolite accumulation might unravel key genes (Krokida et al., 2013). These studies demonstrate that metabolic pathway as well as underlying regulation can be easily elucidated by using multiomic dataset. Some of the important secondary metabolites and genetic machinery underlying their regulation are discussed below.

1.6.1 Carotenoids

Carotenoids are hydrophobic, liposoluble class of 40-carbon terpenoid molecules that can be classified into oxygen devoid, carotenes and the oxygen containing xanthophylls basing on the basic chemical structure and the oxygen presence (Rodrigo-Baños et al., 2015). They are characterized by an extended conjugated π-electron system that can only be synthesized by plants and microorganisms (Bohn 2008). Carotenoids are strong scavengers of singlet oxygen (1O2) and peroxyl radicals because of this conjugated double-bond structure. Over 700 carotenoids have been identified but only 50 have been reported to play a role in the human diet (Mueller and Boehm, 2011). Carotenoids are well known for their vital role in plant

and animal development. For example, β-carotene is a precursor for vitamin A while some carotenoids are also precursors for carotenoid derived volatiles (Fester et al., 2002; Vogel et al., 2010). They are also responsible for the coloration of fruit, flower, and other organs of higher plants (Giuliano et al., 2003; Milborrow et al., 2001; Cunningham et al., 1998).

Carotenoids accumulate during tomato fruit ripening. Correlation analysis between gene coexpression and carotenoid content for *S. pennellii* ILs led to identification of TF SlERF6 that influences carotenoid biosynthesis (Lee et. al., 2012). It has been shown that TFs BZR1-1D (Brassinazole resistant 1-1D) and SlNAC4 [no apical meristem (NAM), ATAF1-2, and CUC2 (cup-shaped cotyledon) - TF family] influences carotenoid accumulation in a brassinosteroids and ethylene dependent pathway respectively (Zhu et al., 2014; Liu et al., 2014). Additionally, other phytohormones such as abscisic acid and jasmonic acid have also been implicated in the regulation of lycopene accumulation in tomato (Galpaz et al., 2008; Liu et al., 2012). Recently, it has been found that SlSGR1 regulate lycopene accumulation through direct interaction with *Solanum lycopersicum phytoene synthase 1* (SlPSY1), a key carotenoid biosynthetic enzyme; while TDR4 works in light dependent manner (Luo et al., 2013; Hsu-Liang Hsieh 2016). Moreover, recent study by Kou et al., (2018) has found that pathways dependent on ethylene and ABA are regulated by TFs SlNAC48 (SNAC4) and SlNAC19 (SNAC9).

1.6.2 Steroidal glycoalkaloids

Steroidal glycoalkaloids (SGAs) are characteristic terpenoids and are common constituents of solanaceous plants. At least 90 structurally unique steroidal alkaloids have been identified in over 350 Solanum species (Friedman et al., 1997; Milner et al., 2011). Chemically SGAs consist of aglycone unit and hydrophilic carbohydrate side chain unit attached at the 3-OH position. Aglycone unit consists of hydrophobic C27 steroid skeleton of cholestane with nitrogen incorporated into the F ring (Milner et al., 2011). These two structural components render glycoalkaloids amphiphilic nature. A significant portion of the biological activity of glycoalkaloids derives from an oligosaccharide moiety. Levels of SGAs are dependent on genotype, tissue, and growth conditions (Friedman and Levin, 1998; Kozukue et al., 2004; Iijima et al., 2008, Mintz-Oron et al., 2008; Itkin et al., 2011). Furthermore, it has been shown that

ethylene production or signaling modulates levels of esculeoside A and lycopene during tomato fruit ripening (Iijima et al., 2009).

In potato (*Solanum tuberosum*), transgenic overexpression of 3-Hydroxy-3-methylglutaryl coenzyme A reductase1 (HMG1) and squalene synthase1 (SQS1) have supported the association between elevated expression of these genes and increased accumulation of SGAs (Ginzberg et al., 2012). It has been found that enzyme GLYCOALKALOID METABOLISM1 (GAME1) catalyzes glycosylation of steroidal alkaloids and regulates their toxicity (Itkin et al. 2011). Recent studies in tomato have found the involvement of gene GAME9 (otherwise known as JRE4) in the regulation of SGAs levels (Ca´rdenas et al. 2016). Moreover, Jasmonate-Responsive ERF TFs have also been shown to regulate SGA biosynthesis in tomato (Thagun et al., 2016).

1.6.3 Chlorogenic acid

Chlorogenic acid (CGA), a ester of caffeic acid and quinic acid, is the major soluble polyphenol in the phenylpropanoid pathway and is probably more accessible than that of many other flavonoids as a potential antioxidant from plants (Chang et al., 2009). The precursor molecule phenylalanine undergoes a series of hydroxylation, methylation and dehydration reactions and synthesise low molecular weight polyphenols such as flavonols, flavones, flavanones, catechins, anthocyanins, isoflavonoids, phenolic esters and dihydroflavonols. In plants, chlorogenic acid is involved in defense against pathogens and UV stress signalling (Dixon and Paiva, 1995; Treutter, 2005); while in case of both plants and humans, it act as strong antioxidants (Rice-Evans et al., 1997). By using transgenics approach, Niggeweg et al. have shown that HQT is the major enzyme and its activity can determine the rate of flux to CGA synthesis in tomato (Niggeweg et al., 2004). Moreover it has been shown that HQT suppression not only decrease PAL (key enzyme in the pathway) expression and enzyme activity but also increase biosynthesis of caffeoyl polyamines in potato (Payyavula et al., 2015).

1.6.4 Flavonoids

Flavonoids are a class of plant and fungus secondary metabolites and are chemically a 15-carbon skeleton structure consisting of two phenyl rings (A and B) and heterocyclic ring (C). Rutin was extracted from leaves and stem epidermis of the

tomato plant (Blount, 1933; Fontaine et al., 1947). Among other flavonoids, naringenin belongs to the first class of flavonoids, which consists of a three-ring molecular system and therefore it is a primer for more advanced flavonoid structures and also a substrate in glycosylation reactions (Slimestad and Verheul 2009). First time naringenin and quercetin (quercetin-3-*O*-rhamnoside) were extracted from the outer epidermis of three tomato cultivars (Wu and Burrell, 1958).

Heterologous overexpression of the maize TFs LC and C1 in tomato has resulted in 10-fold increase in total flavonoid glycoside content of red ripe tomatoes (Gall et al., 2003). Recently, gene stacking of arabidopsis PAP1 (production of anthocyanin pigment 1) with an onion CHI using transgenic and crossing approach have found 130 and 30 times more rutin and total anthocyanin content respectively (Lim and Li 2017). Recent study by Wahyuni et al., (2014) used candidate gene approach by combining eQTL analysis based on gene expression data of 14 flavonoid genes and flavonoid mQTL data. This has led to identification of new QTL for naringenin chalcone on chromosome 1, with its QTL maximum at the Ca-MYB12 gene in *Capsicum annuum*.

1.6.5 Acyl-sugars

Plant epidermal outgrowth structures such as stomata, root hairs and trichomes carry out carbon fixation, nutrient uptake and secondary metabolite synthesis respectively and therefore these epidermal outgrowth structures plays vital role in plant development. Among these structures trichomes are especially attractive system because of their ability to synthesize vital secondary metabolites such as acyl-sugars, flavonoids, phenolics, food flavors from mint (Mentha spp.) and basil; alkaloids artemisinin and tetrahydrocannabinol; and isoprenoids (Schilmiller et al., 2009; Schilmiller et al., 2010; Schmidt et al., 2011; Kim et al., 2012; Brückner et al., 2014; Rios-Estepa et al., 2008; Xie et al., 2008; Li et al., 2014; Zhang et al., 2008; Flores-Sanchez and Verpoorte, 2008). These secondary metabolites are of commercial interest because of their medicinal, antifeedant, oviposition deterrent, and in some cases food flavour properties. Trichomes term derived from the Greek word “trichos”, which means hair –are specialized cells; and can be glandular or nonglandular, unicellular or multicellular and species specific. For example, arabidopsis has only unicellular non-glandular trichomes while tomato have four (i.e.,

type I, IV, VI and VII) glandular capitate trichomes and four (i.e., type II, III, V and VIII) non-glandular (Mathur and Chua 2000; Glas et al., 2012).

Acyl-sugars are polyesters of sugars that are secreted by the glandular trichomes of many species in the nightshade family (*Solanaceae*), including *S. pennellii*, potato (*Solanum berthaultii*), tobacco (*N.* tabacum), petunia (*Petunia hybrida*), *Physalis nicandroides*, and *Datura metel* (Fobes et al., 1985; Severson et al., 1985; King et al., 1986, 1988; King and Calhoun, 1988; Ohya et al., 1996; Maldonado et al., 2006). Besides methyl ketones and terpene derived volatiles, acyl-sugars also plays vital role in defense against a variety of insects including whiteflies, aphids, spider mites, and leaf miners (Hawthorne et al., 1992; Rodriguez et al., 1993; Liedl et al., 1995; Alba et al., 2009). They are also used as antibiotics and emulsifiers for food and cosmetic products (Chortyk et al., 1993, Hill and Rhode, 1999). Collectively, the different acyl chain lengths (C2-C12) and the variety of iso, anteiso and straight chains give rise to many different possible acyl-sugar structures (Schilmiller et al., 2015). Using *S. pennellii* ILs, many genes in the acyl-sugar pathway have been characterised. For example, BAHD type acyltransferases such as Sl-ASAT1 (Solyc12g006330), Sl-ASAT2 (Solyc04g012020) and Sl-ASAT3 (Fan et al., 2015; Schilmiller et al., 2015); putative carboxylesterase enzymes of the a/b-hydrolase superfamily acylsugar acylhydrolase1 (ASH1) and (ASH2) (Schilmiller et al., 2016) and inhibition insensitive version of isopropylmalate synthase SlIPMS3 (Solyc08g014230) (Ning et al., 2015).

1.7 Recent technical development of Virus Induced Gene Silencing (VIGS) system

Biotechnological tools such as zinc-finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system can generate specific gene knockouts in plants, but requires time-consuming stable plant transformation (Senthil-Kumar and Mysore, 2014). Virus Induced Gene Silencing (VIGS) is a powerful post transcriptional gene silencing (PTGS) method that is used widely for high-throughput gene function analysis (Burch-Smith et al., 2006; Wang et al., 2006; 2007; Nelson et al., 2007; Wege et al., 2007; Zhang et al., 2009). It is easier to perform and is not time consuming. A partial sequence of a host gene can be cloned into recombinant virus vectors. Direct inoculation (agroinjection) of VIGS (or RNA interference)

constructs into developing fruit creates successful “local” silencing effects shortening the experimental procedure (Fu et al., 2005; Hoffmann et al., 2006; Orzaez et al., 2006). After inoculation virus spreads systemically and the endogenous gene transcripts which are homologous to the insert in the viral vector (VIGS-vector), are degraded by post-transcriptional gene silencing (PTGS) (Baulcombe, 1999).

There are 22 commonly used VIGS vectors that have gene silencing ability in more than one plant species. Tobacco rattle virus (TRV)-based VIGS vectors are widely used in several plant species, especially in Solanaceae (Senthil-Kumar and Mysore, 2014). TRV drives a highly efficient silencing in Nicotiana *benthamiana* (*N. benthamiana*) (Ruiz et al., 1998; Ratcliff et al., 2001), but less efficiently in potato and tomato (Liu et al., 2002b; Brigneti et al., 2004) and thus impede metabolomics on silenced parts. To avoid these hurdles, internal reporter was required. Tomato plants ectopically expressing transcription factors Rosea1 and Delila in their fruit under the control of the ripening-induced E8 promoter have been shown to accumulate high levels of anthocyanin pigments, not only in the peel but also in the pericarp (Butelli et al., 2008). Therefore Orzaez et al., (2009) used these purple tomato plants to conduct VIGS study in order to have internal easily scorable marker. Doing this, they have shown that fruit VIGS, enhanced by anthocyanin monitoring, can be a powerful tool for reverse genetics in the study of the metabolic networks operating during tomato fruit ripening (Orzaez et al., 2009).

Aim of the thesis

The general purpose of my doctoral thesis is to investigate metabolic regulation and key genes of tomato secondary metabolism. The work in this thesis is reported in three major results chapters. The aim of the first chapter was to understand metabolic linkage between leucine (primary metabolism) and its secondary metabolic products 3-methylbutanal, 3-methylbutanol and iC5 acyl-sucrose. For this, antisense lines for key genes in branched chain amino acids pathway were analysed by GC-MS, SPME-GC-MS, LC-MS and stable isotope study. Obtained results showed that KARI might be a rate limiting enzyme for iC5 acyl-sucrose synthesis only in young leaf. Additionally, transcript analysis of 2 isoforms of DHAD suggested that these isoforms might be functionally different; and therefore might have resulted in only DHAD lines having significantly changed levels of 3-methylbutanal, iC5 acyl-sucrose and α-tomatine in red ripe tomato fruits. Second chapter is focused on investigating key genes that are involved in secondary metabolism and fruit ripening in tomato. Here, potential candidate genes were found by combined eQTL and TF approach, and functionally validated by VIGS. Furthermore, transgenics developed for 3 different candidates were analysed by metabolite profiling. In the third chapter, GC-MS and LC-MS were used for metabolomic analysis on young leaf tissues of 57 *S. pennellii* ILs that were exposed to drought stress treatment. Primary and secondary metabolite data was analysed for significantly affected mQTLs.

Chapter 2. Analysis of metabolic linkage between branched chain amino acids and secondary metabolism in tomato

2.1 Introduction

Large number of insect pests attacks cultivated crops, causing severe reduction in yield. For tomato crop, whitefly (*Bemisia tabaci*) attack is severe because whitefly acts as a host for more than 60 different viral vectors. Crop protection has traditionally relied on the use of pesticides. Today, the trend in pest control is decreased use of pesticides, not only due to cost; but to minimize environmental impact and to avoid the development of pesticide resistance in pest populations. Therefore developing innovative bio-based pest control strategies is the necessity of time. In this attempt, trichomes have been studied very intensively because of their ability to synthesize various secondary metabolites such as sesquiterpenes and acyl-sugars; and even volatile organic compounds with antifeedant and repellent activity (Nonomura et al., 2009; Bleeker et al., 2011; Schilmiller et al., 2009; Schilmiller et al., 2010; Flores-Sanchez and Verpoorte, 2008; Balcke et al., 2017). Acyl-sugars are lineage-specific secondary metabolites that can be found in many genera of Solanaceae such as Solanum (King et al., 1990; Schilmiller et al., 2010; Ghosh et al., 2014), Petunia (Kroumova and Wagner, 2003; Liu et al., 2017), Datura (Forkner and Hare, 2000) and Nicotiana (Kroumova and Wagner, 2003; Kroumova et al., 2016). Earlier reports have found that trichomes of wild type *S. pennellii* can synthesize acyl-sugars that can impart resistance to whitefly (Kishaba et al., 1992). It has also been found that defense against whitefly can be affected by leaf-trichome densities, concentration of acyl-sugars in the trichomes as well as the type of trichomes (Williams et al., 1980; Kisha, 1981; Berlinger, 1986; Simmons, 1994). Acyl-sugars are synthesised in head cells of trichomes on leaf and stem surface. Acyl-sugars may act by preventing oviposition and feeding by arthropods resulting in reduced fecundity and fertility values (Dias et al., 2013). Moreover, it has been shown that sucrose esters damage insect cuticle by disrupting the integrity of cellular membranes and uncoupling oxidative phosphorylation (Walia et al. 2014, Putreka and Severson 1995).

Though *S. pennellii,* a wild species of tomato synthesize acyl-sugars in high amount, present day tomato cultivars synthesise them in less amount because of

domestication syndrome (Fobes et al., 1985; Frary and Doganlar, 2003; Bai and Lindhout 2007). Hence obtaining tomato plants with high acyl-sugar content in the leaves and high levels of resistance to major pests is of great importance in the management of whiteflies. Besides its role in plant defense, acyl-sugars also have commercial uses such as antibiotic, food additives and in cosmetic products. Despite this economic importance of acyl-sugars, their pathway regulation still remains poorly understood.

Branched chain amino acids (BCAAs) performs various functions that are vital for maintaining growth and development of not only plants but also animals (Harris et al., 2005; Nair and Short, 2005; Brosnan and Brosnan, 2006; Avruch et al., 2009; Fujii et al., 2011, Kochevenko et al., 2012). For example, recent studies implicate BCAAs not only in the activation of mTOR (mammalian target of rapamycin) signaling pathway and in the development of insulin resistance in case of mammals; but also in the development of symbioses of *Rhizobium leguminosarum* with *Pisum sativum* and *Pisum vulgaris* (Kimball and Jefferson, 2006a, 2006b; Hannah et al., 2010; Valerio et al., 2011, Prell et al., 2010). Based on currently available information, following schematic representation has been given for BCAAs catabolism leading to acyl-sugar and branched-chain volatile synthesis (Figure 2.1) (Slocombe et al., 2008, Kochevenko and Fernie, 2011; Kochevenko et al., 2012). Importantly, in this pathway, pyruvate links to valine and leucine as their precursor.

Acyl-sugars are esters of fatty acids and sugars. There are mainly two types of acyl-sugars as acyl-glucose and acyl-sucrose. Tomato acyl-sugars generally consist of sucrose esters that often contain branched-chain fatty acids in normal, iso or anteiso configuration (Ning et. al., 2015). But based on acyl chain composition, they can be named as 2-methylpropanoic acid (iC4) and 3-methylbutanoic acid (iC5) acyl-sugars (Fan et al., 2015). Catabolism of BCAAs isoleucine (Ile), valine (Val) and leucine (leu) leads to synthesis of corresponding branched chain keto acids (BCKAs) such as α-ketomethylvaleric acid (KMV), α-ketoisovaleric acid (KIV) and α-ketoisocaproic acid (KIC) respectively. These BCKAs serves as a key branching point where pathway branches off for corresponding volatile and acyl-sugar synthesis. For example, KIC is a common substrate for iC5 acyl-sucrose and 3-methylbutanol synthesis (Beck et. al., 2004, Ning et al., 2015). It has been found that ketol-acid reductoisomerase (KARI), dihydroxy-acid dehydratase (DHAD) and

isopropylmalate dehydratase (IPMD) i.e.

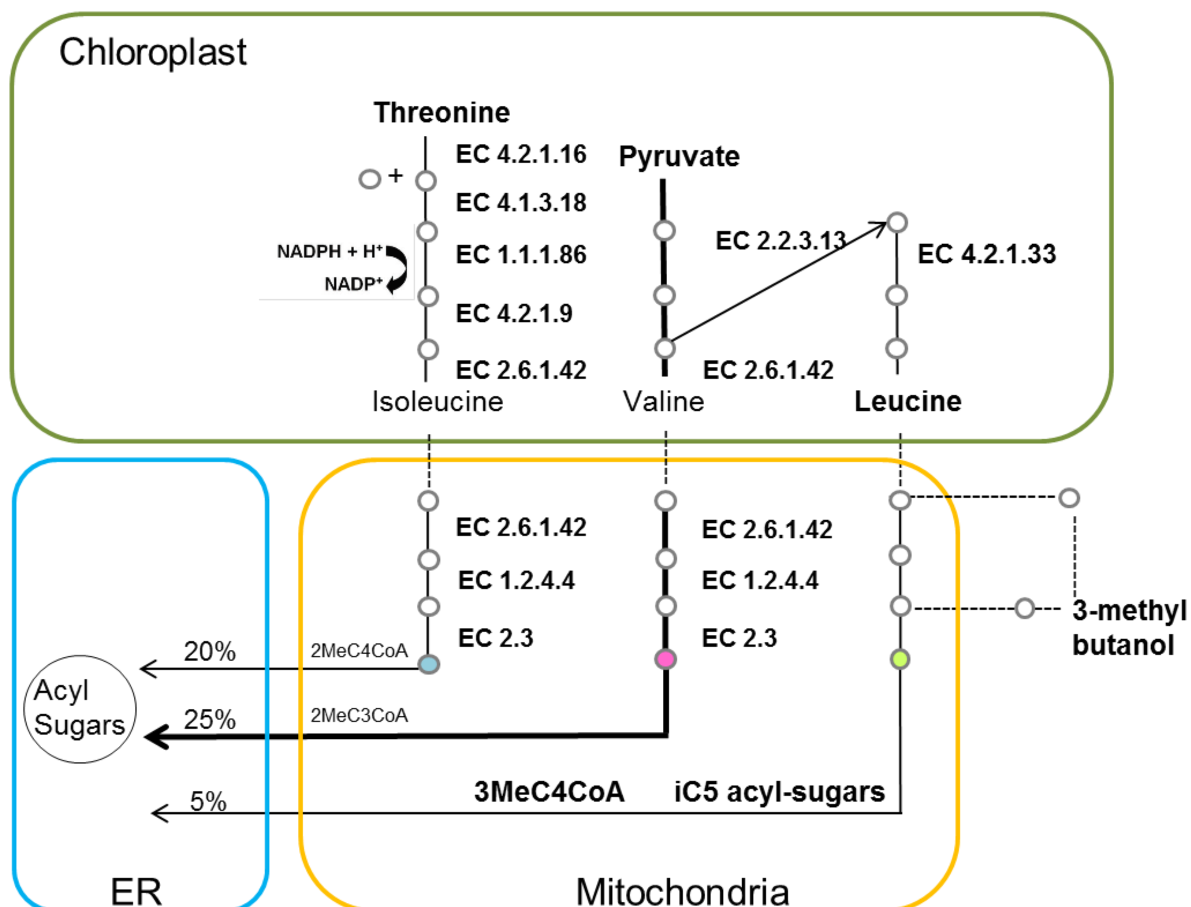


Figure 2.1 Proposed model of acyl-sugar and branched-chain volatile synthesis in tomato plants (Slocombe et al., 2008, Kochevenko et al., 2012, Binder 2010). EC 4.2.1.16: threonine dehydratase, EC 4.1.3.18: acetolactate synthase, EC 1.1.1.86: ketolacid reductoisomerase, EC 4.2.1.9: dihydroxyacid dehydratase, EC 2.2.3.13: 3-isopropylmalate synthase, EC 4.2.1.33: isopropylmalate dehydratase, EC 2.6.1.42: branched-chain aminotransferase1/3/4, EC 1.2.4.4: branched-chain keto acid dehydrogenase, EC 2.3: acyl CoA transferase. The size of an arrow indicates its contribution to the final production of acyl-sugars. Solid arrows depict major metabolic routes and broken arrows indicate transport of those metabolites. Light blue, pink and olive green colour to nodes indicates 2-methylbutanoic Co-A, 2-methylpropanoic Co-A and 3-methylbutanoic Co-A respectively.

enzymes involved in BCAAs biosynthetic pathway co-localized, or mapped close to BCAAs QTL; and major coordinate QTL (i.e. those in which all the three BCAAs changed) co-localized with BCAT1, IPMD and DHAD genes (Table 2.1) (Maloney et al., 2010). BCAAs content in the leaves of antisense lines of KARI, DHAD and IPMD were reduced while those in fruit were unchanged for both the KARI lines but were increased for one of the DHAD and IPMD line (Kochevenko et al., 2012). Tomato volatiles plays important role not only in imparting flavour to the red ripe tomatoes (Rambala et al., 2017) but also in whitefly-tomato relationship.

Though one of the mQTLs study on flavour volatiles in tomato (Table 2.1) have

Table 2.1. Location of mQTLs and BCAAs pathway specific key genes in *S. pennellii* ILs.

	Key metabolites in the pathway					Key genes in the pathway			
IL	**Iso**	**Leu**	**Val**	**ILV**	**Branched chain volatiles**	**BCAT1**	**DHAD**	**IPMD**	**KARI**
IL 2-3					**				
IL 2-4		*			**				
IL 2-5			*		***				
IL 2-6					***				
IL 3-2				*					
IL 3-5					***				
IL 3-4		*	*						
IL 4-1				*					
IL 4-4					****				
IL 5-1					**				
IL 5-2				*					
IL 6-2					***				
IL 6-3					***				
IL 6-2-2				*				#	
IL 7-1				*					
IL 7-2	*								#
IL 7-3			*		**				#
IL 7-4					**				#
IL 7-4-1					**				
IL 7-5					**				
IL 7-5-5					**				
IL 8-1			*						
IL 8-1-1									
IL 9-3					***				
IL 9-3-1					***				
IL 9-3-2					***				
IL 10-1				*	***				
IL 10-3					*				
IL 11-2					***				
IL 12-3	*	*		*		#	#		

(* represent mQTL; # represent co-localization; Iso-Isoleucine; Leu-Leucine; Valine-Valine; ILV-BCAAs).

identified 12 loci affecting the emission of leucine- and/or isoleucine-derived volatiles possibility of such a locus affecting TF or enzyme or precursor pools makes gene identification and annotation difficult (Tieman et al., 2006a). Therefore, the fact that

some key enzymes in BCAAs metabolism and the volatile synthesis pathway are not known, it is challenging to elucidate metabolic linkage thereof. There are only two recent studies that have tried to decipher metabolic linkage between primary and secondary metabolism. One of those studies had attempted to upregulate flux through primary metabolism to induce volatiles synthesis but failed. Dal Cin et al., (2011) could not induce levels of phenylalanine derived volatiles by overexpressing ODO1, a MYB TF even though they succeeded in inducing levels of phenylalanine and phenylpropanoids. In the second study, Kochevenko et al., (2012) had attempted to induce branched chain volatiles synthesis by overexpressing BCAT3 (BCAAs anabolic enzyme) and BCAT1 (BCAAs catabolic enzyme), but found no larger effects; instead they found that catabolism of BCAAs supports respiration in young tomato fruit. Both studies suggest that regulation of volatile synthesis occurs at the level of commitment to another secondary metabolite pathway i.e. in this case respiratory or acyl-sugar pathway. Acyl-sugar pathway has been studied in *S. pennellii* and *N. benthamiana* with isotopic labelling and also by using a set of 76 *S. pennellii* ILs (Slocombe et al., 2008; Schilmiller et al., 2010, 2012; Alseekh et al., 2015). These mQTLs studies have identified ILs producing different acyl chain substitutions on acyl-sugar metabolites (IL1-3/1-4 and IL8-1/8-1-1) and genomic regions influencing the quantity of acyl-sugars (IL5-3, IL11-3 and IL8-2). However, metabolic regulation underlying acyl-sugar pathway or metabolic linkage between BCAAs and acyl-sugar pathway has not been studied in detail.

In this chapter, I focused on elucidating metabolic linkage between leucine (primary metabolism) and its secondary metabolic products 3-methylbutanal and iC5 acyl-sucrose. Here, I analysed antisense lines of KARI, DHAD, IPMD and BCAT1 for their effect on branched chain volatile and iC5 acyl-sugar content. Obtained results indicate that KARI might be a rate limiting enzyme for iC5 acyl-sucrose synthesis in young leaf. Moreover, via stable isotopic study, I have also found that in tomato, major flux for synthesis of BCAAs downstream products is through leucine catabolism and not glucose. Additionally, this study shows inductive metabolic linkage between BCAAs, branched chain volatiles and other secondary metabolic pathway at DHAD transcriptional levels in fruit.

2.2. Materials and Methods

2.2.1 Plant materials

The transgenic plant antisense constructs of KARI, DHAD, IPMD and BCAT1 have been previously described (Maloney et al., 2010, Kochevenko and Fernie 2011). The antisense construct contains a 1327 bp fragment of KARI, the 1897 bp fragment of DHAD, the 875 bp fragment of IPMD small subunit and 1138 bp fragment of SlBCAT1 in the antisense orientation under the control of the 35S promoter. Transgenic plants from 2 independent lines per each genotype (total 8 transgenic lines) were selected on kanamycin containing MS medium (50 mg/l). *S. lycopersicum cv.* Moneymaker (WT) was germinated on MS medium without kanamycin. After seed germination, 6 plants for each of above mentioned 8 lines and WT were selected and transferred to soil pot for cultivation under long-day conditions (250 μmol photons/m^2/s, 16/8 h day/night cycle) at 22^0C and 50% humidity, as described previously in the literature.

2.2.2 Unlabelled leaf acyl-sugar and branched-chain volatile analysis

Leaf samples were taken 90 days after germination. Samples were then processed in a cryogenic grinding robot (Labman, North Yorkshire, UK). Whole leaf powder was used for volatile analysis with solid phase micro extraction-gas chromatography-mass spectrometry (SPME-GC-MS) (Fuentes et al., 2016). For acyl-sugar analysis, leaf tissues were processed as per the protocol mentioned in one of the following section. Peak annotation was performed based on updated literature survey (Slocombe et al., 2008, Schilmiller et al., 2010, 2012).

2.2.3 Metabolite feeding of fruit pericarp

Red ripe fruits from WT, KARI, DHAD, IPMD and BCAT1 lines were harvested at 40 days post-anthesis (dpa) and infiltrated with substrate [U-^{13}C] leucine the same day. [U-^{13}C] Glucose was used only for BCAT1 lines. Fruits were cut in half, cored, and pericarp discs were cut out with a 1 cm cork borer. Discs were trimmed horizontally to a depth of 0.5 cm to ensure uniformity. For each sample, three pericarp discs were frozen with liquid nitrogen immediately as a control and other three discs were placed single layered in sterile plastic petri dishes as a treatment. Thirty μl of 10 mM

amino acid and 10 mM a-keto acid or de-ionized water were pipetted onto the surface of each treatment pericarp disc, after which the plates were sealed and incubated at 25 ^{0}C in darkness for 6 hours. [U-^{13}C] Glucose was applied the same way. Two discs from each sample were powdered, weighed and then transferred in glass tubes and branched-chain volatiles were collected and analysed as described earlier. The other pericarp disc was used for GC-MS and LC-MS analysis of primary metabolites and acyl-sugar content, respectively and processed as per the standardized protocol.

2.2.4 Metabolite feeding of young leaf

Seeds of above mentioned WT and antisense lines were germinated and plants were grown as per the protocol mentioned in one of the previous sections. Young leaves were harvested 63 days after germination and infiltrated with substrate [U-^{13}C] leucine for WT, KARI, DHAD and IPMD lines; and [U-^{13}C] glucose only for WT and BCAT1 lines, the same day. For each sample, one young leaf was collected and frozen with liquid nitrogen immediately, as a control and stored at -80^{0}C freezer. Other leaf was placed in sterile 2 ml eppendorf tube containing 1.3 ml of 10 mM [U-^{13}C] leucine and incubated at 25 ^{0}C in light for 6 hrs. [U-^{13}C] glucose was applied the same way. Leaf samples were powdered, weighed and then transferred in glass tubes and branched-chain volatiles were collected and analysed as described earlier. Leaf powder was also used for GC-MS and LC-MS analysis of primary metabolites and acyl-sugar content, respectively and processed as per the standardized protocol.

2.2.5 Analysis of [U-^{13}C] leucine and [U-^{13}C] glucose labelled samples

Samples were processed as described above. The ^{12}C and ^{13}C spectral fragments of non-labelled control incubations were compared with that of the [U-^{13}C] leucine and [U-^{13}C] glucose fed tomato pericarp discs as detailed before (Roessner-Tunali et al., 2004). Molecular masses, retention time and associated peak intensities were extracted from the raw files using the Xcalibur software (Thermo Fisher Scientific, Bremen, Germany). Metabolite identification and annotation were performed using standard compounds. Relative abundance and percent enrichment was calculated for iC5 acyl-sucrose and 3-methylbutanal.

2.2.6 Metabolite profiling – Primary metabolites

Leaf and red ripe fruit pericarp samples were harvested, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Samples were then powdered by using retschmill at 30 l/s, for 30s. Metabolite extraction for GC-MS was carried out as previously described by Lisec et al. (2006) with minor modifications. For this purpose, stock solution for extraction was prepared by mixing 20 ml of 80% methanol with 1 ml of stock ribitol (0.2 mg/ml in water) as internal standard for relative quantification. Then 50 mg of ground material was extracted in 1200 µL of above mentioned stock solution for extraction. The mixture was then homogenized by using retschmill at 23 l/s, for 2 minutes (min) and then centrifuged for 10 min at 14,000 rpm. After centrifugation, the supernatant was transferred to a new tube and centrifuged again for 5 min at 14000 rpm. Sample aliquots of 600 µL for LC-MS and 150 uL for GC-MS of the upper phase was transferred to new tubes and dried in a centrifugal vacuum concentrator. The pellet was resuspended in 40 µL of methoxyaminhydrochlorid (20 mg/mL in pyridin) and derivatized for 2 hours by shaking at 900 rpm, at 37°C. Afterwards, 100 µL of *N*-methyl-*N*-[trimethylsilyl] trifluoroacetamide (MSTFA) was added containing 25 µL/mL FAMEs (fatty acid methyl esters mixture) as retention time standards. The mixture was incubated by shaking at 900 rpm for 30 min at 37°C. A volume of 1 µL of this solution was used for injection. The GC-MS system comprised a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph and a LECO Pegasus III TOF-MS running in EI positive mode. Metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005). Chromatograms and mass spectra were evaluated using Chroma TOF 1.0 (Leco) and TagFinder 4.0 software (Luedemann et al., 2008; 2012).

2.2.7 Metabolite profiling – Secondary metabolites

Leaf and red ripe fruit pericarp samples were harvested, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Samples were then powdered by using retschmill at 30 l/s for 30s. Metabolite extraction for LC-MS was carried out as mentioned in previous section. Dried samples were then resuspended in 200 uL of stock solution for extraction. UPLC separation was performed using a Waters Acquity UPLC system (Waters, Mildford, MA, U.S.A.), using a HSS T3 C18 reverse

phase column (100 × 2.1 mm i.d. 1.8 µm particle size, Waters) at a 40 °C according to the previously published protocol (Giavalisco et al., 2009). The mobile phases consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The flow rate of the mobile phase was 400 µL/min, and 2 µL sample was loaded per injection. The UPLC was connected to an Exactive Orbitrap (Thermo Fisher Scientific, Bremen, Germany) via an heated electro spray source (Thermo Fisher Scientific, Bremen, Germany). The spectra were recorded using full scan mode of negative ion detection, covering a mass range from *m/z* 100–1500. The resolution was set to 25,000 and the maximum scan time was set to 250ms. The sheath gas was set to a value of 60, while the auxiliary gas was set to 35. The transfer capillary temperature was set to 150°C while the heater temperature was adjusted to 300°C. The spray voltage was fixed at 3 kV, with a capillary voltage and a skimmer voltage of 25V and 15V, respectively. MS spectra were recorded from min 0 to 19 of the UPLC gradient. Molecular masses, retention time and associated peak intensities were extracted from the raw files using the Xcalibur software (Thermo Fisher Scientific, Bremen, Germany). Metabolite identification and annotation were performed using standard compounds, literature and tomato metabolomics databases (Moco et al., 2006; Iijima et al., 2008; Tohge and Fernie, 2009, 2010; Rohrmann et al., 2011). Data are reported in a manner compliant with the standards suggested by Fernie et al. (2011).

2.2.8 Metabolic profiling – Volatile organic compounds

For SPME-GC-MS profiling of volatile organic compounds (VOCs), leaf and red ripe fruit pericarp samples were harvested, immediately frozen in liquid nitrogen and powdered by using retschmill at 30 l/s for 30s. Aliquots of 500 ± 10 mg of frozen powdered leaf tissue were weighed in frozen microcentrifuge tubes, and then transferred to frozen 20 mL head-space screw cap vials. These vials were kept at 15°C for at least 1 hr and then incubated for 10 min at 50°C prior to VOCs analysis. StableFlexÔ SPME fiber with 65 mm polydimethylsiloxane/divinylbenzene coating (Supelco, Bellefonte, USA) was used to sample VOCs in a replicated randomized block sequence design. VOCs were then profiled as described previously (Agudelo-Romero et al., 2015; 2013). Column used was DB-624 capillary column of 60 m length, 0.25 mm internal diameter and 1.40 mm film thickness (Agilent Technologies

Deutschland GmbH, Waldbronn, Germany). VOCs were analyzed by gas chromatography coupled to electron impact ionization/quadrupole mass spectrometry (GC-EI/QUAD-MS) using an Agilent 6890N24 gas chromatograph connected to an Agilent 5975B VL mass spectrometer (Agilent Technologies, Böblingen, Germany). Data files were visually controlled, exported in NetCDF file format and baseline-corrected using the Chroma TOF 1.0 (Leco) while TagFinder software was used for data processing into a standardized numerical data matrix and compound identification (Luedemann et al., 2008). Retention time deviation <1.0% and presence of at least three specific and selective mass fragments were the criteria for metabolite identification.

2.3 Results

2.3.1 Metabolite profiling of leaf of WT and transgenic plants

Kochevenko and Fernie 2011 had developed antisense lines of KARI, DHAD and IPMD, key enzymes in BCAAs biosynthetic pathway. By using these lines, they have shown that BCAAs content in source leaves were significantly reduced. However, BCAAs content was significantly increased in fruits of IPMD lines. Antisense inhibition of neither KARI nor DHAD produced a clear effect in fruit BCAA contents. In order to evaluate its effects on BCAA-derived secondary metabolites such as iC5 acyl-sucrose and branched chain volatiles, two independent antisense lines from each construct were chosen. After kanamycin screening transgenic plants from the T_2 generation were grown in the greenhouse and source leaves were analyzed for iC5 acyl-sucrose, 3-methylbutanal and 3-methylbutanol in comparison with WT controls. LC-MS was used for ic5 acyl-sucrose analysis while SPME-GC-MS was used for branched chain volatile analysis. Results showed no significant change for iC5 acyl-sucrose (690.8 *m/z*), 3-methylbutanal and 3-methylbutanol content in KARI, DHAD, IPMD and BCAT1 lines (Figure 2.2 and 2.3; Supplementary Table 2.1).

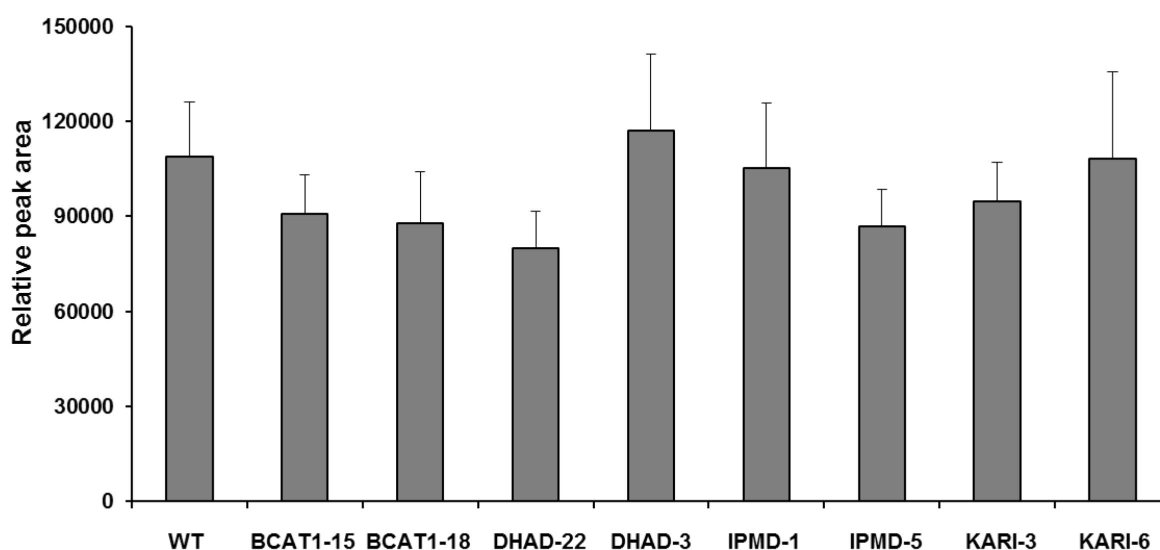


Figure 2.2 LC-MS analysis of iC5 acyl-sucrose (690.8 *m/z*) content in the leaves of WT and antisense transgenic lines. Data represent means ± SE from six independent biological replicates. No statistically significant changes found according to Student’s t test ($P < 0.05$).

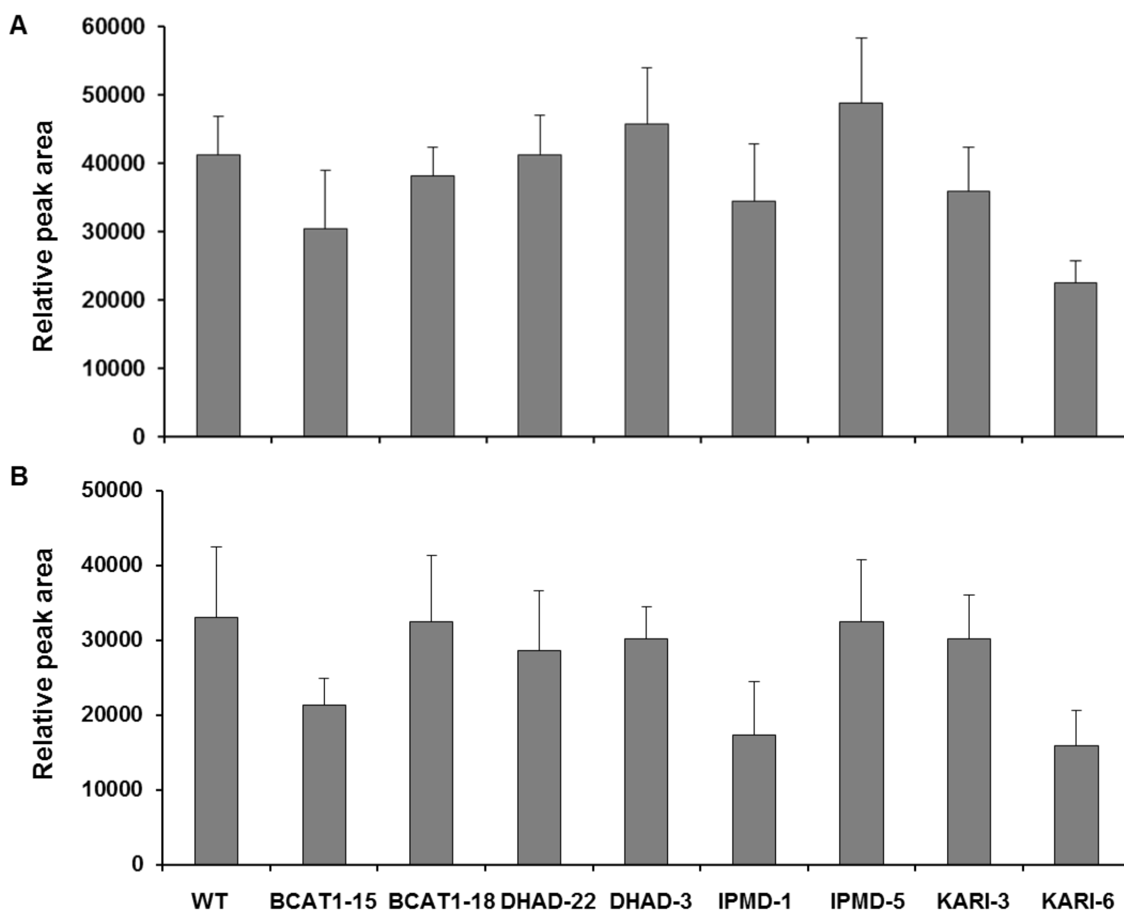


Figure 2.3 SPME-GC-MS analysis of A) 3-methylbutanal and B) 3-methylbutanol content in the leaves of WT and antisense transgenic lines. Data represent means ± SE from six independent biological replicates. No statistically significant changes found according to Student's t test ($P < 0.05$).

These results suggest that alteration in leucine levels in the leaves of tomato does not influence the rate of synthesis of iC5 acyl-sucrose, 3-methylbutanal and 3-methylbutanol; indicating metabolic linkage of BCAAs metabolism with other pathways. Earlier studies have shown that catabolism of BCAAs supports respiration in leaf and green fruits of tomato by fueling the mitochondrial electron transport chain both directly via the electron transfer protein complex and indirectly via the TCA cycle; but not volatile synthesis (Arau´jo et al., 2010, 2011, Kochevenko et al., 2012). In summary, results showed that antisense expression of neither KARI, DHAD, IPMD (BCAAs biosynthetic enzymes) nor BCAT1 (BCAAs catabolic enzyme) had a significant effect on iC5 acyl-sucrose and branched chain volatiles synthesis in mature leaves of tomato.

2.3.2 [U-^{13}C] leucine and [U-^{13}C] glucose enrichment analysis

Total content of BCAAs degradation products iC5 acyl-sucrose and branched chain volatiles were not significantly changed in transgenic leaves led to the assumption that they are synthesized from a different precursor. Branched-chain fatty acids (BCFAs) biosynthesis mainly takes place by using BCAAs as precursors. Studies in *Staphylococcus xylosus* have shown that BCFA 3-methylbutanoic acid can also be synthesised from glycolysis via pyruvate, in case of depletion of BCAAs (Figure 2.4A) (Beck et. al., 2004). Previous work with [U-^{13}C] leucine feeding in tomato fruit pericarp led to the proposed biosynthetic pathway for leucine degradation to 3-methylbutanol (Figure 2.4B) (Kochevenko et al., 2012). Both studies provide direct evidence of proposed pathway to 3-methylbutanoic acid and 3-methylbutanol but not to iC5 acyl-sucrose. In order to further analyze linkage between leucine catabolism, and synthesis of iC5 acyl-sucrose, 3-methylbutanal and 3-methylbutanol in tomato fruit; a separate metabolite feeding experiment was performed in which [U-^{13}C] leucine was added to tomato fruit pericarp discs and young leaves of KARI, DHAD, IPMD and BCAT1 lines while [U-^{13}C] Glucose was added to same of only BCAT1 lines.

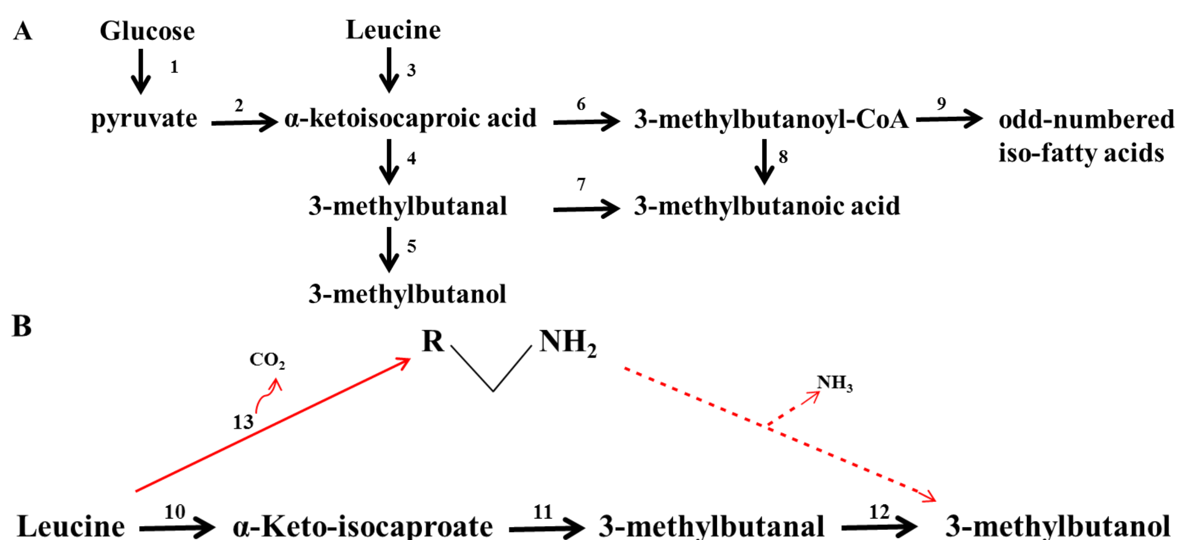


Figure 2.4 A) Biosynthetic pathway for leucine and glucose degradation leading to odd-numbered iso-fatty acids and 3-methylbutanol synthesis in microbes: (1) glycolytic enzymes, (2) the enzymes of the isoleucine–valine biosynthetic pathway, (3) a transaminase, (4) a branched-chain ketoacid decarboxylase, (5 & 7) an aldehyde dehydrogenase, (6) a branched-chain ketoacid dehydrogenase, (8) an acyl-CoA hydrolase or phosphate butyrate CoA transferase and butyrate kinase and (9) enzymes of the fatty acid biosynthetic pathway. Figure presented here was modified from Beck *et al.*, 2004 with updated literature. B) Proposed biosynthetic pathway for leucine degradation to 3-methylbutanol in tomato plants:

(10) branched-chain aminotransferases 1 (BCAT1), (11) keto-acid decarboxylase, (12) aldehyde dehydrogenase and (13) branched-chain decarboxylase. Solid arrows indicate that the enzyme activity and genes have been identified. Dashed arrows indicate that the proposed plant enzymes and genes are yet to be identified. Red route and Black route: Two different routes as partially demonstrated by Tieman *et al.,* 2006b, Gonda *et al.,* 2010 and Kochevenko *et al.,* 2012.

Rational for using [U-^{13}C] Glucose only for BCAT1 lines was that glucose will be preferred substrate over leucine since BCAT1 is silenced. Samples were analyzed with SPME-GC-MS. I could not detect label in 3-methylbutanol for both the [U-^{13}C] leucine and [U-^{13}C] Glucose fed samples, and also in 3-methylbutanal for [U-^{13}C] Glucose fed samples. Relative abundance and percent enrichment for leucine and 3-methylbutanal was calculated for the [U-^{13}C] leucine treated red ripe pericarp samples. Relative abundance and percent enrichment for leucine were not affected in control and treated samples of any of the antisense lines except for significant increase in one of treated KARI line (Figure 2.5A; $P < 0.05$). However, relative abundance for 3-methylbutanal was increased in case of one of IPMD, KARI and both DHAD lines. Interestingly, percent enrichment was significantly lower only for both DHAD lines as compared to WT (Figure 2.5B; $P < 0.05$). Next, relative abundance and percent enrichment for leucine and 3-methylbutanal were calculated for [U-^{13}C] leucine treated young leaf samples.

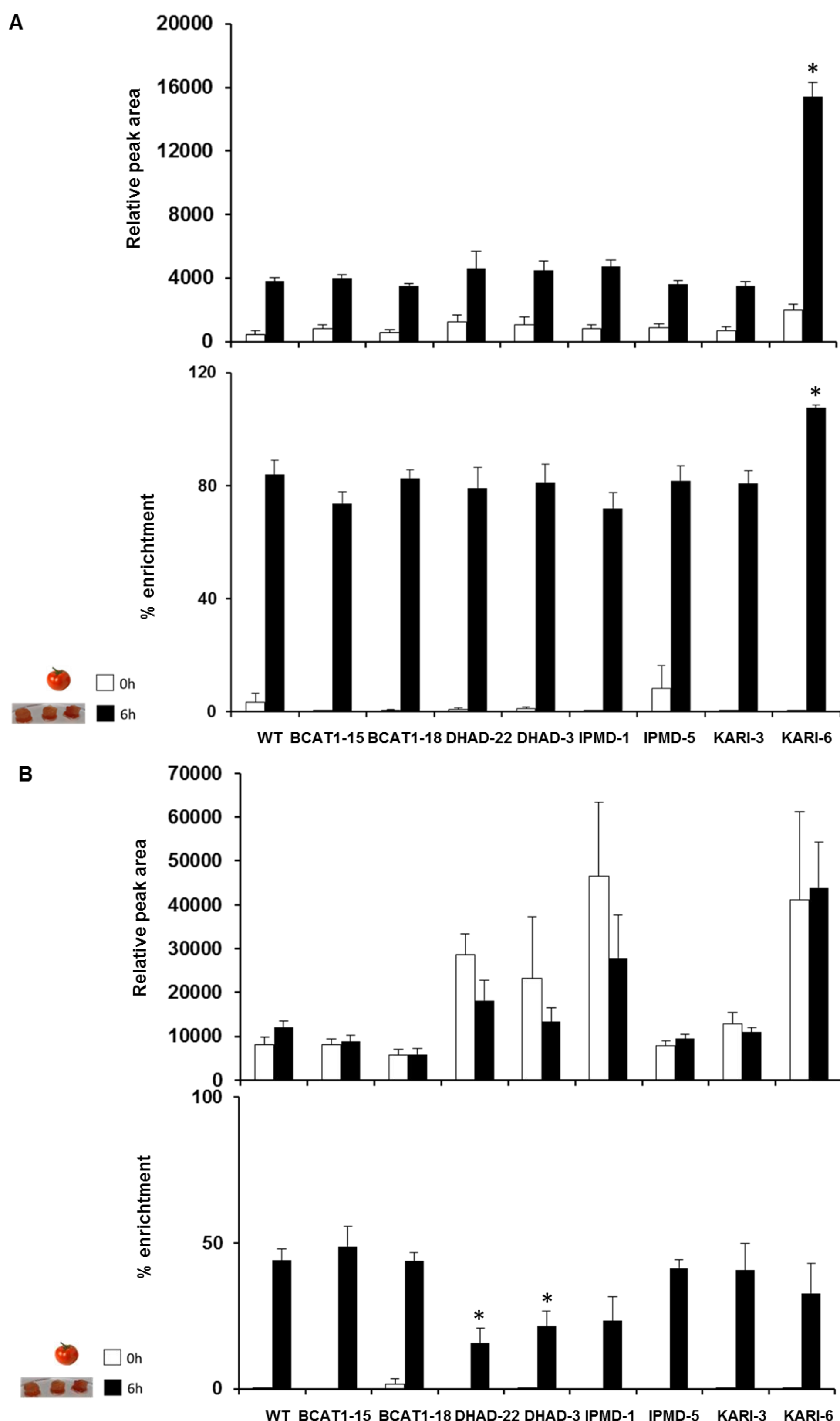


Figure 2.5 A) Relative abundance and percent enrichment for leucine in red ripe pericarp tissues of control (0 hour) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. B) Relative abundance and percent enrichment for 3-methylbutanal for the same. Values represent the mean ± SE of six independent determinations. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

Relative abundance and percent enrichment for leucine and 3-methylbutanal were not affected (Figure 2.6A and B; Supplementary Table 2.3A and B) for [U-^{13}C]

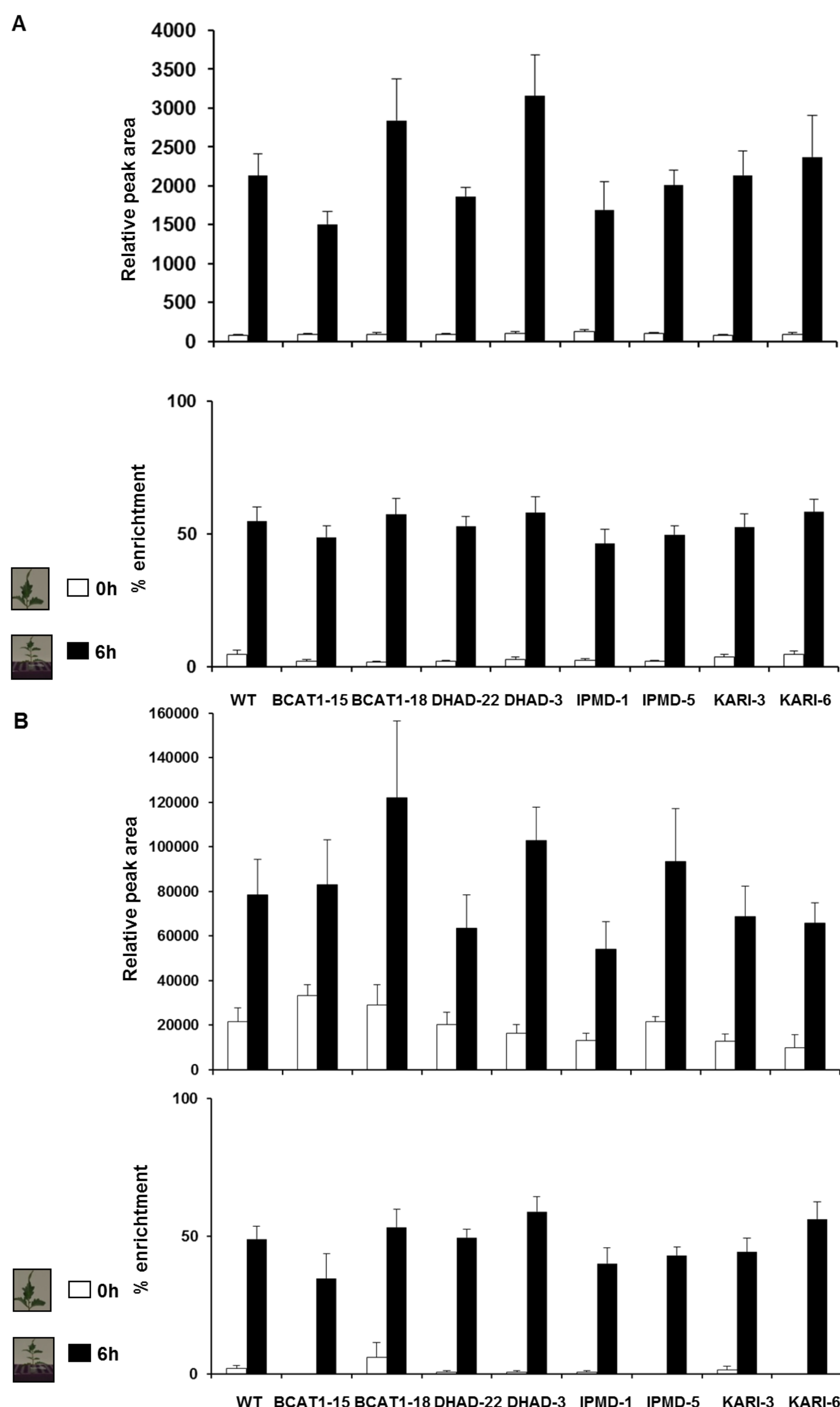


Figure 2.6 A) Relative abundance and percent enrichment for leucine in young leaves of control (0 hour) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. B) Relative abundance and percent enrichment for 3-methylbutanal of the same. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

leucine treated young leaf samples. Having checked percent enrichment for 3-methylbutnal in leaf and fruit tissues of antisense lines, I went further to check the same for iC5 acyl-sucrose in same tissue samples. No significant changes were found for relative abundance and percent enrichment of iC5 acyl-sucrose in red ripe tissues (Figure 2.7A). But total iC5 acyl-sucrose content was significantly increased only for one of DHAD line in case of control over that of WT (Figure 2.7B).

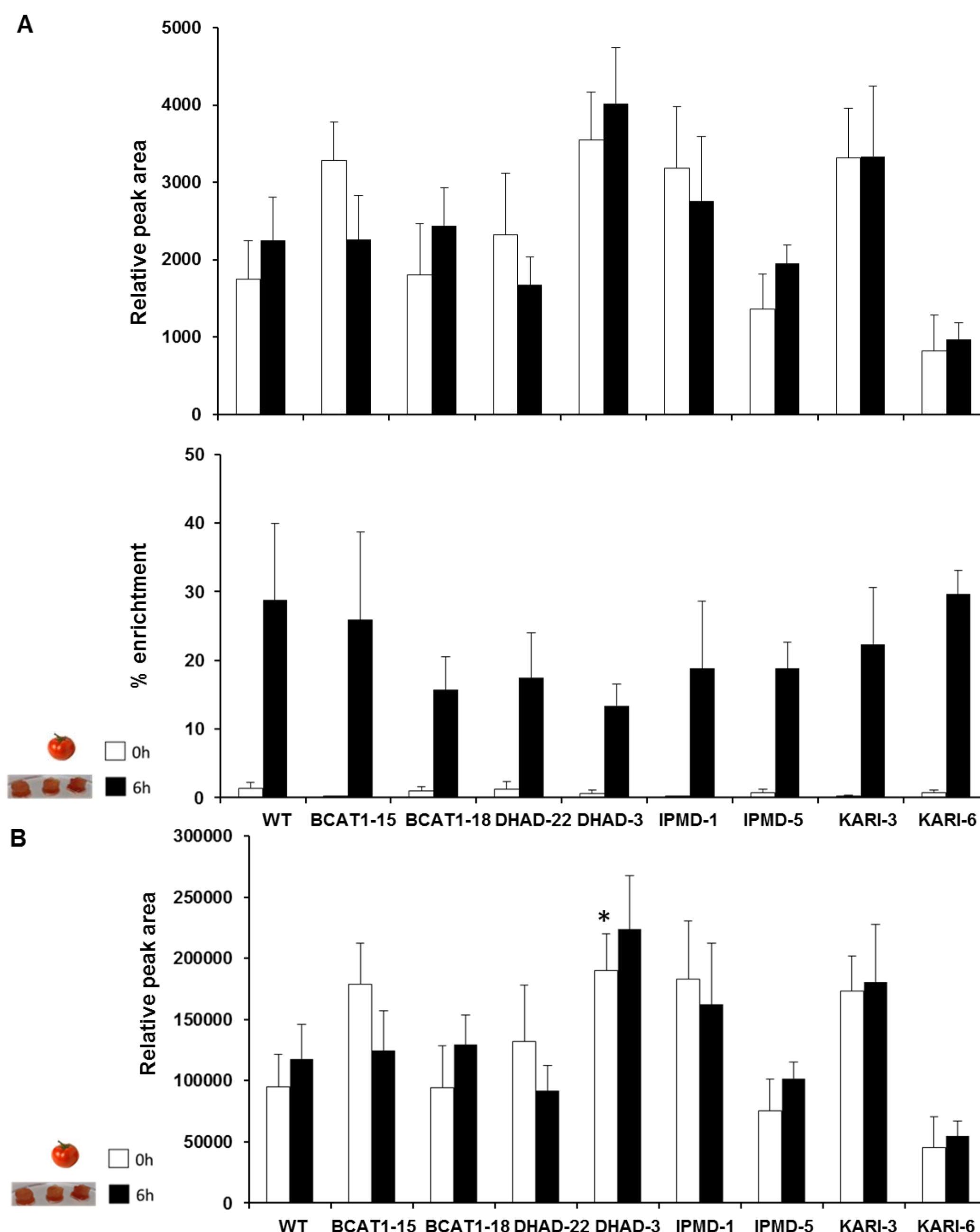


Figure 2.7 A) Relative abundance and percent enrichment for iC5 acyl-sucrose (690.8 *m/z*) in red ripe fruit pericarp of control (0 hour) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. B) Total iC5 acyl-sucrose (690.8 *m/z*) content of the same. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

Interestingly, when I checked the same in leaf tissue, I found relative abundance and total content of iC5 acyl-sucrose was significantly decreased for both the KARI lines and one of IPMD line only for control (Figure 2.8A and B; Supplementary Table 2.3B and C); while percent enrichment was significantly increased only for one of KARI line.

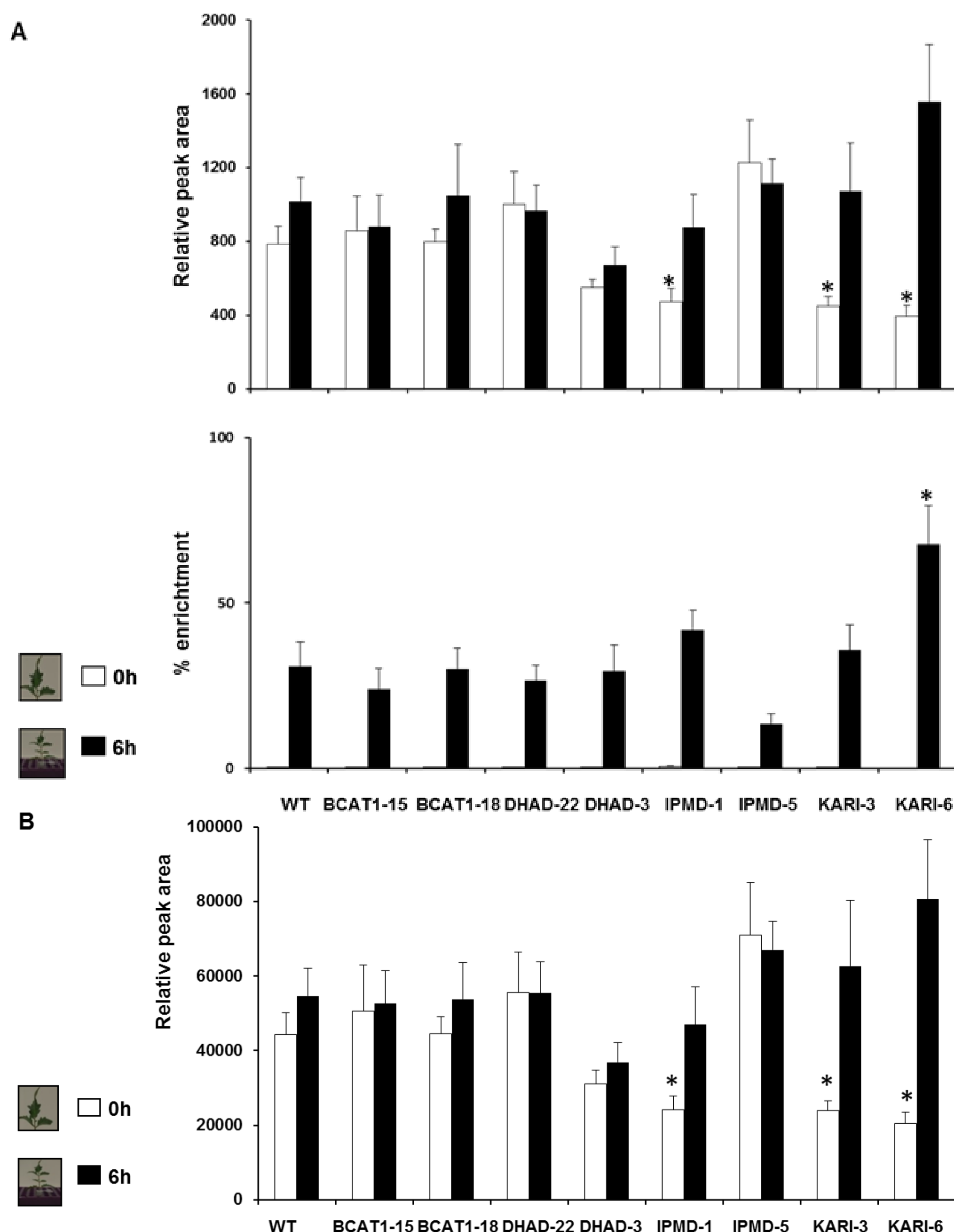


Figure 2.8 A) Relative abundance and percent enrichment for iC5 acyl-sucrose (690.8 *m/z*) in young leaf samples of control (0 hour) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. B) Total iC5 acyl-sucrose (690.8 *m/z*) content of the same. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

Trichome type 4 and 6 synthesizes acyl-sugars. Type 6 also synthesizes flavonoids, monoterpenes and sesquiterpenes (Li et al., 2004; Schilmiller et al., 2009; Kang et al., 2010). Pyruvate is a common precursor for tricarboxylic acid (TCA) cycle and BCAAs biosynthesis. Inhibition of TCA cycle in *Corynebacterium glutamicum* (*C. glutamicum*) by deleting pyruvate dehydrogenase complex (PDHC) induced BCAAs biosynthesis and hence increased demand for NADPH supply by KARI (Bartek et. al., 2011). With the help of transcriptomics, metabolomics, proteomics; and $^{13}CO_2$ and glucose labeling studies; Balcke et al., (2017) have shown that tomato glandular trichomes constitute a strong sucrose sink with low Calvin-Benson-Bassham (CBB) cycle activity but high expression of photosystem genes. Like leaf mesophyll cells, the glandular head cells of type VI trichomes possess their own chloroplasts with intact thylakoid membranes, suggesting they have the capacity to generate the ATP and NADPH required for the assembly of secondary metabolites (Balcke et al., 2017). Though higher activity of photosynthetic machinery might support higher demand for NADPH by KARI for iC5 acyl-sucrose synthesis in young leaf, photorespiration becomes limiting factor. This is evident from significant decrease in iC5 acyl-sucrose content for both the lines of KARI, for control. Here increased demand for NADPH might have met by minor flux through pentose phosphate pathway (PPP) for treatment.

These combined results thus suggest that regulation of metabolic linkage between leucine and its degradation products iC5 acyl-sucrose, 3-methylbutanal and 3-methylbutanol; is tissue specific with KARI being the rate limiting for iC5 acyl-sugars synthesis in young leaf. Additionally, results also suggest that DHAD is a key enzyme for 3-methylbutanal synthesis in fruit.

2.3.3 Effect of altered BCAAs metabolism on pyruvate, photorespiration and TCA cycle

Having speculated photorespiration as a limiting factor for iC5 acyl-sucrose synthesis in leaf, we went further to check the effect of altered BCAAs metabolism on pyruvate and photorespiration as well as TCA cycle metabolites. Pyruvate was decreased in young leaf tissues of most of the antisense lines (Figure 2.9A). Levels of glycine were not affected in young leaf tissues of any of the antisense lines except for one of BCAT1 line (Figure 2.9B). However, serine content in young leaf tissues was increased for one of DHAD and IPMD line (Figure 2.9C). Next, I checked levels of

these metabolites in red ripe fruit tissues. Interestingly, pyruvate was significantly increased in control fruit tissues of one of the DHAD and IPMD line (Figure 2.10A, Supplementary Table 2.5A). Additionally, glyceric acid levels were significantly reduced in young leaf samples of some of control as well as treated DHAD and IPMD lines. Intriguingly, they were slightly changed in those of red ripe fruit tissue (Supplemental Table 2.5B).

Levels of succinate and citrate were increased in red ripe fruit tissues of most of the antisense lines but didn’t change in same of young leaf tissues, for control as well as treatment (Figure 2.11 and 2.12). Aconitate content was significantly decreased in control red ripe fruit tissues of most of the antisense lines but was increased in same of young leaf tissues (Figure 2.11B and 2.12B). These results suggested that levels of other secondary metabolites might have affected.

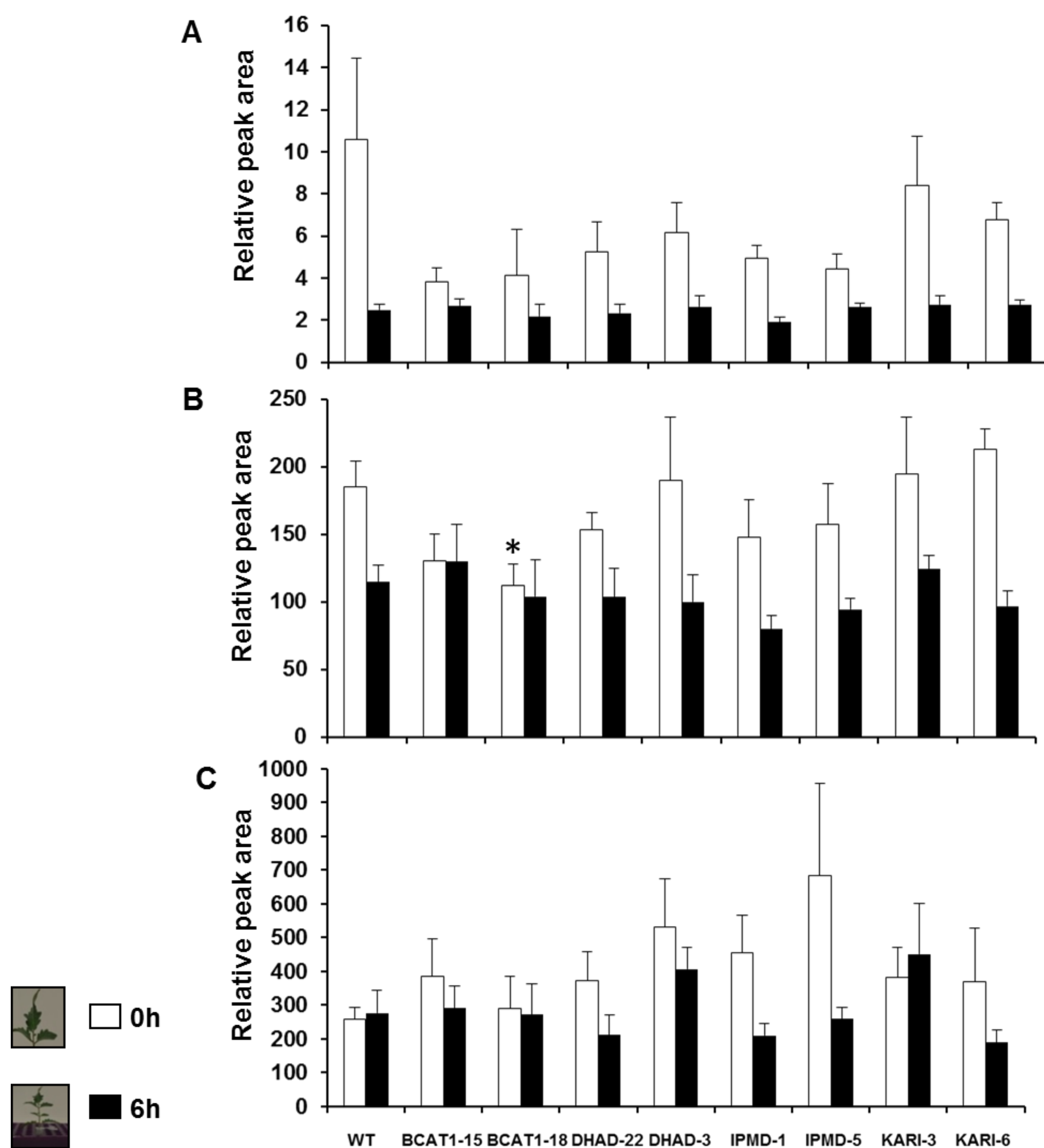


Figure 2.9 Relative contents of A) Pyruvate, B) Glycine and C) Serine in young leaf of control (0 hours) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student’s t-test (P<0.05).

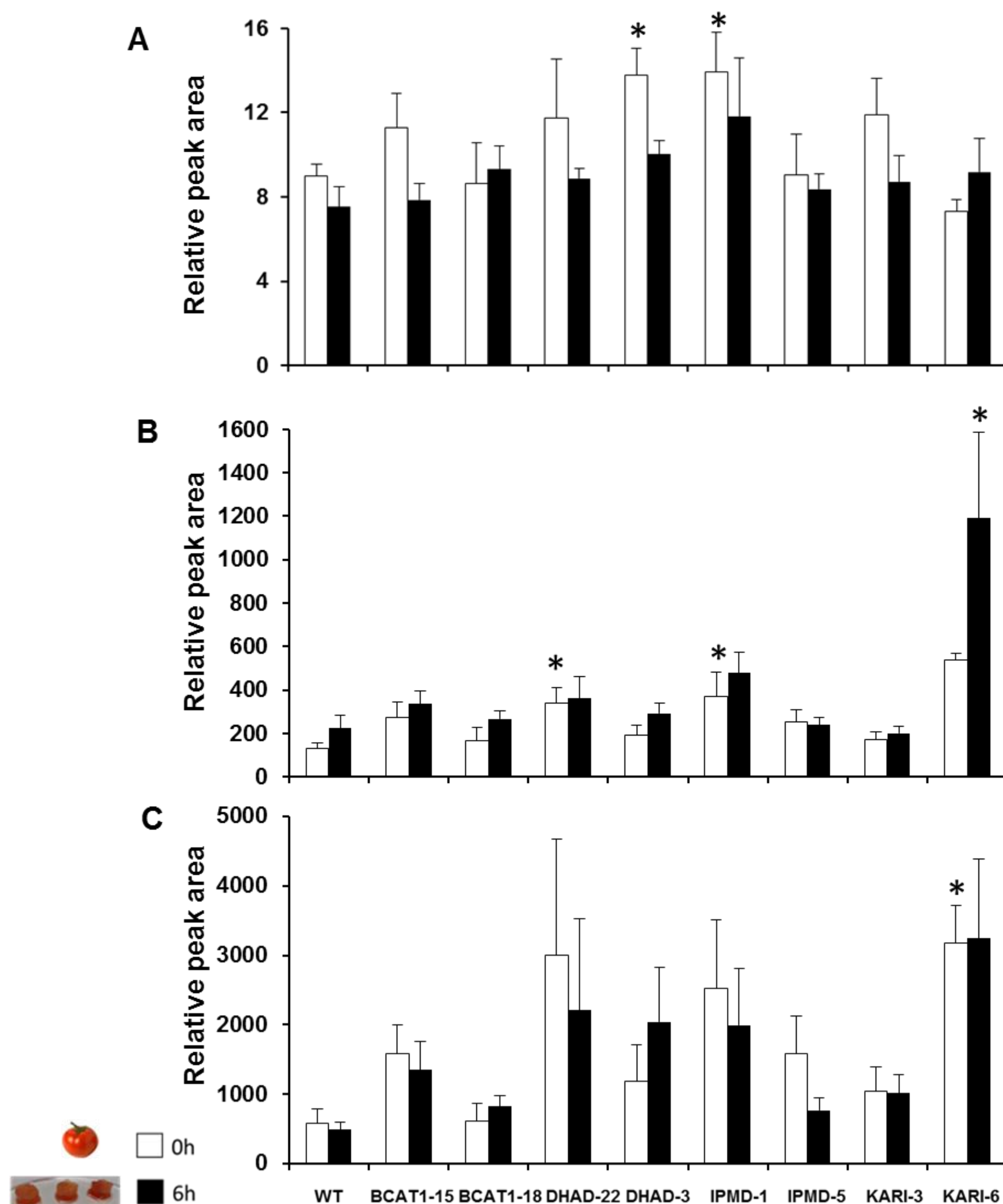


Figure 2.10 Relative contents of A) Pyruvate, B) Glycine and C) Serine in red ripe fruit pericarp of control (0 hours) and [U-13C] leucine labelled (6 hours) WT and antisense transgenic lines. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test ($P<0.05$).

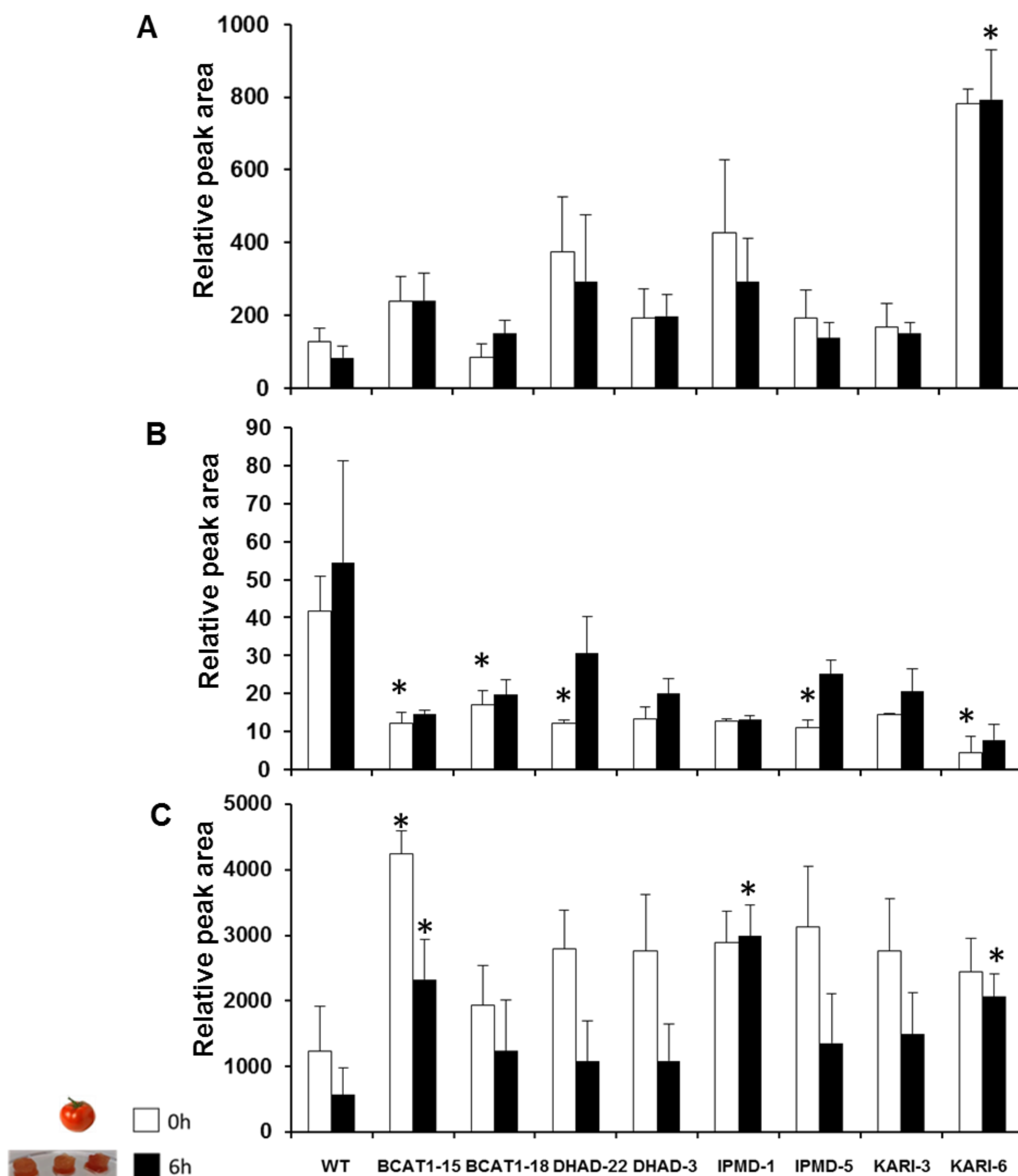


Figure 2.11 Relative contents of A) Succinate, B) Aconitate and C) Citrate in red ripe fruit pericarp of control (0 hours) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

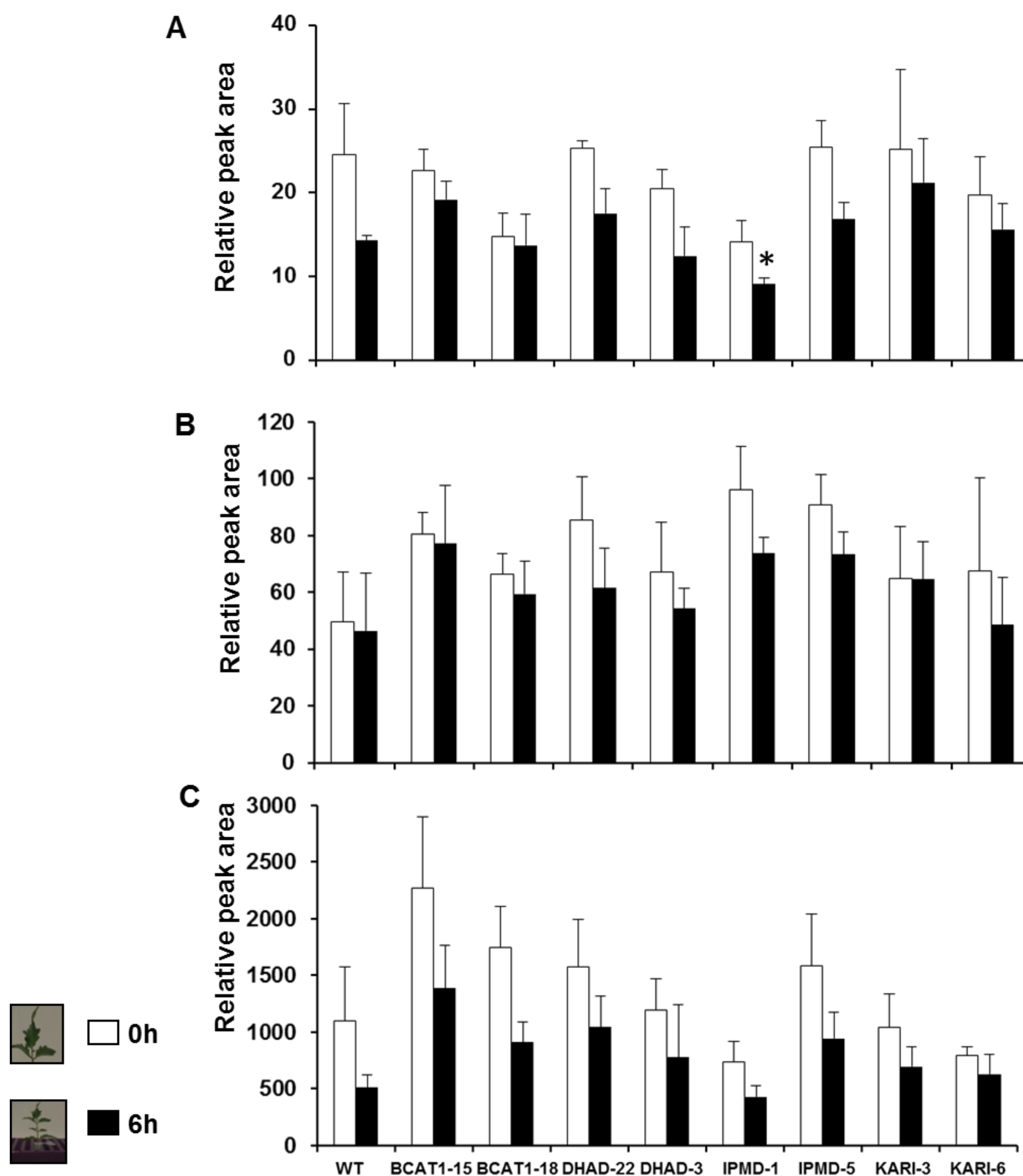


Figure 2.12 Relative contents of A) Succinate, B) Aconitate and C) Citrate in young leaf of control (0 hours) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

2.3.4 Linkage of leucine metabolism with other pathways

Transcript analysis have indicated that both trichome types I and IV, as well as VI, across Solanum species, express many of the genes necessary for acyl- sugar, flavonoid and terpenoid production (McDowell et al., 2011). Here, in order to decipher metabolic linkage between leucine metabolism and other secondary

metabolic pathways such as phenylpropanoid and glycoalkaloid metabolic pathway, we checked the rutin and α-tomatine content in both leaf and fruit tissues of above mentioned antisense lines.

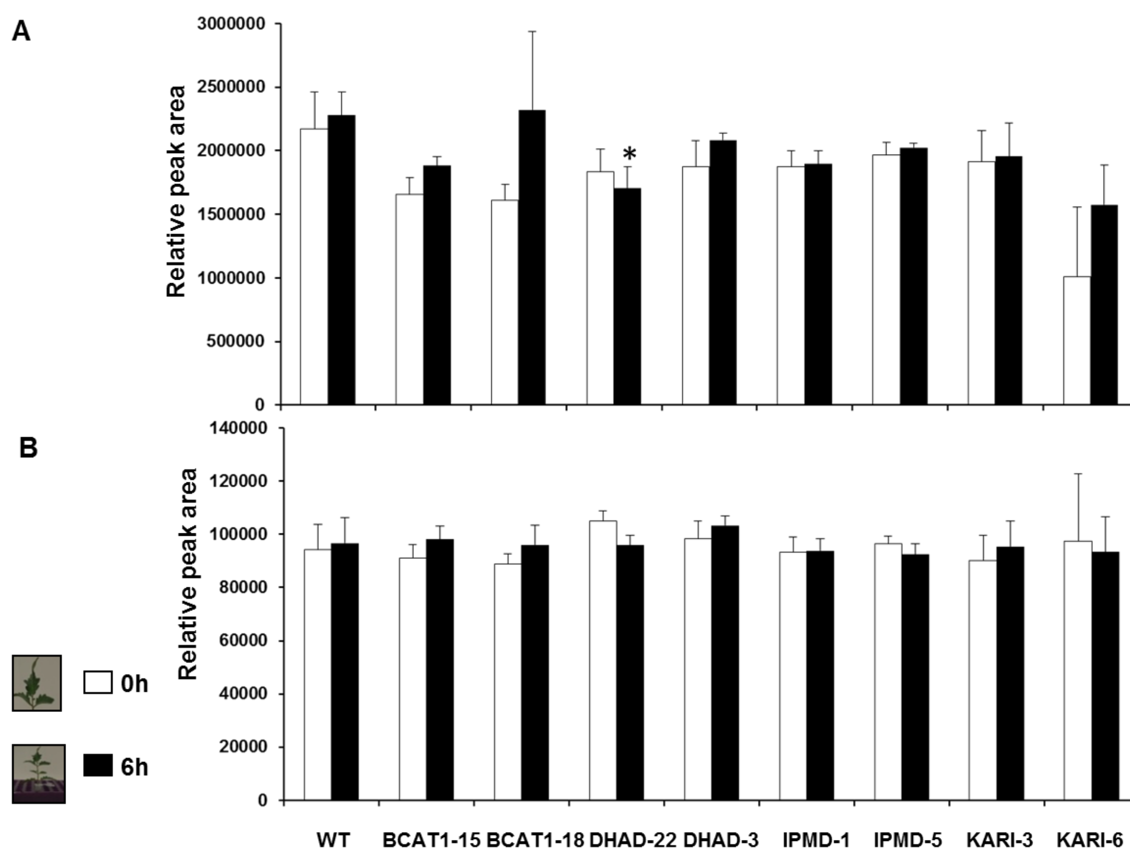


Figure 2.13 Relative contents of A) Rutin and B) α-Tomatine in young leaf of control (0 hour) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test ($P<0.05$).

Levels of both rutin and α-tomatine were not significantly changed in young leaf tissue of any of the antisense lines for both the timepoints, except one of treated DHAD line (Figure 2.13A and B). On the other hand surprisingly, levels of rutin in treated red ripe fruit tissue were significantly decreased for all the antisense lines except for one of the DHAD and IPMD line (Figure 2.14A and B). Moreover I found that DHAD line (line 3) showing no significant change for rutin content, showed significant increase in α-tomatine content.

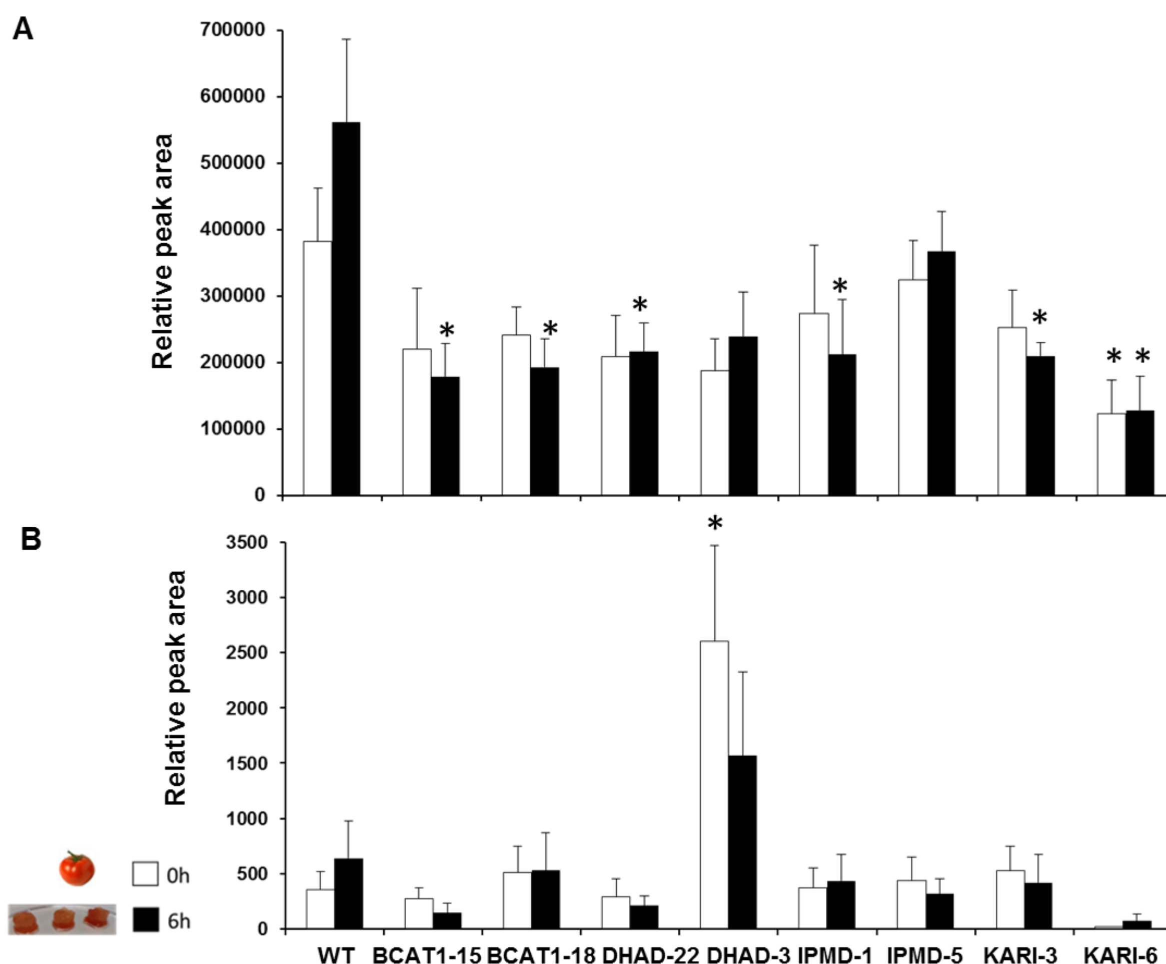


Figure 2.14 Relative contents of A) Rutin and B) α-Tomatine in red ripe fruit pericarp of control (0 hours) and [U-^{13}C] leucine labelled (6 hours) WT and antisense transgenic lines. Values represent the mean ± SE of six independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

Red ripe tomato fruit is heterotrophic and levels of valine, isoleucine, and leucine at 40 days post anthesis (dpa) are four and 10 times lower than those determined in fruits of 10 dpa (Maloney et al., 2010). Phenylalanine levels in leaf samples of antisense lines were significantly reduced while those in red ripe fruit pericarp samples were not changed (Supplementary material Kochevenko and Fernie, 2011). Obtained results showed that despite significant reduction in phenylalanine content in leaf samples of antisense lines, synthesis of phenylalanine derived secondary metabolite rutin was not affected. Contrastingly, even though levels of phenylalanine were not reduced in red ripe fruit tissue, rutin content in most of the treated samples of antisense lines was significantly affected. However, besides this effect on phenylpropanoid metabolism, arginine metabolism was also affected in fruit. Despite significant increase in arginine levels, putrescine content was not changed

(Supplementary material of Kochevenko and Fernie 2011). These combined results therefore indicate that alteration in BCAAs metabolism affect phenylpropanoid and arginine metabolism, and might be regulated in leaf and fruit tissue differentially. Additionally, our results indicate that reduced DHAD transcriptional levels in fruit tissue of one of DHAD line might support α-tomatine synthesis significantly by inducing pyruvate synthesis. Further experiments might shed light on this metabolic linkage.

2.4. Discussion

Secreting glandular trichomes (SGTs) synthesizes species specific secondary metabolites. Secondary metabolite diversity in tomato trichomes has been studied very well (Slocombe et al., 2008; Besser et al., 2009; Schilmiller et al., 2009, 2010). In recent years, there has been an increasing interest in understanding acyl-sugar pathway because of its commercial value and role in plant defense against many insect herbivores. BCAA leucine is a common substrate for synthesis of iC5 acyl-sugars and branched chain volatiles 3-methylbutanol and 3-methylbutanal. The genetic architecture of BCAAs metabolism has been established in plants (Kochevenko and Fernie 2011). There are also few studies that have found metabolite QTLs affecting acyl-sugar and volatile synthesis but the studies concerning their metabolic flux information are still lacking (Tieman et al., 2009; Alseekh et al., 2015). These previous studies provide an interesting context in which to evaluate the results of the current study. Research in trichome metabolomics is challenged by miniscule nature of tomato trichomes and their ability to synthesize not only acyl-sugars but also flavonoids, terpenes and volatiles (McDowell et al., 2011). In order to decipher metabolic linkage between primary and secondary metabolism, here I explored antisense lines impaired in BCAAs metabolism for their effects on BCAAs downstream products as well as on other secondary metabolic pathways.

Previous studies have revealed that BCAAs catabolism supports respiration in leaves by feeding directly into the TCA cycle, and indirectly into mitochondrial electron transport chain via the reactions catalyzed by isovaleryl dehydrogenase and 2-D-hydroxyglutarate dehyrogenase (Ishizaki et al., 2005, 2006; Engqvist et al., 2009; Arau´jo et al., 2010, 2011); during dark period of a normal light–dark cycle (Caldana et al., 2011; Engqvist et al., 2011; Kochevenko et al., 2012). Furthermore, it has been shown that catabolism of BCAAs even in young green tomato fruits supports respiration but not volatile synthesis (Kochevenko et al., 2012). Here, authors have speculated that the volatile production may be influenced by the BCAAs content in red ripe fruits. α-ketoisocaproic acid (α-KIC) is the immediate precursor for 3-methylbutanal, 3-methylbutanol and iC5 acyl-sucrose synthesis and could be present in the chloroplast and mitochondria (Beck et. al., 2004). But nothing is known about the genes encoding α-KIC transporters. Moreover, site of volatile synthesis and some genes in the pathway is still unknown. Research in leucine derived volatile synthesis is hurdled by its extensively polygenic nature. For

example, 12 loci that quantitatively affect the emission of leucine- and/or isoleucine-derived volatiles have been identified. But a locus can encode TFs that coordinately regulate genes or may encode enzymes that catalyze limiting steps in single pathways or may influence the precursor or intermediate pools (Tieman et al., 2006). Moreover, authors have also suggested that leucine degradation also synthesizes isovaleronitrile besides 3-methylbutanal and 3-methylbutanol. In this study, obtained results demonstrate that synthesis of 3-methylbutanal is not affected by leucine content in red ripe fruits and young leaf of tomato. However, results indicate that DHAD is a key enzyme that could be transcriptionally modulated to regulate levels of 3-methylbutanal, iC5 acyl-sucrose and α-tomatine in red ripe tomato fruits.

Metabolism in red ripe fruit is different than that from leaf tissue. Fruit maturation stage itself is accompanied by large changes in primary as well as secondary metabolite content. Glycolysis and respiration are dominant carbon fluxes in the fruit (Rontein et al., 2002; F Carrari and AR Fernie, unpublished results). Moreover, it has been found that most of the TCA cycle intermediates were decreased while levels of glucose and fructose were increased as fruit matures (Carrari and Fernie 2006). Pyruvate is a precursor for BCAAs and sesquiterpenes biosynthesis in plastids and it can be imported from the cytosol or produced through plastidic glycolysis or by plastidic isoforms of malic enzyme (Oliver et al., 2009; Weber and Brautigam, 2013; Eisenhut et al., 2015; Shtaida et al., 2015). BCAAs biosynthesis, glycolysis and TCA cycle are linked by PDHC. Study in *C. glutamicum* found that inactivation of PDHC interrupts the most important connection between glycolysis and the TCA cycle, and increases BCAAs synthesis by inducing PPP (Bartek et. al., 2011). Moreover, it has been shown that in tomato fruits PPP functions mainly as the mechanism for the conversion of glucose to various intermediates for biosynthesis and the overflow of PPP is routed by way of fructose-6-phosphate and fructose-1,6-diphosphate to the glycolytic pathway (Wang et al., 1962). However, metabolic linkage between BCAAs biosynthesis, glycolysis and TCA cycle; and its effects on downstream secondary metabolic pathways have not been explored in tomato plants. With the stable isotope [U-^{13}C] leucine and [U-^{13}C] glucose labelling experiments narrated here I am able to show that in tomato, major flux for synthesis of BCAAs downstream products is through leucine catabolism and not glucose since label was not detected in any of the BCAAs degradation products for [U-^{13}C] glucose labelled samples. Therefore flux through PPP if at all present as per previous reports might have been minor.

Additionally, obtained results showed decrease in glycine content in treated young leaf tissues of most of the antisense lines over that of respective control, supporting the occurrence of photorespiration in them. In BCAAs biosynthetic pathway, reduction reaction catalyzed by KARI depends on NADPH and Mg2+ (Singh 1999). Tomato is a C3 plant and photorespiration incurs additional cost of one ATP and one NADPH, making KARI as a rate limiting enzyme for iC5 acyl-sucrose synthesis in young leaf. Red ripe fruit tissues are able to meet this demand because of lower or no photorespiratory activity and higher levels of glucose and dominant glycolytic flux. Lower photorespiratory activity in tomato fruit tissue is evidenced by one of the study that showed drastic reduction in large number of proteins involved in light reactions (including photosynthesis, Calvin cycle, and photorespiration) and major CHO metabolism (starch metabolism) during chromoplastogenesis (Barsan et al., 2012). Young leaves are photosynthetically active and are source tissue, while red ripe fruits are heterotrophic and are sink tissue. Lower photorespiratory activity, higher amino acids content as well as higher glycolytic and or TCA cycle flux in fruit tissues might meet higher demands for NADPH and pyruvate. It has been shown that free amino acid (particulary glutamate and GABA (gamma-aminobutyric acid)) content of tomato fruit pericarp increases markedly during tomato fruit ripening (Boggio et al. 2000), suggesting a high protein turnover. These amino acids can fuel TCA cycle at different levels, thus increasing the availability of pyruvate for secondary metabolite synthesis. Consistent with this, increased pyruvate, succinate and citrate levels in red ripe pericarp tissues of most of the antisense lines indicate induced protein (or membrane) degradation led synthesis of amino acids and their feeding into TCA cycle. However, based on results, it is clear that amino acid increase in red ripe tomato fruits doesn´t support ic5 acyl-sucrose synthesis.

Tomato flavour is very important when it comes to consumer acceptance and profit to farmers. Volatiles can be derived from primary as well as secondary metabolites. There is an increasing trend in finding out new key genes involved in volatile synthesis pathway (Rambala et al., 2017). In this case study, our interest particularly lies in understanding metabolic linkage between 3-methylbutanal, 3-methylbutanol and 3-methylbutanoic acid acyl-sucrose synthesis. BCAAs biosynthesis is tightly regulated by allosteric regulation of TD, ALS and IPMS. Maloney et al., in 2010 found seven major coordinate QTLs for BCAAs and two of them co-localized with SlBCAT4 (IL3-2) and SlBCAT1 (IL12-3). A latter study found

that polymorphism in BCAT1 is responsible for the quantitative trait variation at the locus of one of the two isoforms of DHAD (Kochevenko and Fernie, 2011). Here, authors had also suggested that DHAD can be a key point for transcriptional regulation and may have other key roles. Surprisingly, and in agreement with this, percent enrichment for 3-methylbutanal content in red ripe pericarp tissue was significantly reduced only for both DHAD lines. Moreover, one of these DHAD lines showed significant increase in iC5 acyl-sucrose and α-tomatine content. Upon checking the transcript profiles of both paralogs of DHAD in tomato, I find that they have contrasting expression profiles i.e. DHAD1 (Solyc05g053540.2) being upregulated in all fruit stages while the DHAD2 (Solyc12g043020.1) in green fruits. It is well known that mutation and gene duplication can create ground breaking molecular novelty required for synthesis of diverse secondary metabolites (Ohno 1970, Kliebenstein et al., 2001). For example, evolution of novel form of the Trp synthase α subunit is the classical example of natural protein engineering (Frey et al., 1997; Melanson et al., 1997; Gierl and Frey, 2001). This novelty has diverted metabolic flux from primary metabolism towards secondary metabolism. In my case study, obtained results and transcript analysis of 2 isoforms of DHAD suggests that these isoforms might be functionally different; and therefore might have resulted in only DHAD lines having significantly changed levels of 3-methylbutanal, iC5 acyl-sucrose and α-tomatine in red ripe tomato fruits.

Looking beyond our immediate aims in this study, further interesting observations were made, namely that despite significant reduction in phenylalanine content, levels of rutin were not affected in young leaf of tomato; except for one of treated DHAD line. But interestingly, rutin content was negatively affected in red ripe pericarp tissue of the antisense lines despite unaffected phenylalanine levels. Besides this effect on phenylpropanoid metabolism, arginine metabolism was also affected in antisense lines. Levels of arginine were significantly increased in fruit tissue of some of the transgenic lines but putrescine content was not affected (Supplementary material Kochevenko and Fernie, 2011). Presently, reason behind the changes in phenylpropanoid and arginine metabolism in transgenic lines is not known.

In summary, analysis of transgenic lines impaired in BCAAs metabolism reveals tissue specific regulation of BCAAs, phenylalanine and arginine derived secondary metabolites. Results from stable isotope study showed that major flux for

synthesis of BCAAs downstream products is through leucine catabolism and not glucose. Photorespiration becomes limiting factor for iC5 acyl-sucrose synthesis in young leaf of tomato. Synthesis of 3-methylbutanal is not affected by leucine content in red ripe fruits and young leaf of tomato. Obtained results also suggests that DHAD could be transcriptionally modulated to define metabolic flux for 3-methylbutanal, iC5 acyl-sucrose and α-tomatine synthesis in red ripe fruits. Further experiments are required to decipher this metabolic linkage in detail.

Chapter 3. Investigation of key genes involved in Tomato secondary metabolism and fruit ripening

3.1 Introduction

Tomato fruit ripening stage is characterized not only by visible breakdown of chlorophyll and build-up of carotenoids, but also contents of other important compounds, such as ascorbic acid and α-tocopherol; and secondary metabolites such as phenolics and glycoalkaloids vary during ripening, thus varying the nutritional value (Giovanelli et al., 1999; Martinez-Valverde et al., 2002; Tohge et al., 2014) (Figure 3.1). There is a high level of intra as well as interspecific variation for the content of these metabolites, for example, acyl-sugars and steroidal glycoalkaloids (Schilmiller et al., 2010; Iijima et al., 2013). Naringenin chalcone as well as tomatine-type and esculeoside-type glycoalkaloids was found to be highly accumulating in *hp1* (high pigment 1) mutant than in *S. lycopersicum* cv. Ailsa Craig (Rohrmann et al., 2011). Therefore, studying phenotypic and genetic diversity found among species is the prerequisite for identification of the genes responsible for such differences (Lippman and Zamir 2007; Gascuel et al., 2017; Cocaliadis et al., 2014). There are around 13 distinct wild species of tomato worldwide. It has been found that human interest led selective breeding during crop domestication and evolution has resulted in the loss of around 95% of the genetic variation and modification of traits (Miller and Tanksley, 1990; Perez-Fons et al., 2014). These modified traits are collectively called as ``Domestication Syndrome´´ (Frary & Doganlar et al., 2003; Bai and Lindhout 2007). There are number of examples of domestication syndrome that can be found in nature easily. For example, tomato species over the years developed the characteristic of production of lycopene compounds but lost the ability to produce defense related compounds such as acyl-sugars. Fortunately, we can cross wild species with the domesticated in order to regain the lost genetic variation. In order to investigate key genes involved in secondary metabolism and fruit ripening in tomato, several genetic resources have been developed. For example, *S. lycopersicoides* ILs (Canady et al., 2005), *S. sitiens* ILs (Pertuze et al., 2003), *S. chmielewskii* ILs (Frary et al., 2003), *S. habrochaites* LA1777 ILs (Monforte and Tanksley, 2000), *S. neorickii* LA2133 BILs (Fulton et al., 2000), *S. cheesmaniae* LA483 RILs (Paran et al., 1995), *S. cheesmaniae* ILs (Robert et al., 2001, Peleman and van der Voort 2003) and *S. pennellii* BILs (Ofner et al., 2016). A set of *S.*

pennellii ILs have been developed by crossing *S. lycopersicum* cv. M82 (hereafter referred as M82) with *S. pennellii* 716 and hence represent complete coverage of *S. pennellii* genome in the genetic background of M82 (Eshed & Zamir 1994). Moreover, mutant collections and TILLING (Targeting Induced Local Lesions in Genomes) platforms have also been developed (Liu et al., 2003; Minoia et al., 2010; Gascuel et al., 2017). Availability of genome, transcriptome, proteome, metabolome and the enzyme activity data resources for some of the tomato species as well as for

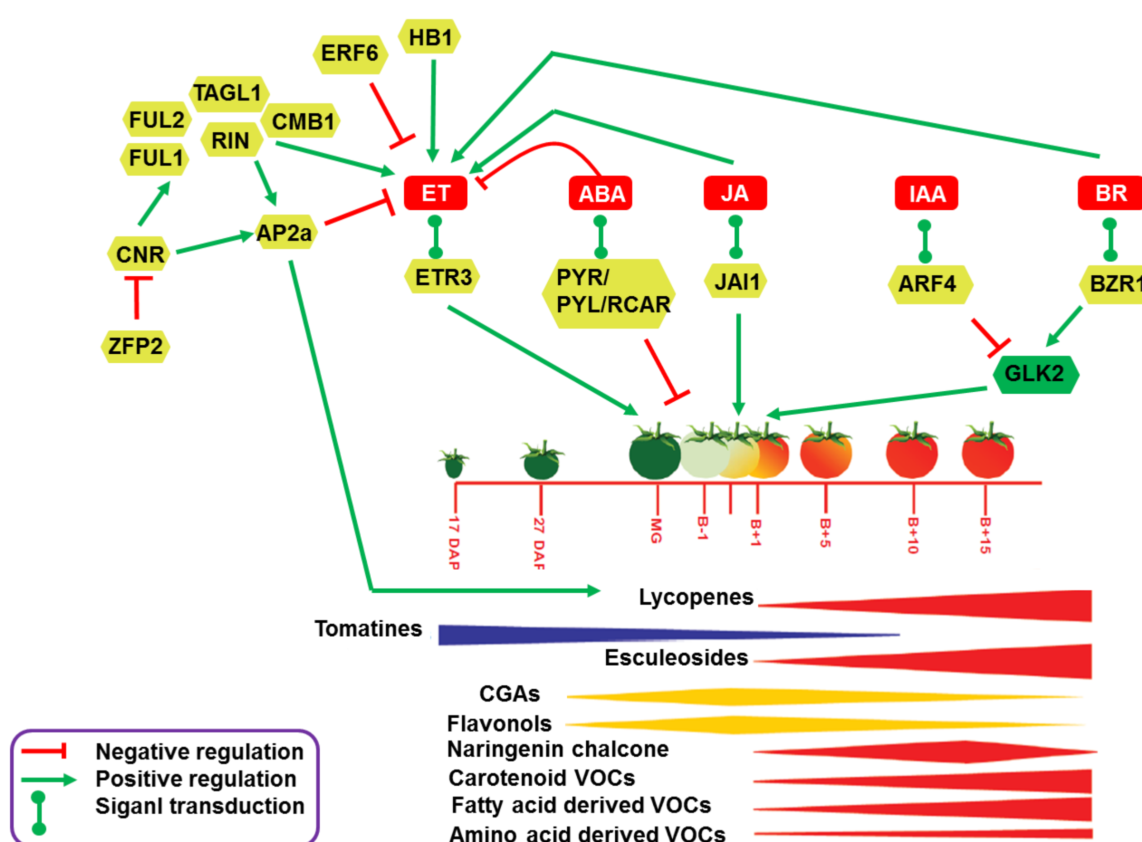


Figure 3.1 Schematic overview of updated network of regulatory factors controlling metabolic change during tomato fruit ripening. Fruit ripening stages (MG, mature green; B, breaker stage; B+5, 5 days after breaker; B+10, 10 days after breaker; B+15, 15 days after breaker are presented). Modified from Tohge et al., 2014 and Liu et al., 2014. Abbreviations: ET, ethylene; RIN, ripening inhibitor; FUL1/2, FRUITFULL1/2; TAGL1, AGAMOUS-LIKE1; CMB1, Carnation MADS Box protein 1; CNR, Colorless non-ripening; ZFP2, Zink finger protein 2; AP2a, APETALA2a; ETR3, ethylene receptor; ERF6, ethylene response factor 6; HB1, homeodomain binding protein 1; PYR/PYL/RCAR, Pyrabactin Resistance1/pyr1-like/Regulatory Components of Aba Receptors; JAI1, Jasmonic acid insensitive 1; ARF4, Auxin response factor 4; GLK2, Golden like 2; BZR1, Brassinazole resistant 1; ABA, abscisic acid; JA, jasmonic acid; IAA, indole acetic acid; BR, brassinosteroid.

the above mentioned ILs can be exploited by metabolomics assisted breeding to shorten the time required for the development of improved tomato line (Faurobert et al., 2007; Steinhauser et al., 2010; The Tomato Genome Consortium 2012; Bolger et

al., 2014; Perez-Fons et al., 2015; Fernie et al., 2006; Fernie and Schauer 2009; Klie et al., 2014, Schauer et al., 2008, Alseekh et al., 2015; Fernandez-Moreno et al., 2016).

Wild tomato species have adapted to highly diverse habitats. Crop domestication and evolution have led to differentiation of todays domesticated species from their wild ancestors by random selection of morphological and physiological characteristics. Ability of cross between wild and domesticated species; and hormonal and genetic control of transition from partially photosynthetic to fully heterotrophic metabolism during development, makes tomato a model system for ``Fruit Omics´´(Lytovchenko et al., 2011; Klee and Giovannoni 2011; Tohge et al., 2014). Previous studies have demonstrated the importance of transcriptomics and metabolomics in unravelling metabolic regulation underlying plant secondary metabolism. Earlier studies of metabolomics were focused on single metabolite for example carotenoid content in tomato, anthocyanin content in maize, starch content in potato and rice; and tocopherol levels in arabidopsis (Liu et al., 2003; Chandler et al., 1989; Fernie and Willmitzer 2004 and Gilliland et al., 2006). This pattern of study then shifted towards the detailed dissection of the particular pathways such as pathways of glucosinolate biosynthesis, seed oil synthesis, seed-soluble oligosaccharide metabolism in arabidopsis and flavonoid biosynthesis in populus (Heidel et al., 2006; Hobbs et al., 2004; Bentsink et al., 2000; Morreel et al., 2006). But nowadays, experiments are much more focused on studying complete complement of cellular metabolite level (Keurentjes et al., 2006; Schauer et al., 2006; Harrigan et al., 2007; Meyer et al., 2007). Schauer et al., (2008) studied the mode of inheritance of primary metabolic traits and revealed that most of the metabolic QTL (mQTL) (174 of 332) were dominantly inherited and a negligible number displayed the characteristics of overdominant inheritance.

Besides these metabolomic studies, there are also several reports of transcriptomic studies in tomato. Combined use of TOM1 cDNA microarray and a time-series loop design identified 869 genes that are differentially expressed in tomato pericarp and 37% of these genes are regulated by ethylene hormone (Alba et al., 2005). With the rise of transcriptomic studies, it was found that coexpression modules represent genuine transcriptional coordination between genes and thus can facilitate the inference of tomato gene function (Ozaki et al., 2010). By using

coexpression and several other techniques, various reports have well documented that plant secondary metabolism is under TF control (Butelli et al., 2008; Luo et al., 2008; Adato et al., 2009). TFs, such as CNR, RIN, FUL1, FUL2, ZFP2, AP2a, ERF6, CMB1, ETR3, HB1, BZR1, ARF4, GLK2, JAI1, TAGL1, SlMADS1 and SGR have been shown to be involved in regulating tomato fruit ripening (Figure 3.1) (Dong et al., 2013; Fortes et al., 2017; Liu et al., 2014; Fujisawa et al., 2013; Lin et al., 2008; Vrebalov et al., 2009). Additionally, TFs that regulate tomato secondary metabolism have also been found. For example, CSN5B, AtERF98, ABI4, and AMR1 have been found to regulate ascorbic acid levels (Wang et al., 2013; Zhang et al., 2012; Kerchev et al., 2011; Zhang et al., 2009). Furthermore, in an attempt to combine metabolomics and transcriptomics data, Mounet et al., (2009) detected up to 37 direct gene-metabolite correlations involving regulatory genes (e.g. the correlations between glutamine, bZIP, and MYB TFs). Combined systems-based analysis of transcriptome, genetic diversity of ILs and metabolite profiling in tomato fruit elucidated important role of SlERF6 in ripening and carotenoid accumulation (Lee et al., 2011). Metabolite profiling on surface of leaves of *S. pennellii* ILs led to identification of genomic regions affecting acyl chain substitutions (IL1-3/1-4 and IL8-1/8-1-1) and quantity (IL5-3 and IL11-3) of acyl-sugar metabolites (Schilmiller et al., 2010). Many attempts are also being made to annotate genes and identify key regulators of secondary metabolism, by integrating mQTL analysis with expression QTL (eQTL) studies. For example, by integrating mQTL analysis with eQTL study in an arabidopsis Bay-06 × Sha RIL population, Sonderby et al. (2008) identified MYB28 as a candidate regulator of aliphatic glucosinolates. Little studies of such integration approaches are being conducted in tomato. Recently, Alseekh et al., (2015) have carried out metabolic correlation network analysis in tomato fruit by using primary and secondary metabolite data. Doing this, they have narrowed down two candidate genes for glycoalkaloid mQTLs. Furthermore, they have also exemplary evaluated these candidates via the use of virus-induced gene silencing (VIGS) technique.

In this study, candidate genes that are involved in secondary metabolism and fruit ripening in tomato were filtered with eQTL and TF approaches. Combined eQTL and TF approach allowed me to finalize 20 potential genes. Furthermore, functional validation of 16 candidates by VIGS, 1 candidate by transient

overexpression and 3 candidates by transgenic approach was carried out. Doing this, I identified SlWD40 TF to be involved in regulating fruit ripening while SlADH (*S. lycopersicum* aldehyde dehydrogenase) and *S. pennellii* allele SopenCYP450 in inducing ic5 acyl-sucrose synthesis. These results demonstrate that our approach could be viable for narrowing down new key genes involved in creating interspecific variation in secondary metabolite content and at the level of fruit ripening.

3.2. Materials and Methods

3.2.1 Literature and data resource survey for transcriptomic and metabolomic data of *S. lycopersicum*, *S. pimpinellifolium* and *S. pennellii*

Genome sequence data is available for all the three species (The Tomato Genome Consortium 2012; Bolger et al., 2014). RNA-seq data were downloaded for *S. lycopersicum* and *S. pimpinellifolium*; for 10 different tissues including tissues for different fruit ripening stages. For M82 and *S. pennellii* (Penn) fruit, RNA-seq data were available (Bolger et al., 2014). This data also include 35 different tissues of *S. pennellii* that were subjected to different biotic and abiotic stresses. Moreover, RNA-seq data for *S. pennellii* ILs leaf and fruit were also available (Unpublished data Giovannoni et al.). With the advent of GC-MS and LC-MS technologies, metabolomic data resources are increasing not only for tomato but also for other plants. Primary metabolite data for leaf and fruit for 5 different wild species (*S. lycopersicum*, *S. pimpinellifolium*, *S. pennellii*, *S. chmielewskii* and *S. habrochaites*) were downloaded (Schauer et al., 2005). Rohrmann et al., (2011) published GC-MS and LC-MS data for different fruit ripening stages. Additionally, primary as well as secondary metabolite data for *S. pennellii* 76 ILs, for leaf and fruit were also available (Schauer et al., 2006; Alseekh et al., 2015). I took RNA-seq data for M82 and Penn fruit (Bolger et al., 2014). All the 34,727 genes in the transcriptome were sorted by two ways. Firstly, by ratio of their expression value of M82 to that of Penn and secondly, by ratio of their expression value of Penn to that of M82 in order to get genes that are highly expressing in M82 and Penn respectively. From each category ``Top 100´´ candidates were focused and further classified based on their function.

3.2.2 Narrowing down candidate genes involved in secondary metabolism and fruit ripening

Candidates from above mentioned two main categories were filtered by using three different filters such as relative fold change in the expression at least >5, eQTL and lastly as annotable genes. Furthermore, these candidates were then sorted into specific and nonspecific eQTLs based on their expression in respective IL. Here, specific and nonspecific eQTL were defined based on expression of particular *S. pennellii* candidate in specific IL or several ILs respectively. Specific eQTL

candidates were then focused and classified based on their function.

3.2.3 Prediction and assessment of putative function of candidate genes

In order to predict putative function for a candidate; a single candidate was chosen based on its available annotation. Expression pattern of this candidate in 10 different tissues of tomato was then checked by using RNA-seq data for *S. lycopersicum* and *S. pimpinellifolium* (The Tomato Genome Consortium 2012). Arabidopsis homolog of this candidate of interest was searched and then its functional annotation, coexpression network as well as expression pattern were checked with the help of TAIR website (https://www.arabidopsis.org/), ATTED-II (https://atted.jp/) and arabidopsis eFP browser (https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) respectively. Same protocol was used for all the candidates from all the functional categories of interest and 12 candidates were finalized. Lastly, some of these candidates were also verified by updated target pathway specificity, phylogenetic tree analysis between *S. lycopersicum*, *S. pennellii* and *A. thaliana,* and amino acid sequence analysis between *S. lycopersicum* and *S. pennellii*. As a one example, protein sequences of selected *S. lycopersicum* CYP450 (SlCYP450), *S. pennellii* CYP450 (SpCYP450) and all the *A. thaliana* CYP450 (AtCYP450) were extracted from Sol Genomics Network (https://solgenomics.net/) and The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/) respectively. Phylogenetic tree was constructed using MEGA 6.0 using neighbour-joining method. Based on species specific gene expression, literature survey for arabidopsis and available annotation; functions for these genes were predicted. After analyzing the tissue specific expression of all the 12 candidates, it was found that expression of candidate number 5 i.e. TF WD-40 hereafter referred as Solyc04g005020 increases exponentially during breaker stage. Based on results from correlation analysis, carotenoid biosynthetic pathway was targeted and its regulatory mechanism was updated with literature survey.

3.2.4 Narrowing down candidate genes encoding TFs

All the tomato TF genes annotated from current gene models in tomato genome (ITAG release 3.10) were sorted as per the protocol mentioned in one of previous sections. Subsequently, TFs that are highly expressing in M82 were filtered with

eQTL mapping. Lastly, candidates were arranged by ratio of average of expression value in breaker and breaker+10 to that of other tissues.

3.2.5 VIGS

Vector construction, infiltration, and fruit harvesting procedures were performed as previously described (Orzaez et al., 2006; 2009). Briefly, around 300 bp fragments of finalised candidates were amplified from tomato M82 fruit cDNA using gateway compatible primers and recombined into the GATEWAY vector pDONR207 (Invitrogen, http://www.invitrogen.com/) by the BP reaction following the manufacturer's protocol to generate an entry clone. An error free entry vector was confirmed by sequencing and then recombined with the pTRV2-Ros/Del/GW destination vector using an LR reaction to produce the expression clones pTRV2-Ros/Del/GW-Respective Gene ID. *Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV3101:pMP90 was then transformed with sequenced expression vectors by electroporation. In order to infiltrate fruit for VIGS, purple MicroTom tomato was used and agroinfiltration was done as previously described (Alseekh et al., 2015).

3.2.6 Transient overexpression study

The sequence encoding Sopen03g031040 was amplified from *S. pennellii* cDNA by using gene specific primers and inserted into the pDONR207 by attB recombination to generate entry clone. Primer sequences are provided in Supplemental Table 3.1. An error-free entry clone was confirmed by sequence analysis and then recombined into destination (35S overexpression) vector pK2GW7 (http://www.vib.be/). LR plasmid was then introduced into *A. tumefaciens* strain GV3101 by electroporation. A recombinant agrobacterium colony was cultured in 5 ml of LB culture containing antibiotics spectinomycin (50 mg/ml) and rifamycin (25 mg/ml) and grown overnight (28^0C at 180 rpm). One ml of this starter culture was used to inoculate 50 ml of LB media containing antibiotics spectinomycin (50 mg/ml) and then grown overnight at 28^0C. The cells were harvested by centrifugation at 3000 rpm for 20 min and resuspended in 5 ml infiltration buffer containing 10 mM MES (pH 5.7), 10 mM MgCl2 and 100 mM acetosyringone. An OD600 of 0.4 was adjusted with infiltration medium. 0.2-0.5 ml of this culture was infiltrated into breaker stage fruit of *S. lycopersicum* cv. MicroTom (MT) through the base of peduncle with 1 ml syringe.

After 2 weeks, infiltrated fruits were harvested; pericarp was isolated and immediately frozen in liquid nitrogen and stored at -80ºC until further analysis.

3.2.7 Development of overexpression and RNAi lines

The sequence encoding Solyc04g005020 and Solyc12g010950 was amplified *S. lycopersicum* cv. Moneymaker (MM) cDNA by using gene specific primers and inserted into the pDONR207 by attB recombination to generate entry clone. Primer sequences are provided in Supplemental Table 3.1. An error-free entry clone was confirmed by sequence analysis before recombination into destination vector. These 2 and Sopen03g031040 (from transient overexpression) genes were finally cloned into pK2GW7. Candidate Solyc04g005020 was also cloned into pK7GWIWG2(I),0 vector (http://www.vib.be/). Generated vectors were then named as pK2GW7SlWD, pK7GWIG2SlWD, pK7WG2Sopen940 and pK2GW7SlADH.

Candidate Solyc04g005020 was also cloned into vector B33BinAR for fruit specific overexpression and named as B33BinAR_SlWD40. Additionally, artificial miRNA cassette (amiRNA) was designed for Solyc04g005020. For this, Solyc04g005020 cDNA sequence was used as target sequence, employing the WMD3 program (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) to design corresponding amiRs. An overlapping PCR (polymerase chain reaction) strategy was employed with in-hand precursor DNA, following the WMD3 protocol (http://wmd3.weigelworld.or/downloads/CloningofartificialmicroRNAs.pdf). The pre-amirs obtained from overlapping PCR (using the athmir-319a backbone) were cloned into the pENTR/D-TOPO vector, and the clones were confirmed by DNA sequencing. Subsequently, these sequences were cloned into B33BinAR via Asp718 and BamHI digestion and cohesive end ligation. Primer sequences are provided in Supplemental Table 3.1. This and other final LR plasmids were then introduced into *A. tumefaciens* strain GV2260 by electroporation and subsequently submitted for transformation into MM plants using the leaf disc transformation method (McCormick et al., 1986). *A. tumefaciens* strain GV2260 transformed with pK2GW7SlADH was also submitted for transformation into *N. tobaccum* cv. SNN (SNN) plants using the leaf disc transformation method.

3.2.8 Plant material and growth conditions

T_0 transgenic plants for each genotype were selected on kanamycin containing MS medium (50 mg/l). SNN and MM (WT) was germinated on MS medium without kanamycin. Every line for each of constructs, SNN and WT were selected and transferred to soil pot for cultivation under long day conditions (16/8 h day/night cycle) at 22^0C and 50% humidity, as described previously in the literature (Carrari et al., 2003). Upon anthesis, flowers were labelled with that particular date.

3.2.9 Metabolic profiling – Primary metabolites

Three biological replicates of leaf and fruit pericarp samples were harvested, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Samples were then powdered by using retschmill at 30 l/s, for 30s. Metabolite extraction for GC-MS was carried out as previously described by Lisec et al., (2006). with minor modifications. For this purpose 50 mg of ground material was extracted in 700 µL methanol supplemented with 30 µL of ribitol (0.2 mg/mL in H_2O) as internal standard for relative quantification. The mixture was shaken at 1000 rpm for 15 min at 70°C and centrifuged for 10 min at 20,800 *g*. After centrifugation, the supernatant was transferred to a new tube and mixed with 350 µL chloroform and 750 µL H_2O and centrifuged for 15 min at 20,800 *g* to separate the polar and apolar phases. 150 µL of the polar (upper) phase was dried in a centrifugal vacuum concentrator. The pellet was resuspended in 40 µL of methoxyaminhydrochlorid (20 mg/mL in pyridine) and derivatized for 2h at 37°C. Afterwards, 70 µL of *N*-methyl-*N*-[trimethylsilyl] trifluoroacetamide (MSTFA) was added containing 20 µL/mL FAMEs (fatty acid methyl esters mixture) as retention time standards. The mixture was incubated for 30 min at 37°C at 400 rpm. A volume of 1 µL of this solution was used for injection. The GC-MS system comprised a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph, and a LECO Pegasus III TOF-MS running in EI+ mode. Metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005). Chromatograms and mass spectra were evaluated using Chroma TOF 1.0 (Leco) and TagFinder 4.0 software (Luedemann et al., 2008).

3.2.10 Metabolic profiling – Secondary metabolites

Three biological replicates of leaf and fruit pericarp samples were harvested, immediately frozen in liquid nitrogen and stored at -80ºC until further analysis. Samples were then powdered by using retschmill at 30 l/s for 30s. For extraction, 50 mg of ground material was aliquoted and then extracted in 200 µL of 80% methanol supplemented with isovitexin (4 µg/mL) as internal standard for relative quantification. The mixture was then homogenized for 2 min at 23l/s. Samples were then centrifuged for 10 min at 10,000 rpm. After centrifugation, the supernatant was transferred to a new tube and then centrifuged again for 10 min at 10,000 rpm. Supernatant was then dried in a centrifugal vacuum concentrator. Dried samples were then resuspended in 200 uL of stock solution for extraction. UPLC separation was performed using a Waters Acquity UPLC system (Waters, Mildford, MA, U.S.A.), using a HSS T3 C18 reverse phase column (100 × 2.1 mm i.d. 1.8 µm particle size, Waters) at a 40 °C according to the previously published protocol (Giavalisco et al., 2009). The mobile phases consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). acetonitrile (Solvent B). The flow rate of the mobile phase was 400 µL/min, and 2 µL sample were loaded per injection. The UPLC was connected to an Exactive Orbitrap (Thermo Fisher Scientific, Bremen, Germany) via an heated electro spray source (Thermo Fisher Scientific, Bremen, Germany). The spectra were recorded using full scan mode of negative ion detection, covering a mass range from *m/z* 100–1500. The resolution was set to 25,000 and the maximum scan time was set to 250ms. The sheath gas was set to a value of 60, while the auxiliary gas was set to 35. The transfer capillary temperature was set to 150°C while the heater temperature was adjusted to 300°C. The spray voltage was fixed at 3 kV, with a capillary voltage and a skimmer voltage of 25V and 15V, respectively. MS spectra were recorded from min 0 to 19 of the UPLC gradient. Molecular masses, retention time and associated peak intensities were extracted from the raw files using the Xcalibur software (Thermo Fisher Scientific, Bremen, Germany). Metabolite identification and annotation were performed using standard compounds, literature and tomato metabolomics databases (Moco et al., 2006; Iijima et al., 2008; Tohge and Fernie, 2009, 2010; Rohrmann et al., 2011). Data are reported in a manner compliant with the standards suggested by Fernie et al. (2011).

3.2.11 Metabolic profiling – Volatile organic compounds

For SPME-GC-MS profiling of volatile organic compounds (VOCs), leaf samples were harvested, immediately frozen in liquid nitrogen and powdered by using retschmill at 30 l/s for 30s. Samples were then processed as per the protocol mentioned in chapter 2.

3.2.12 Heat Maps

Metabolite data was normalized by internal standard and fresh weight. Average of three replicates was taken. Then fold change values were calculated over WT. Heat maps were generated based on log2-transformed fold change values using the "pheatmap" R package version 1.0.8 in statistical software environment R, version 3.3.2 (Kolde R. 2015). Package also helped to perform clustering by complete-linkage clustering using euclidean distances.

3.3. Results

3.3.1 Narrowing down candidate genes using eQTL approach

In order to filter potential candidate genes involved in fruit ripening and secondary metabolism, comparative phenomics and transcriptomics was carried out for M82 and *S. pennellii* mature fruits*.* Figure 3.2 and 3.3 shows difference between M82 and *S. pennellii* at the level of fruit phenotype, metabolite as well as at transcript level.

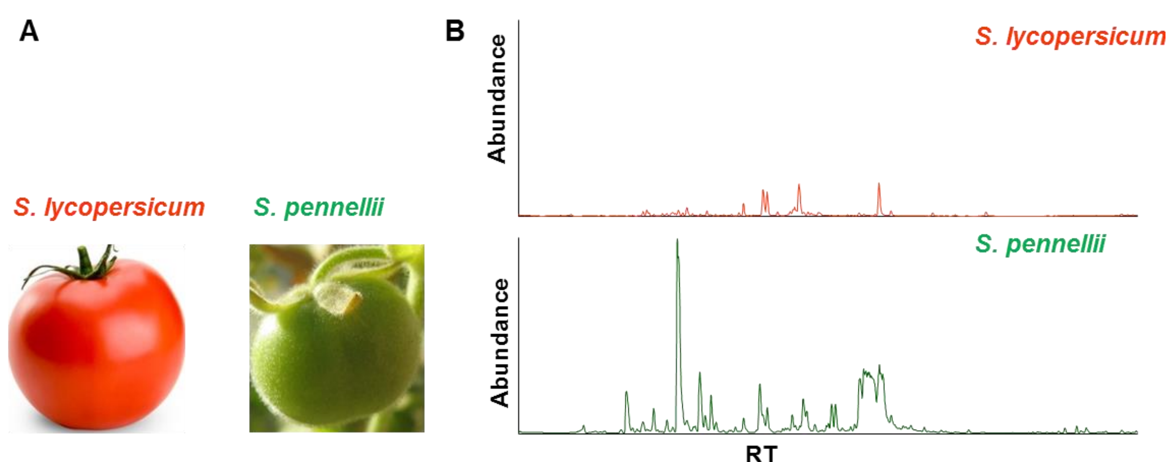


Figure 3.2 Differential phenotype and metabolite profile for mature fruits of *S. lycopersicum* cv. M82 and *S. pennellii*. A) Mature fruits. B) LC-MS profile (X-axis represents RT (retention times) and Y-axis represents metabolite abundance.

For transcript analysis, transcriptome data for M82 and *S. pennellii* mature fruits was sorted as per the protocol mentioned in section 3.2.1. Next, I compaired 2 datasets that contained top 100 highly expressed genes in M82 and *S. pennellii*, separately. For this, all 100 genes were sorted into different functional categories. Though I found some of the candidates related to secondary metabolism and TFs, I also found enrichment of both datasets with candidates related to post translational modifications, photosystem and also with unassigned function. Many oxidoreductases and cytochrome P450s (CYP540s) have been shown to play key role in secondary metabolism (Inoue et al., 2005). However, enrichment of these candidates was not found in above datasets. So in order to narrow down new key genes involved in secondary metabolism and fruit ripening in tomato, filtration method was optimized by using whole fruit RNA-seq data for both the species and 76 ILs.

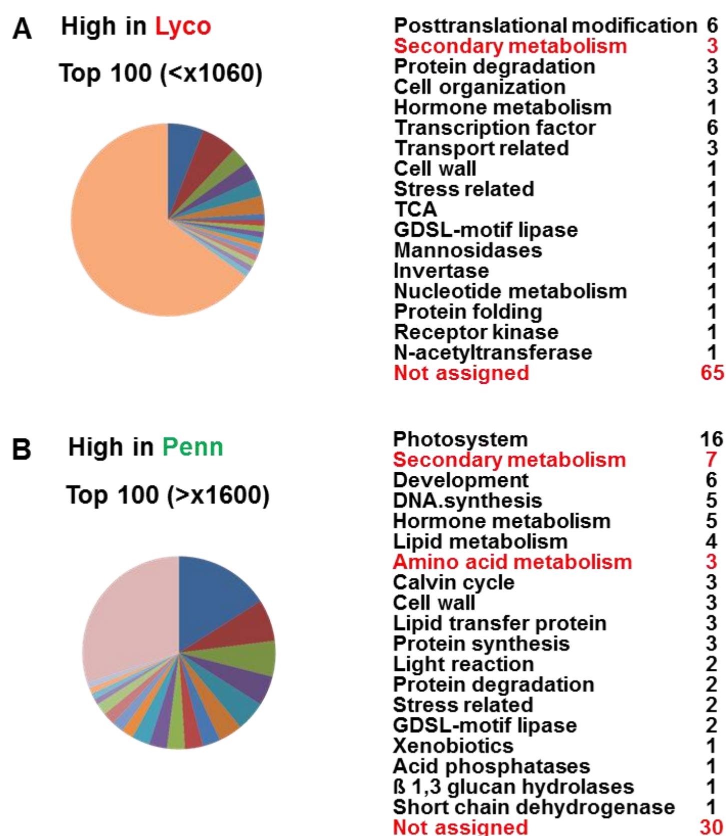


Figure 3.3 Classification of genes that are differentially expressed in *S. lycopersicum* cv. M82 and *S. pennellii* fruits at mature stage; according to their gene function. Lyco, S. *lycopersicum* cv. M82 and Penn, *S. pennellii* (Bolger et al., 2014). Numbers in bracket indicates Lyco/Penn and Penn/Lyco ratio. Pie chart shows number of candidates in each functional category. Important functional categories are indicated by red colour. Numbers indicate number of candidates belonging to that functional category.

Total 34727 genes in transcriptome could be sorted into 2 different datasets as the one (referred as Lyco) containing genes that are highly expressing in M82 (13521) and the other (referred as Penn) containing genes that are highly expressing in *S. pennellii* (11781) (Figure 3.4). I observed that around 5040 genes were not expressed in both M82 and *S. pennellii* mature fruits. For Lyco and Penn category,

around 300 candidates could be narrowed down by using three different filters as ratio (expression 5 times higher), eQTL and annotable genes. Next, based on their expression into specific IL, these candidates were classified into specific and nonspecific eQTLs (Unpublished data Giovannoni et al.).

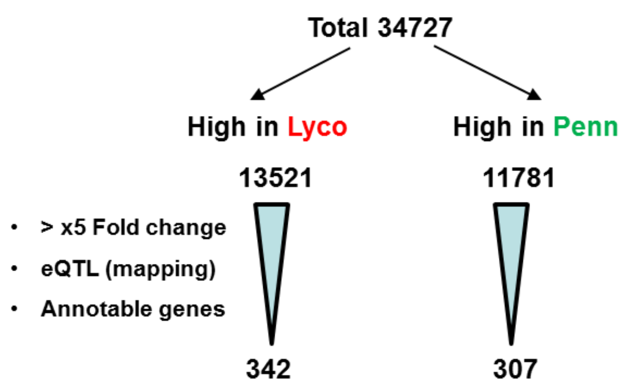


Figure 3.4 Flow chart for narrowing down candidate genes with eQTL approach. *S. lycopersicum* cv. M82 referred as Lyco and Penn referred *S. pennellii*. Filters are shown in bullet points.

Around 110 specific and 210 nonspecific eQTLs were obtained in the finally filtered candidates from both the categories (Figure 3.5). Genes showing nonspecific eQTL

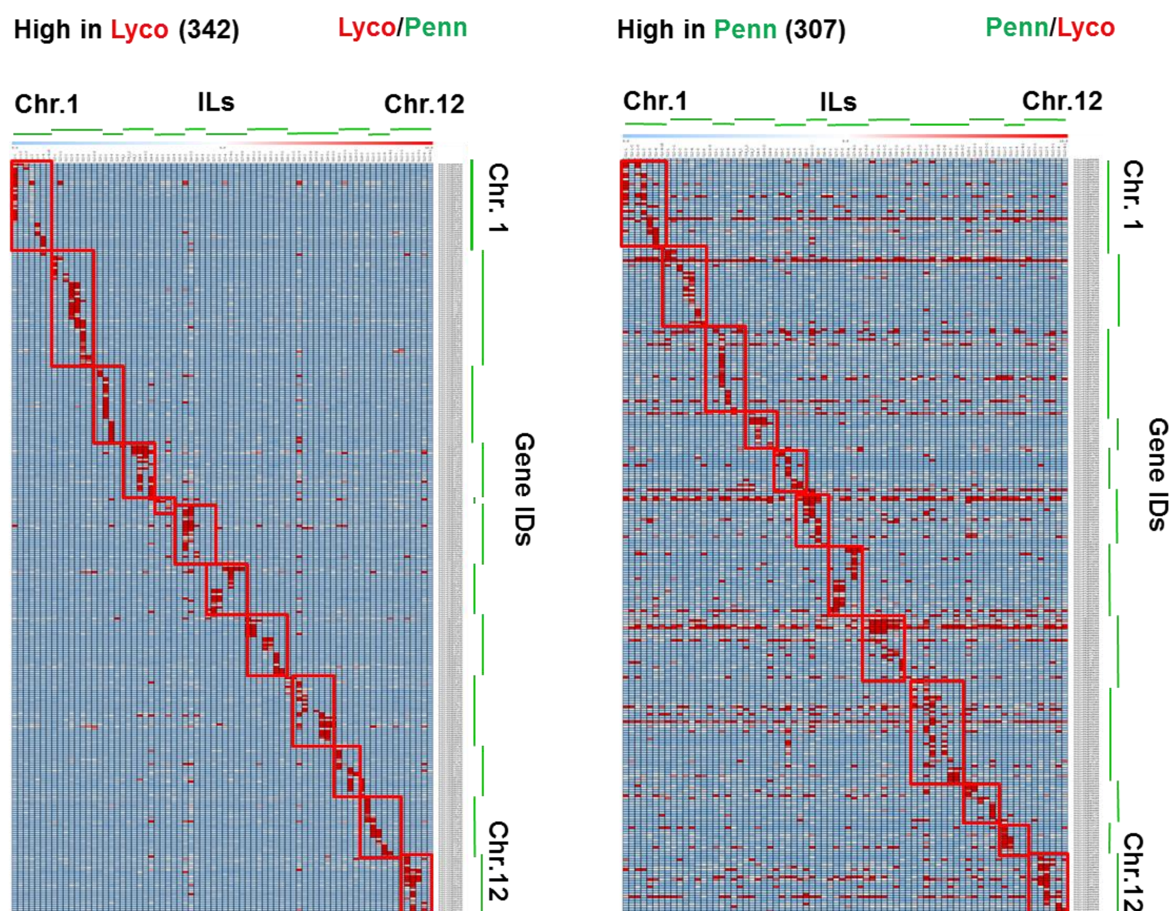


Figure 3.5 Heat map of relative expression level of filtered candidates. Lyco/Penn, Genes were sorted by ratio of expression value for *S. lycopersicum* and *S. pennellii*. ILs are arranged as per the number of chromosome (X-Axis). Genes are arranged according to their Gene IDs (Y-Axis). Regions of red or blue indicate that the gene expression is increased or decreased respectively, over that of M82.

from Penn dataset showed expression across most of the ILs, might be due to epistatic interactions to lower the effect of highly expressing *S. pennellii* genes. This was not observed for genes from Lyco dataset.

Furthermore, specific eQTL candidates were then sorted based on their functional annotation. Around 20 candidates with unassigned function could be found in each category (Figure 3.6). Potential candidates of interest could be easily distinguished from those that were related to primary metabolism. I focused 6 functional categories of interest from both datasets (Figure 3.6). Previous studies have implicated these 6 functional categories in regulating tomato fruit ripening and secondary metabolism.

A

Category	No.	Category	No.
Alcohol dehydrogenase superfamily	1	Nucleotide metabolism	2
Amino acid metabolism	3	Nitrilases	1
Cell organisation	6	Oxidases	2
Cell wall	2	O-methyl transferases	1
CHO metabolism	3	Photosynthesis related	1
Cytochrome P450	5	Protein related	16
Development	1	RNA processing	2
Dienelactone hydrolase	1	Secondary metabolism	2
Glutathione S transferases	4	Signalling	5
GDSL-motif lipase	1	Stress	2
Glucosidases	1	Short chain dehydrogenase	3
Hormone metabolism	10	Transport	6
Lipid metabolism	5	Transcription factor	8
Mitochondrial electron transport	2	UDP glycosyl transferases	4
		Not assigned	19

B

Category	No.	Category	No.
Amino acid metabolism	1	Protein related	4
Cell organisation	2	Photosynthesis related	5
Cell wall	6	Secondary metabolism	2
CHO metabolism	1	Short chain dehydrogenase	1
DNA	5	Signalling	5
Cytochrome P450	4	Stress	3
Development	11	Tetrapyrrole synthesis	1
Glutathione S transferases	2	Transcription factor	2
Hormone metabolism	8	Transport	2
Lipid metabolism	7	UDP glycosyl transferases	6
Metal handling	1	Not assigned	21
Myrosinases	1		
Oxidases	3		
Plastocyanin like	1		

Figure 3.6 Classification of specific eQTL candidate genes that are differentially expressed in A) *S. lycopersicum* cv. M82 and B) *S. pennellii* fruits at mature stage; according to their gene function. Important 6 functional categories are indicated by red colour. Numbers indicate number of candidates belonging to that functional category.

For example, glycosyltransferases have been shown to be involved in maintainance of cell homeostasis, plant growth, development and defense responses (Jones and Vogt 2001; Lim and Bowles, 2004). Additionally, interest in fatty acid derived volatiles

and the fact that all fatty acid derived VOCs increase during fruit ripening makes lipid metabolism category important (Klee and Giovannoni 2011). My primary focus was on CYP450s, oxidases, secondary metabolism and TFs. As discussed in introduction, various TFs plays key role in regulating tomato fruit ripening. Additionally, various CYP450s have been implicated in flavonoid metabolism. For example, CYP93B have been shown to catalyze naringenin to apigenin conversion (Akashi et al., 1999). This literature survey helped me to filter total 12 candidates from both lyco and penn datasets (Table 3.1 and 3.2).

Table 3.1 List of candidate genes from Lyco

No.	**Gene ID.**	**Gene Annotation**	**Expression in Lyco**	**Ratio* (Lyco/Penn)**
1	Solyc01g094080	Cytochrome P450	Mainly in fruit	**692**
2	Solyc03g095900	2-ODD	In fruit after breaker	**348**
3	Solyc01g080410	Transcription factor	In fruit after breaker	**347**
4	Solyc12g010950	Alcohol dehydrogenase	Induced by insect	**294**
5	Solyc04g005020	Transcription factor (WD40)	Only in fruit	**180**
6	Solyc03g111970	Cytochrome P450	Mainly in fruit after breaker	**56**
7	Solyc12g008660	Transcription factor	In all tissues	**19**

Table 3.2 List of candidate genes from Penn

No.	**Gene ID.**	**Gene Annotation**	**Expression in Lyco**	**Ratio* (Penn/Lyco)**
8	Solyc09g092580	Cytochrome P450	Only in green tissues	**3592**
9	Solyc03g111940	Cytochrome P450 71 A4	Mainly in 1 cm fruit	**1163**
10	Solyc02g078380	Ammonia ligase	Mainly in green fruit	**81**
11	Solyc07g045090	Oxidases	In green fruits	**45**
12	Solyc06g076160	Cytochrome P450	In all tissues	**41**

*- Ratio is calculated by expression value for *S. lycopersicum* and *S. pennellii.*

Next, phylogenetic tree analysis was performed for CYP450 candidates. For this, protein sequences of selected *S. lycopersicum* CYP450 (SlCYP450), *S. pennellii* CYP450 (SpCYP450) and all the *A. thaliana* CYP450 (AtCYP450) were downloaded and phylogenetic tree was constructed using MEGA 6.0 using the neighbour-joining method. Phylogenetic analysis clearly indicated location of selected SlCYP450 and SpCYP450 in four different clades with AtCYP450 (Figure 3.7). Based on expression of tomato specific genes, literature survey for arabidopsis and available annotation; function for one of the CYP450 candidate could be predicted tentatively. I distinguished that candidate number 9 i.e. Solyc03g111940 shown in Figure 3.7 (here after called as SopenCYP450) is specific to tomato. SopenCYP450 is presently annotated as a CYP450 71A4 like protein. Upon

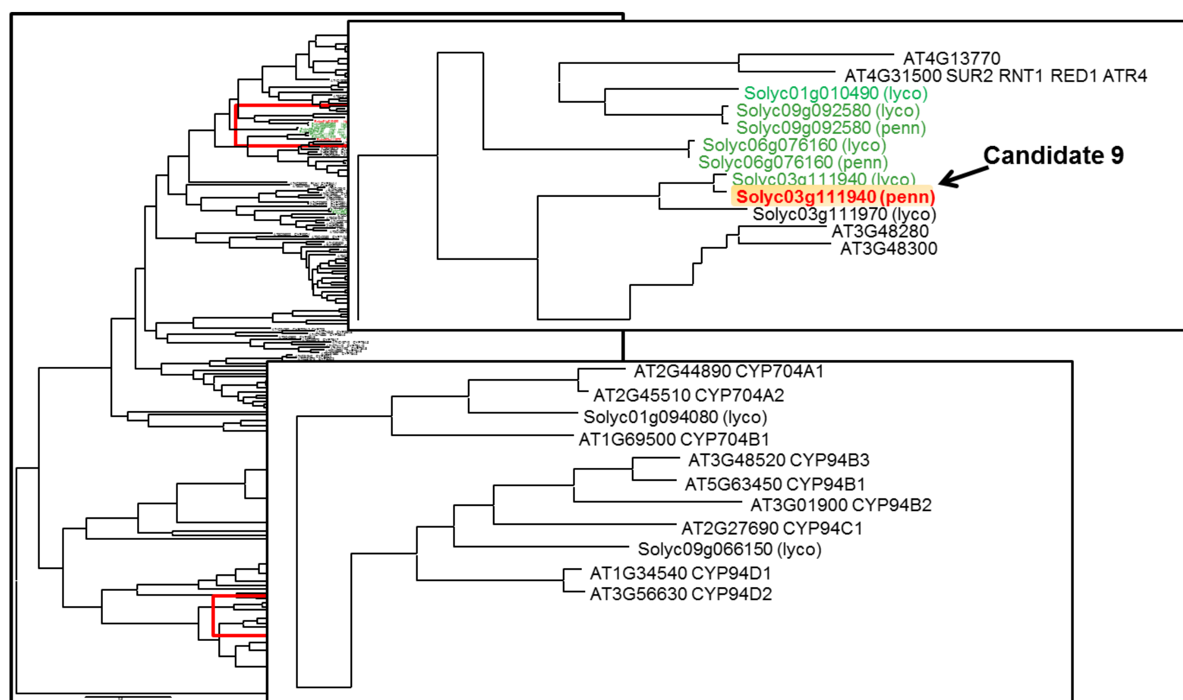


Figure 3.7 Phylogenetic tree of selected *SlCYP450* and *SpCYP450* with all the *AtCYP450* constructed using MEGA 6.0 using neighbour-joining method.

pursuing interest in this candidate by checking its expression in ILs, excitingly I found that this candidate is highly expressed only in IL3-4 (Figure 3.8A), for which valine and leucine mQTL had already been shown (Figure 3.8B) (Kochevenko and Fernie 2011).

Next, I checked species specificity for all 12 candidates. I found that candidate number 5 i.e. Solyc04g005020 to be tomato specific. Additionally, tissue specific expression of all the 12 candidates was also analyzed using RNA-Seq data available

from ``The Tomato Genome Consortium 2012´´. Interestingly, I observed that expression of Solyc04g005020, hereafter referred as SlWD-40 TF was found to increase exponentially during breaker stage.

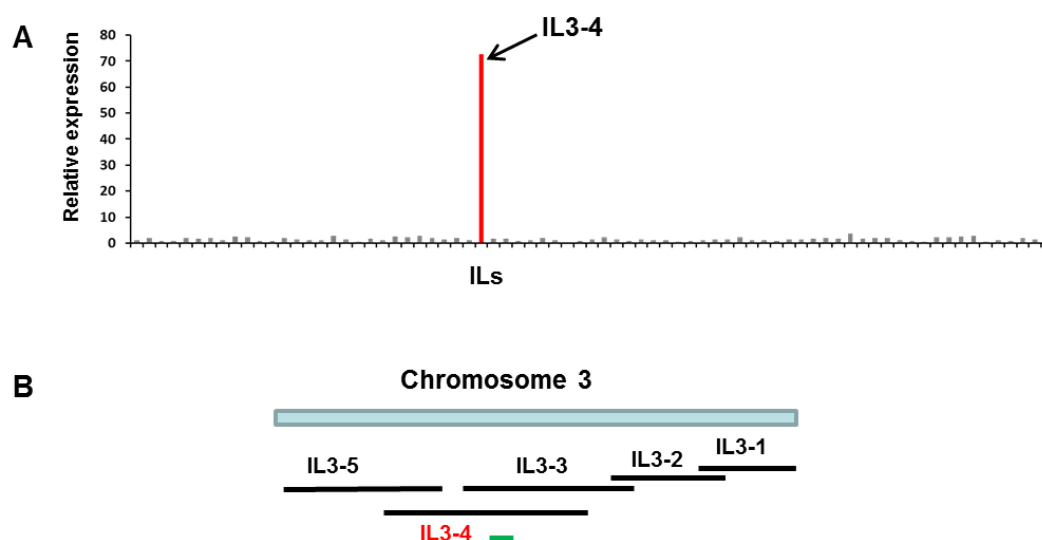


Figure 3.8 A) Relative expression of SopenCYP450 in IL3-4 is shown in red. B) The introgressed fragments in ILs are shown on the bottom of the chromosome 3. mQTL for valine and leucine is shown in green (Kochevenko and Fernie 2011).

Hence, carotenoid biosynthetic pathway was focused and updated it´s regulatory mechanism based on literature survey (Figure 3.9). Upon differential coexpression correlation analysis between SlWD-40 TF and functionally characterized genes in the carotenoid biosynthetic pathway, SlWD-40 TF was found to be highly correlating with Psy1, ELIP2 and Rin, key regulators in this pathway.

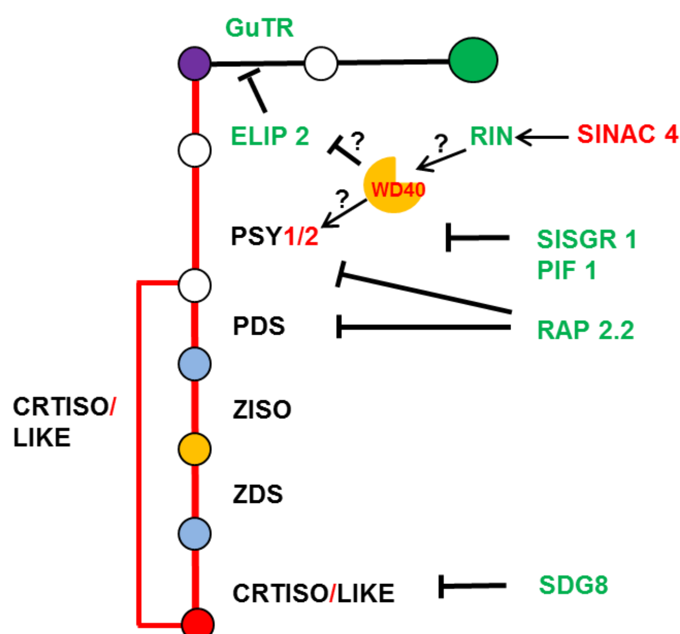


Figure 3.9 Carotenoid biosynthetic pathway and it´s regulatory mechanism.
Metabolites are indicated by different colours to nodes. Geranyl geranyl diphosphate as Purple; Chlorophyll, Green; Intermediate metabolites, White; Phytoene, Blue; Carotene, Orange and Lycopene, Red. Abbreviations: GuTR, glutamyl tRNA reductase; ELIP2, early light-induced proteins; PSY1/2, phytoene synthase 1/2 (Solyc03g031860 and Solyc02g081330); PDS, phytoene desaturase (Solyc03g123760); ZISO, zeta-carotene isomerase (Solyc12g098710); ZDS, zeta-carotene desaturase (Solyc01g097810); CRTISO/like, carotenoid isomerase/like (Solyc10g081650, Solyc02g085250 and Solyc05g010180); RIN, ripening inhibitor (Solyc05g012020); SlNAC4, *S. lycopersicum* NAM/ATAF1/2/CUC2 family 4 protein; SlSGR1, *S. lycopersicum* stay green protein 1 (Solyc08g080090 and Solyc12g056480); PIF 1, phytochrome interating factor 1; RAP2.2, ERF (ethylene response factor) subfamily B-2 of ERF/AP2 TF family (Solyc03g123500, Solyc06g063070 and Solyc12g049560) and SDG8, Set Domain Group 8 (Solyc06g059960 and Solyc04g057880).

3.3.2 Narrowing down candidate genes using TF approach

It is well documented that secondary metabolism and fruit ripening is under strict control of TFs (Butelli et al., 2008; Luo et al., 2008; Adato et al., 2009, Liu et al., 2014). Therefore, in addition to filtration of candidate genes mentioned in last section, 20 TF candidates were also filtered (Figure 3.10).

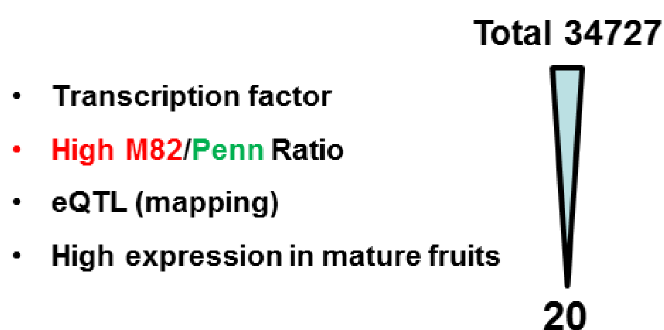


Figure 3.10 Flow chart for narrowing down candidate genes with TF approach. *S. lycopersicum* referred as M82 and Penn referred to *S. pennellii*. Filters are shown in bullet points.

Lastly, both 12 candidates from eQTL (Lyco dataset Table 3.1) approach and 20 candidates form TF (Figure 3.10) approach were narrowed down to 20 potential candidates and are shown in Table 3.3.

Table 3.3 Finalised potential candidates from both eQTL and TF approaches*

Gene ID	Function	Phenotypic Score
Solyc05g015370	Putative transcription regulator	Yellowish
Solyc05g015380	Putative transcription regulator	Green
Solyc05g015360	Putative transcription regulator	Green
Solyc03g095900	2-ODD	Red
Solyc03g044460	BHLH transcription factor	Green
Solyc04g005020	**WD-40 repeat family protein**	**Yellowish red**
Solyc11g010710	AP2-like ethylene-responsive TF	Green
Solyc01g080410	Peptide msrB 5	Red
Solyc03g116440	Zinc finger, FYVE/PHD-type	Red
Solyc12g009050	CCAAT box binding factor	Red
Solyc12g010950	**ADH**	**Whitish Green**
Solyc01g094080	Cytochrome P450	Red
Solyc07g066160	C2H2 zinc finger	Red
Solyc07g052700	MADS-box	Yellowish
Solyc12g098620	BHLH TF	Red
Solyc0g090310	C2C2(Zn) DOF zinc finger	Red
Solyc08g065300	Mutator-like transposase	
Solyc12g099120	Myb TF	
Solyc10g049720	BHLH TF	
Solyc06g050160	Homeobox TF	

*-Candidates that are highly expressing in Lyco.
Dark columns represents that those candidates have been validated by VIGS.
Phenotypes were scored visually after 15 days of agrobacterium infection.
Candidates in bold were further used for creating permanent transformations.

3.3.3 Functional validation of candidates by VIGS

We used purple tomato approach, the technique developed by Orzaez et al., (2009) (Figure 3.11). Out of 20 candidates (shown in Table 3.3), partial fragments for 16 candidates were cloned into VIGS vector. Total 16 candidates were then validated by VIGS.

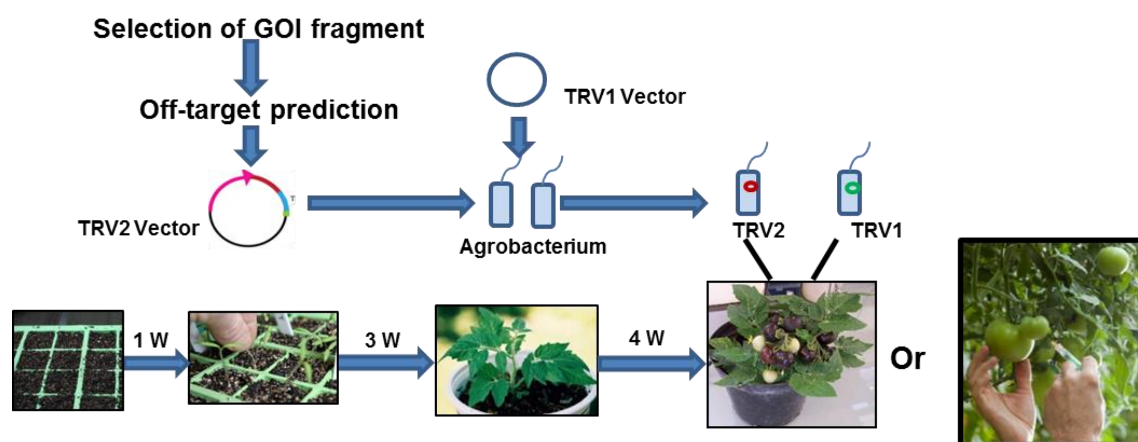


Figure 3.11 Methodology used for VIGS experiment.
Abbreviations: GOI- Gene of Interest, TRV1 & TRV2- Tobacco rattle virus 1 and 2, W-Weeks.

Three purple MicroTom tomato plants were used as biological replicates. Around 10-15 fruits per plant were infected with agrobacterium carrying respective VIGS vector. After silencing Del/Ros1 (empty vector) in Microtom Del/Ros1 fruits, there was depletion in purple pigments but not in lycopene content and silenced part could be easily dissected out from nonsilenced tissues (Figure 3.12A). Phenotypes were scored visually after 15 days of infection for all the 16 validated candidates (Table 3.3). Among total number of infected fruits, approximately 60% were silenced. Interestingly, yellowish phenotype obtained for SlWD-40 TF (Figure 3.12B) is a same phenotypic change in VIGS for phytoene desaturase (a well-known enzyme in carotenoid biosynthetic pathway), earlier reported by Orzaez et al., (2009).

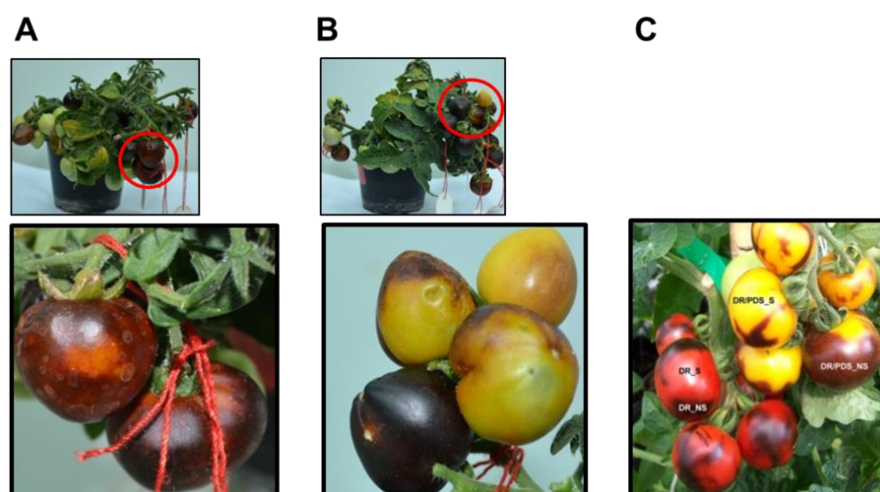


Figure 3.12 Silencing of A, *Del/Ros1* (empty vector) and B, SlWD-40 TF in Microtom Del/Ros1 fruit (Purple fruit). C) Silencing of *phytoene desaturase* (DR/PDS_S) as reported by Orzaez et al., (2009).

This result together with correlation analysis strongly suggested involvement of SlWD-40 TF in regulating tomato fruit ripening and carotenoid biosynthesis.

Next, VIGS silenced parts of pericarp were cut and processed for metabolite profiling by GC-MS. This was done for all the 16 candidates and their respective empty vector. Metabolite profiling for these VIGS silenced parts revealed that chlorogenic acid was significantly decreased only for candidate Solyc05g015370 (Figure 3.13; P<0.05). Presently, cloning of this gene is in progress.

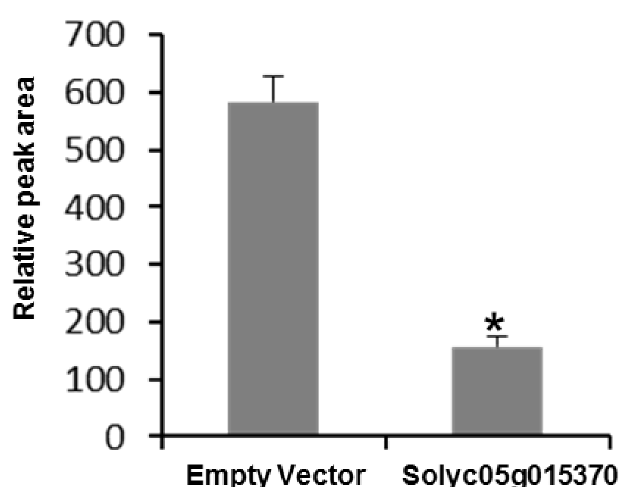
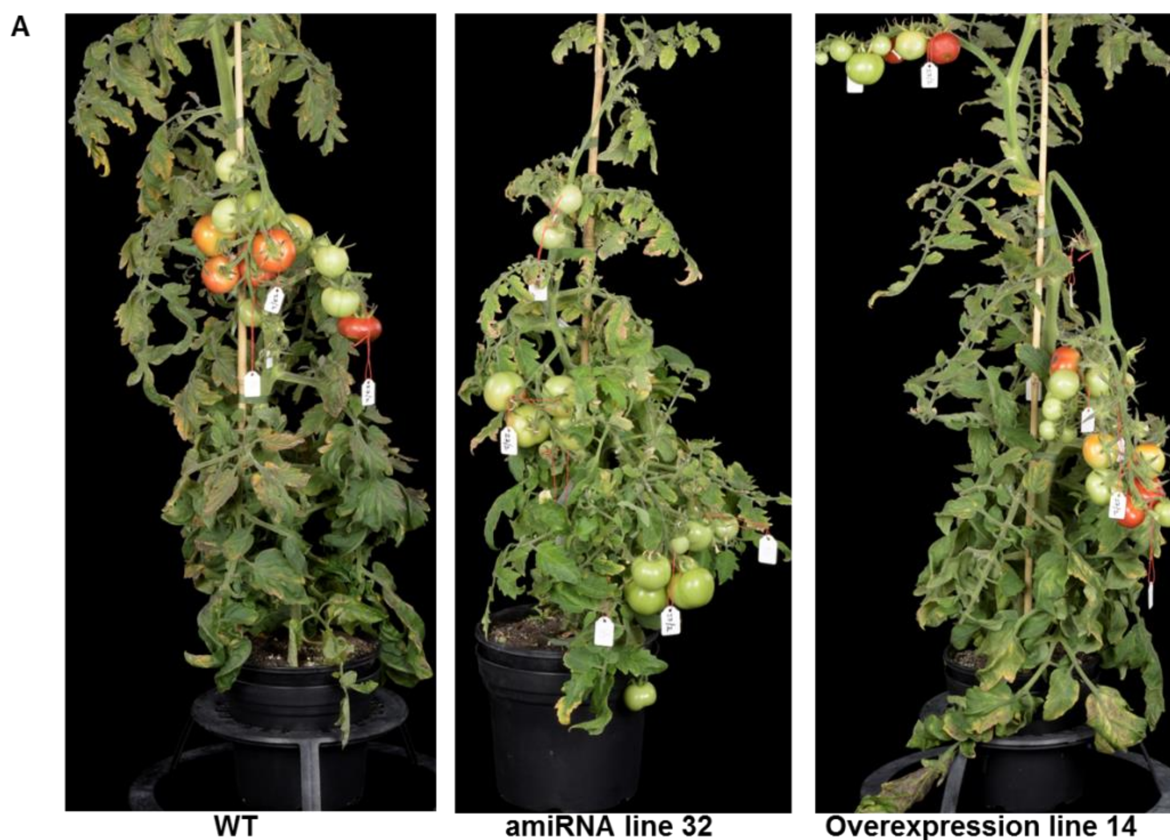


Figure 3.13 Relative content of chlorogenic acid in silenced parts of VIGS samples of empty vector and Solyc05g015370. Data represent the mean ± SE of four independent determinations. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05).

3.3.4 Analysis of transgenic lines

3.3.4.1 SlWD-40 TF regulates tomato fruit development, ripening and amino acid metabolism

Using agrobacterium-mediated tomato transformation, 14 NPT-II PCR-positive overexpression and amiRNA transformants were generated for candidate SlWD-40. Here, I have used B33 (fruit) specific overexpression vector (Rocha-sosa et al., 1989). In order to analyze fruits with identical stage, flowers were labelled upon anthesis. All the fruits having identical date of anthesis were analyzed by phenotyping and metabolite profiling. Results from phenotyping study clearly showed impairment in fruit development and ripening process in case of 2 amiRNA lines (line 9 and 32; Line 32 is shown in Figure 3.14). Additionally, VIGS results and correlation analysis had also suggested involvement of this TF WD-40 in tomato fruit ripening and carotenoid biosynthesis. Combined results thus show that SlWD-40 TF regulates tomato fruit development and ripening.



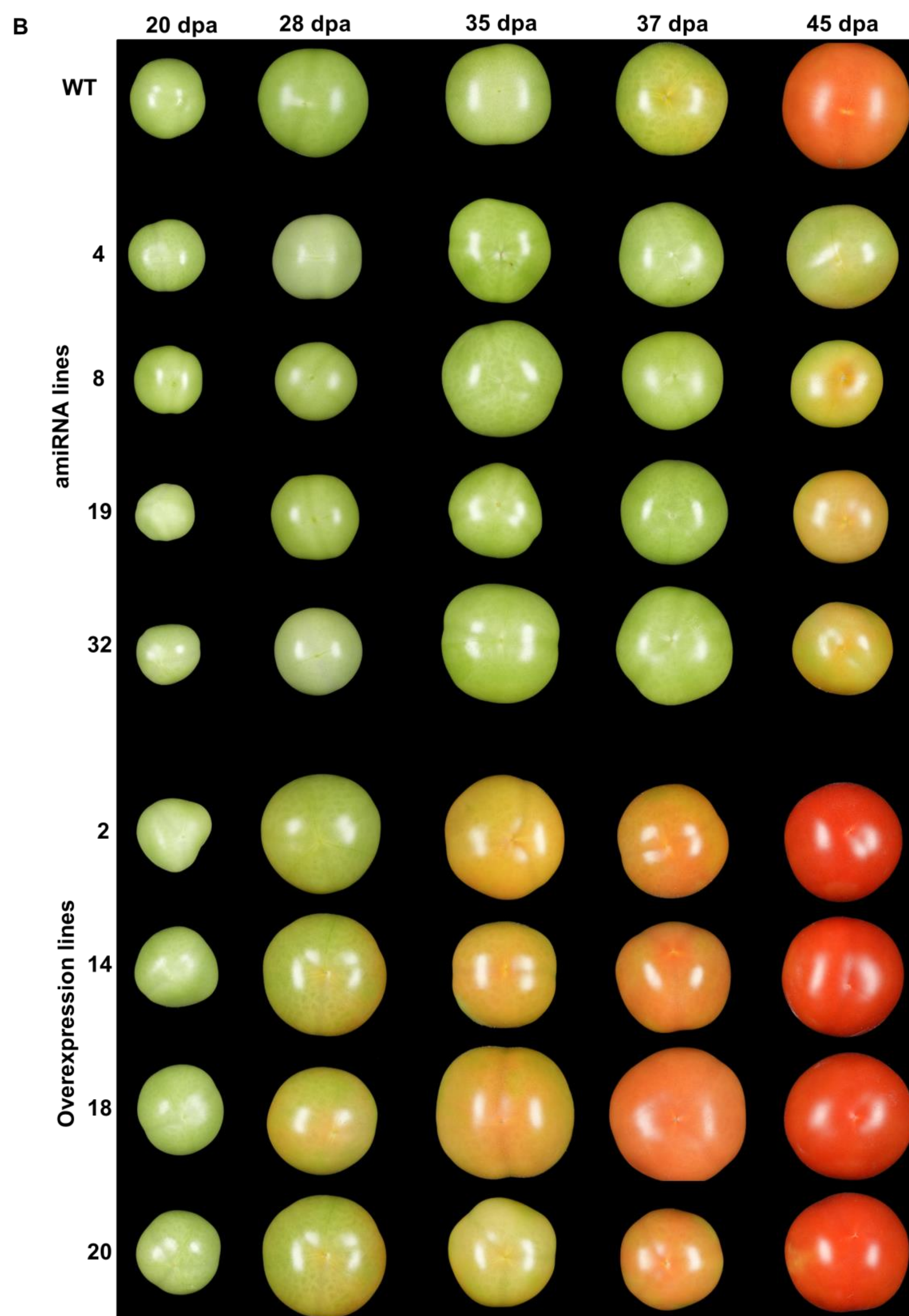


Figure 3.14 Photographs of A) WT, amiRNA line 32 and overexpression line 14 plants (T_0 generation). B) Photographs of 20, 28, 35, 37 and 45 dpa fruits for WT and four T1 generation amiRNA (lines 4, 8, 19 and 32) and overexpression (lines 2, 14, 18 and 20) lines revealed delay in fruit ripening. Effect of SlWD40 on fruit pigmentation and development. Fruits from amiRNA lines showed decreased fruit size over that of WT and overexpression lines.

Furthermore, I found that one of the amiRNA line (Line 9) was unable to produce any seeds. Next, I investigated metabolic profiles of WT and transgenics for different fruit ripening stages as 2 centimeter (cm), mature green and red ripe stage.

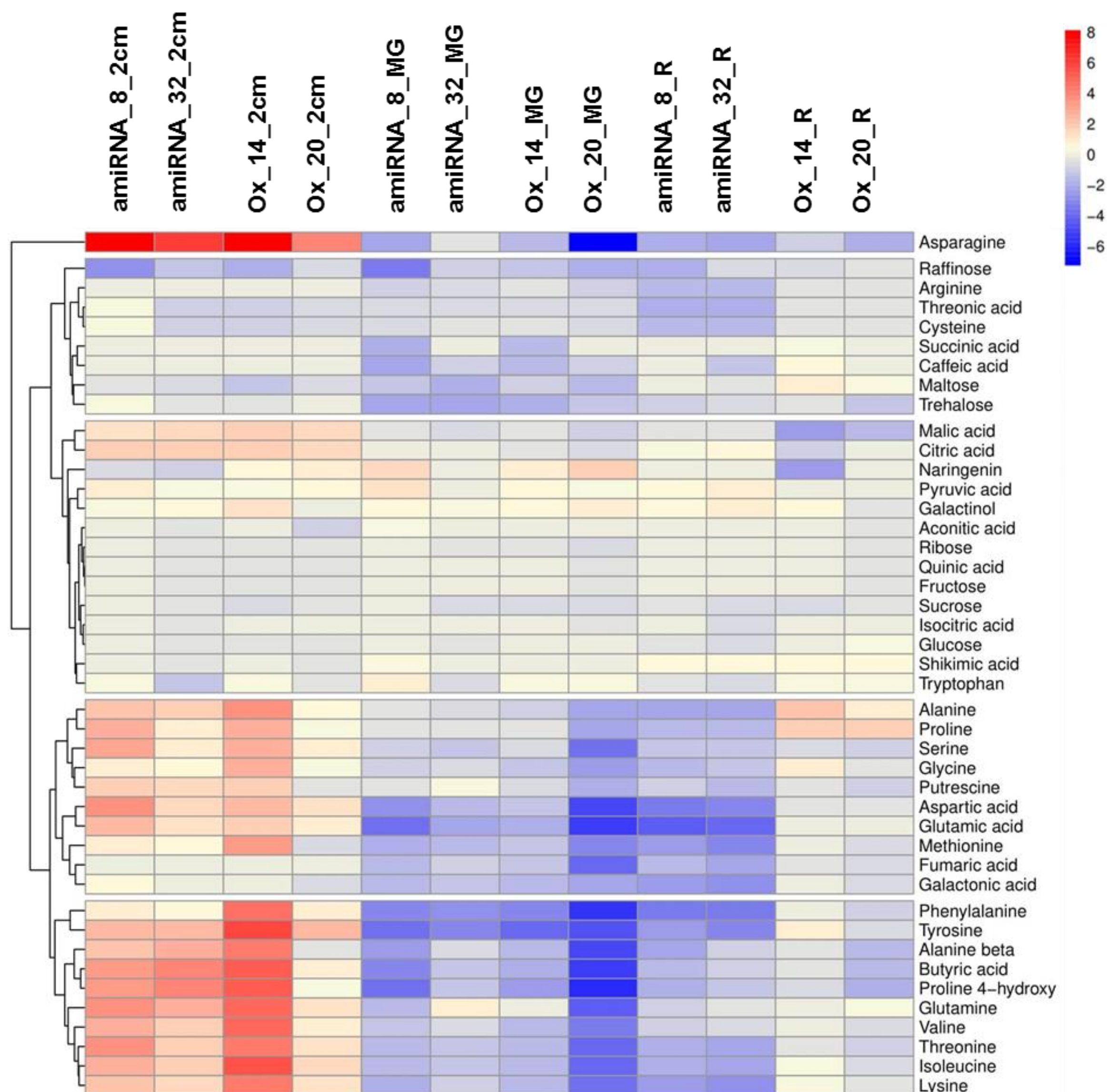


Figure 3.15 Heat map of metabolite profiles of pericarp tissues of T_0 SlWD40 amiRNA lines 8 and 32 as well as overexpression lines 14 and 20, at different fruit ripening stages as 2 cm, MG (mature green) and R (red). Regions of red or blue indicate that the metabolite content is increased or decreased relative to WT. X-axis indicates different transgenic lines alongwith different fruit ripening stages. Y-axis indicates metabolites.

I observed that amino acid metabolism was mainly affected in all transgenics, at all fruit ripening stages. After having close look on significantly affected metabolies, I found that alanine and tyrosine were significantly increased in 2 cm fruits of both of the amiRNA lines (Figure 3.15). On the other hand valine, glutamine and lysine were significantly increased in same tissues of overexpression lines. Ribose content was significantly decreased in mature green fruits for all transgenics.

3.3.4.2 SlADH affects iC5 acyl-sugar biosynthesis in tobacco

VIGS study showed whitish green phenotype for candidate SlADH. Expression analysis of this candidate in *S. pennellii* RNA-seq data showed that this candidate was induced by insect attack. Pursuing interest in this candidate, I then checked its ortholog in *N. attenuata* and expression with the help of *N. attenuata* data hub (http://nadh.ice.mpg.de/NaDH/). Excitingly, I found that its expression is strongly induced upon insect attack (Figure 3.16).

Figure 3.16 Expression of NIATv7_g07158.t1 (*N. attenuata* ortholog for Solyc12g010950) under biotic stress treatment (http://nadh.ice.mpg.de/NaDH/).

Therefore, full length gene was cloned for SlADH, in sense orientation in the vector pK2GW7 under the control of the constitutive CaMV 35S promoter. This construct was then used for generating constitutive overexpression lines in both SNN and MM. 3 promoter PCR-positive overexpression lines were selected for secondary metabolite and volatile profiling.

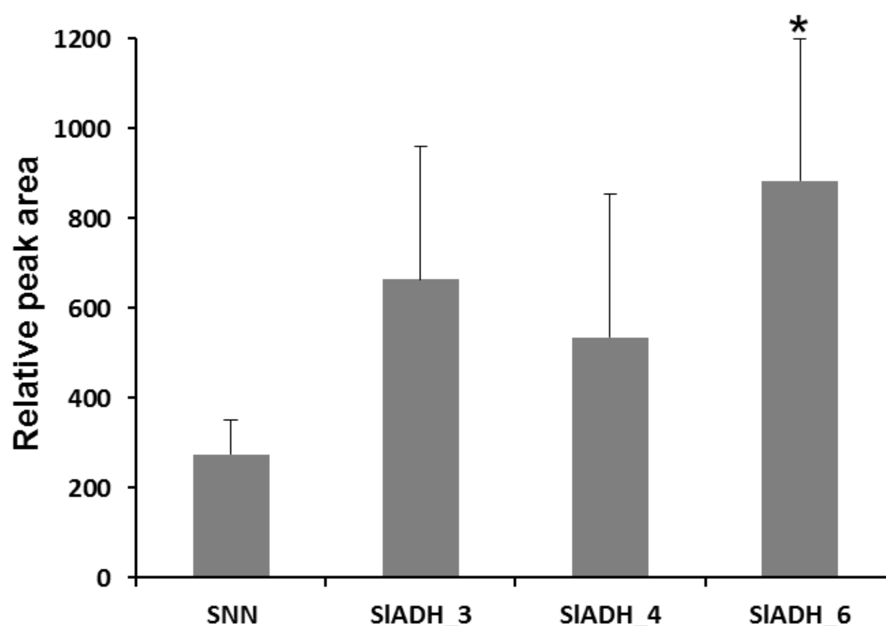


Figure 3.17 Relative content of iC5 acyl-sugars in leaf tissues of SNN and T_0 SlADH overexpression lines. Data represent the mean ± SE of three independent determinations. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.1).

Excitingly, I found that iC5 acyl-sugars were highly accumulated in transgenics over that of SNN. Line 6 showed significant increase at permissive threshold (Figure 3.17). Catabolism of leucine leads to synthesis of α-ketoisocaproic acid (KIC) and then to iC5 acyl-sugars, 3-methylbutanal and 3-methylbutanol. Therefore, I analyzed branched chain volatiles 3-methylbutanal and 3-methylbutanol in these lines. I found that 3-methylbutanal was decreased for line 3 and 6 while 3-methylbutanol was decreased only for line 3 (Figure 3.18; Supplemental Table 3.3). However, these changes were not significant. Line 4 showed significant increase for 3-methylbutanol.

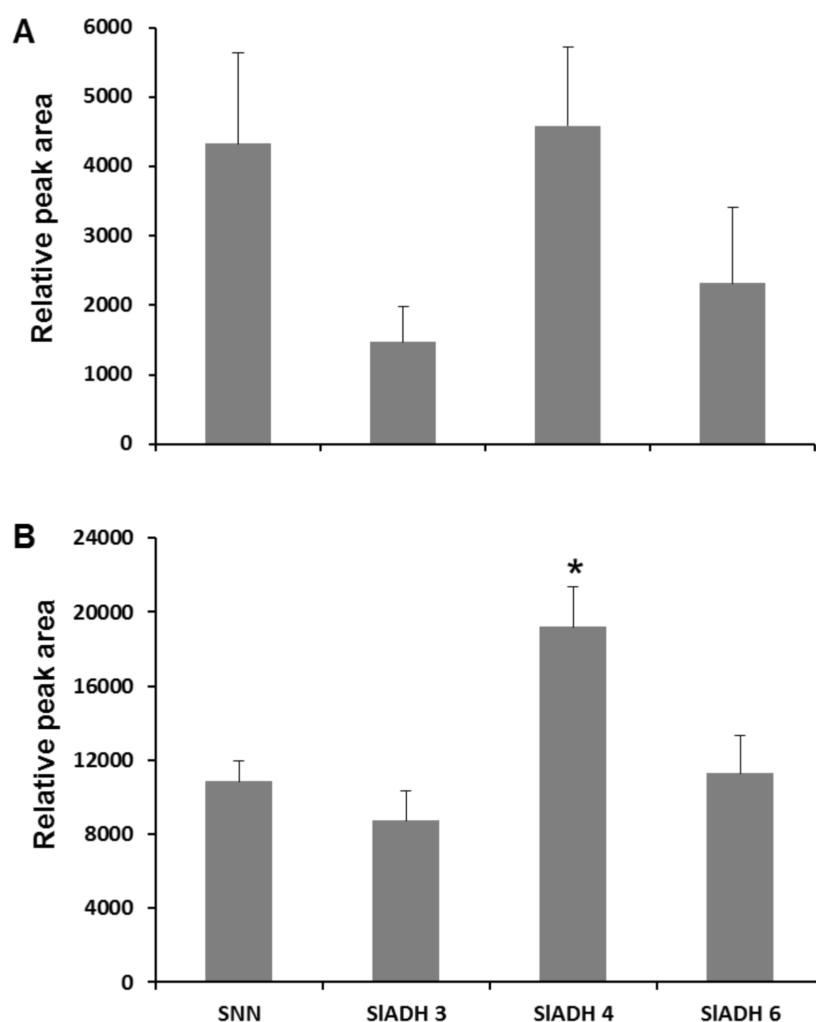


Figure 3.18 Relative contents of A) 3-methylbutanal and B) 3-methylbutanol in leaf tissues of SNN and T_0 SlADH overexpression lines. Data represent the mean ± SE of three independent determinations. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test ($P<0.1$).

Line 3 and 6 were then further used for primary metabolite analysis. Levels of tyrosine, lysine, ornithine, isoleucine and valine were significantly decreased only for line 6 (Figure 3.19; $P<0.1$). On the other hand, content of serine and glycine were significantly increased for line 3. Sugars were not affected except fructose in both lines.

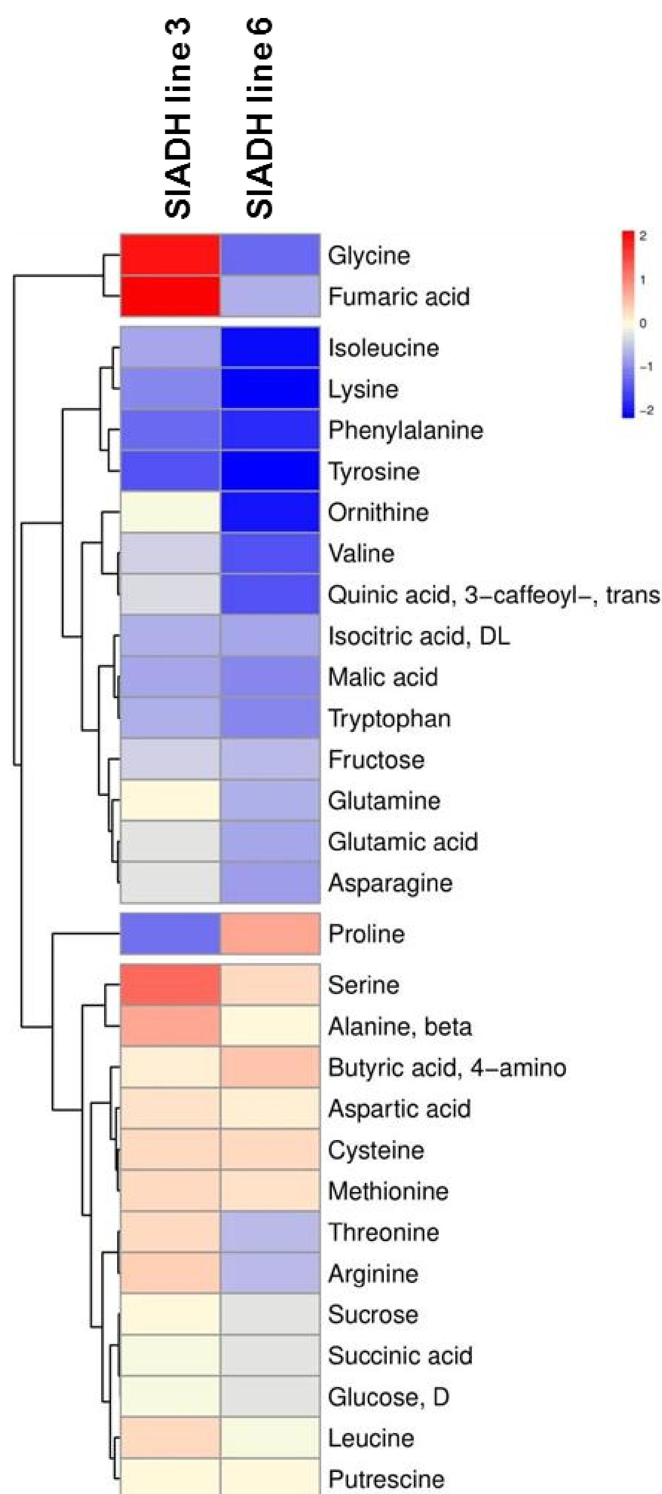


Figure 3.19 Heat map of metabolite profiles of leaf tissues of T_0 SlADH overexpression lines 3 and 6. Regions of red or blue indicate that the metabolite content is increased or decreased relative to WT. X-axis indicates different transgenic lines. Y-axis indicates metabolites.

3.3.4.3 SopenCYP450 positively affects ic5 acyl-sucrose biosynthesis

SopenCYP450 showed specific eQTL in IL3-4. Moreover, IL3-4 harboured mQTL for leucine and valine (discussed earlier, Figure 3.8). Therefore, I decided to transiently overexpress SopenCYP450 (*S. pennellii* allele of Solyc03g111940) in MT. For this, I followed the protocol described by Bhaskar et al., (2009). Excitingly, as shown by Kochevenko and Fernie 2011, we found expected results i.e. levels of leucine and iC5 acyl-sucrose were elevated (Figure 3.20). Furthermore, in order to validate these results, stable constitutive overexpression lines were created in MM. Young leaf tissue samples of 6 promoter PCR-positive overexpression lines were used for secondary metabolite profiling.

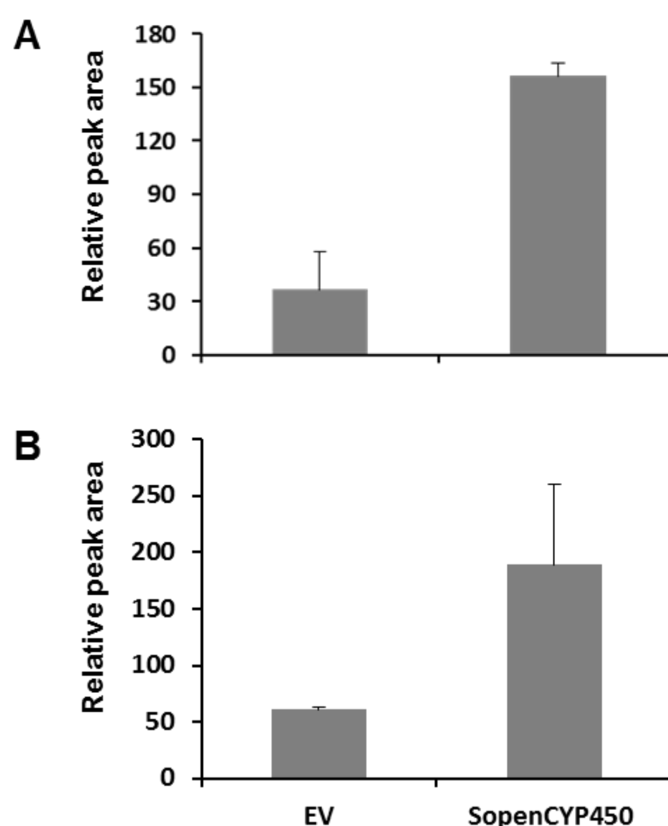


Figure 3.20 Relative contents of A) leucine and B) iC5 acyl-sucrose in transiently overexpressed parts of MT pericarp. EV indicates empty vector. Data represent the mean ± SE of two independent determinations.

As was found for transient study, results obtained by LC-MS analysis clearly showed significant increase in iC5 acyl-sucrose content for 2 transgenic lines (Figure 3.21; P<0.1; Supplemental Table 3.4).

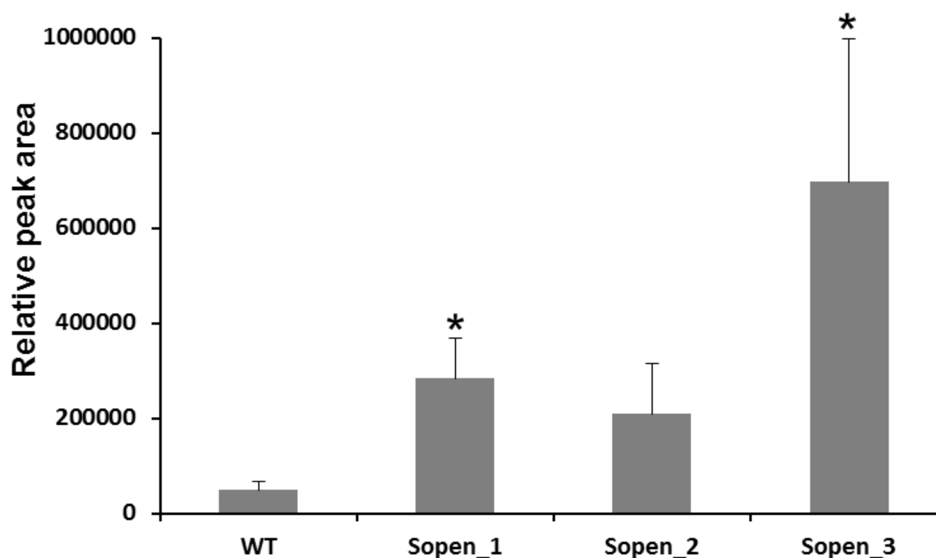


Figure 3.21 Relative content of iC5 acyl-sucrose in leaf tissues of WT and T_0 Sopen03g031040 overexpression lines. Data represent the mean ± SE of three independent determinations. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.1).

Line 1 and 2 were then next used for primary metabolite analysis. Levels of aspargine and sucrose were significantly elevated for line 1 (Figure 3.22; P<0.1). Putrescine content was significantly decreased for line 2. Additionally, levels of glucose, isocitric acid, butyric acid 4-amino, lysine, ornithine, glycine, threonine, malate and putrescine were effectively dropped for line 2. Moreover, levels of chlorogenic acid were strongly elevated for both transgenic lines. However, these changes were not statistically significant.

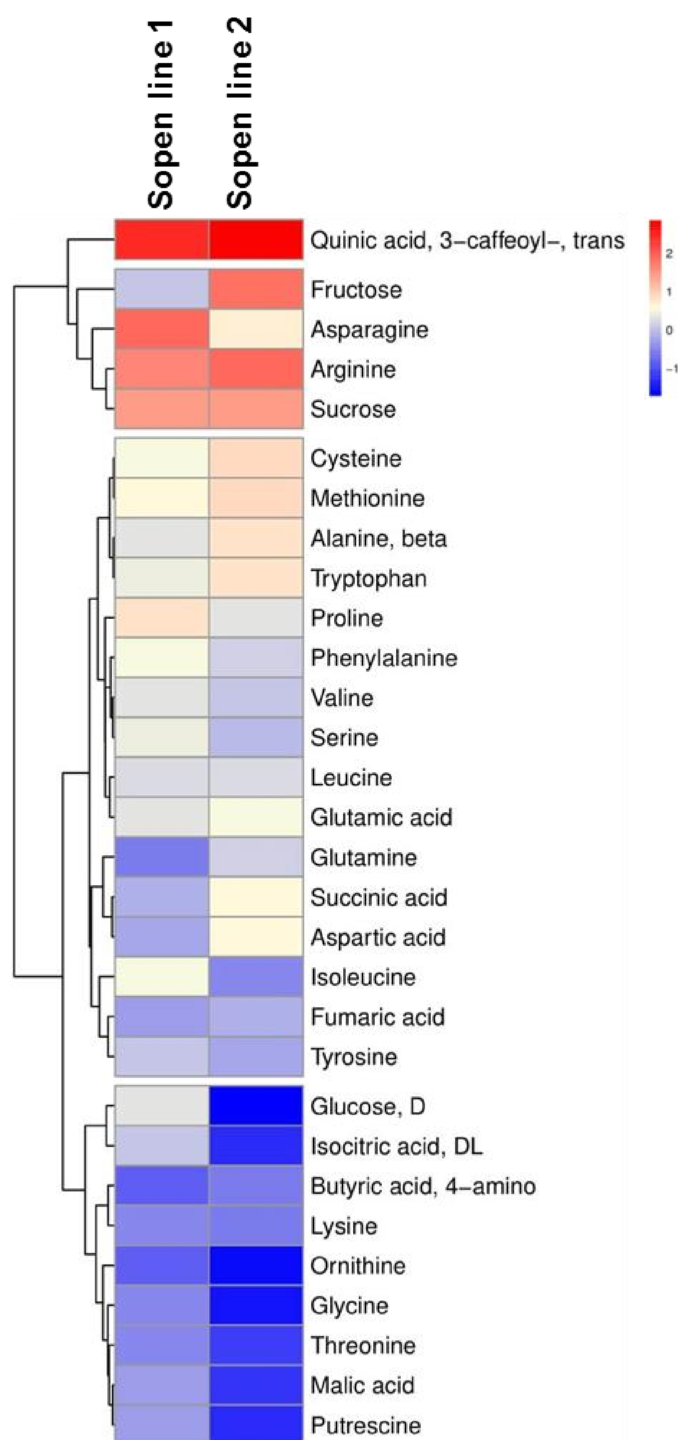


Figure 3.22 Heat map of metabolite profiles of leaf tissues of T_0 SopenCYP450 overexpression lines 1 and 2. Regions of red or blue indicate that the metabolite content is increased or decreased relative to WT. X-axis indicates different transgenic lines. Y-axis indicates metabolites.

3.4 Discussion

Advancement in the field of next generation sequencing and metabolomics has resulted in modification of traditional QTL mapping and association mapping approaches (two most commonly used QTL mapping methods) into much more focused and defined eQTL, mQTL, GWAS and mGWAS approaches (Rambla et al., 2015; Zhu et al., 2018; Ranjan et al., 2016; Wu et al., 2018). Each of these studies is highly effective strategy not only to identify key regulators underlying organismal development but also to functionally annotate genes. Additionally, modulation of expression levels of TFs by transgenic approaches can also aid in identifying key genes and metabolic pathways that they regulate (Tamagnone et al., 1998; Grotewold et al., 2000; Tohge et al., 2005; Liu et al., 2014; Tohge et al., 2015). Several studies have implicated Myb, BHLH, TCP and WD40 TFs as playing key roles in plant secondary metabolomics (Sonderby et al., 2007; Dal Cin et al., 2011; Kong et al., 2012; Pillet et al., 2015). To get an insight into the tomato fruit ripening and metabolic regulation underlying tomato secondary metabolomics, in this study, I filtered 20 potential candidates by combining eQTLs (Unpublished data Giovannoni et al.) and TFs approaches. Furthermore, validation by VIGS and transient overexpression study helped me to predict putative function to some of them. In order to functionally characterize 3 candidates, overexpression lines were developed and metabolite profiling was carried out. Obtained results strengthened our confidence on candidate genes and approach we used.

3.4.1 SlWD40 TF regulates tomato fruit development and ripening

Expression of SlWD40 TF markedly elevates after mature green (MG) stage (Figure 3.23). So in order to understand its fruit stage specific effects, T_0 fruits of fruit (B33) specific overexpression and amiRNA lines were analyzed phenotypically and also by metabolite profiling. Fruits of amiRNA lines showed deceased fruit size over that of WT. Green and slightly yellowish (instead of red) phenotypes obtained for most of the fruits of 2 amiRNA lines at breaker and red ripe stage respectively; strongly suggested that SlWD40 might be involved in regulation of fruit ripening process in tomato. This is also supported by VIGS results and correlation analysis. Metabolite profiling on fruits of transgenic lines showed that amino acid metabolism was mostly affected. Generally, this TF does not express in 2 cm stage fruits. Overexpressing this SlWD40 in 2 cm stage fruits resulted in significant increase in valine, glutamine

and lysine content.

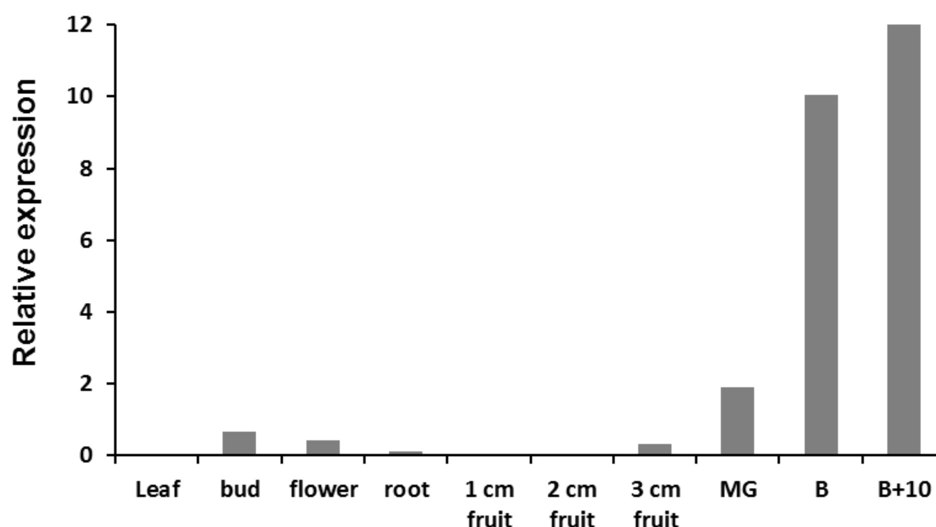


Figure 3.23 Relative expression of SlWD40 in different tissues of *S. lycopersicum* cv. Heinz. MG- mature green, B-breaker and B+10-breaker+10 stage. Data represent average of two replicates (The Tomato Genome Consortium 2012).

Previous studies have implicated malate in the physiology of fruit ripening (Lance et al., 1967; Jeffery et al., 1984; Goodenough et al., 1985; Nunes-Nesi et al., 2005, 2007). Moreover, I observed that result obtained for decrease in malate content in both overexpression lines at red ripe stage is a similar metabolic change in fumarase antisense lines obtained by Centeno et al., (2011). Overall results suggested that SlWD40 TF tightly controls primary metabolism and have fruit developmental stage specific effects. ABA-regulated TF SlAREB1 has been shown to be involved in stress-induced responses (Orellana et al., 2010). This TF is expressed in seeds and fruit tissues in tomato. Furthermore, it has also been shown that SlAREB1 control primary metabolism in tomato fruit and participates in the regulation of the metabolic programming that takes place during fruit ripening (Bastias et al., 2014). Inability of one of amiRNA line to produce seeds and the fact that SlWD40 normally expresses in seeds, indicates that SlWD40 TF might regulate other developmental processes besides ripening. Identification of SlWD40 interacting partner might unravel regulatory mechanism underlying tomato fruit ripening.

Transcriptomics and metabolite profiling on fruits (20, 28, 35, 37 and 45 dpa) from T_1 generation transgenics, is in progress and might give some insights into the regulation of fruit ripening by SlWD40.

3.4.2 Heterologous overexpression of SlADH induces iC5 acyl-sugar biosynthesis in tobacco

Expression of SlADH (Solyc12g010950) was strongly upregulated under biotic stress. This gene was putatively annotated as alcohol dehydrogenase. These results suggested that it could be involved in last steps of acyl-sugar biosynthesis (Figure 3.24). Three constitutive overexpression lines for this gene were developed in SNN and analyzed. It has been proposed that in microbes, alcohol dehydrogenase (ADH) can catalyze 3-methylbutanal to 3-methylbutanoic acid conversion (Beck et. al., 2004). Additionally, authors had suggested that, ADH can also catalyze reversible reaction of 3-methylbutanal to 3-methylbutanol conversion. Results obtained by analyzing overexpression lines for their branched chain volatiles and iC5 acyl-sugar content suggests that SlADH might catalyze 3-methylbutanal to 3-methylbutanoic acid or 3-methylbutanol conversion (Figure 3.24).

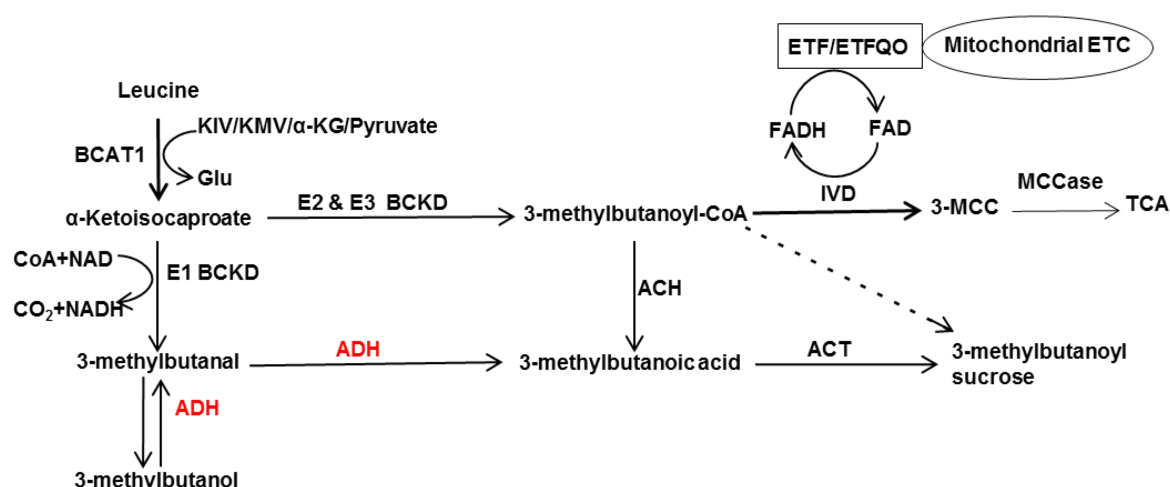


Figure 3.24 Updated pathway of iC5 acyl-sugar (3-methylbutanoyl sucrose) biosynthesis in tomato (Wallace et al., 1995, Beck et. al., 2004). KIV-Ketoisovalerate, KMV-ketomethylvalerate, α-KG- α-Ketoglutarate, BCKD- branched-chain ketoacid dehydrogenase, ADH- alcohol dehydrogenase, ACH-acyl-CoA hydrolase, ACT-acyl-CoA transferase, IVD-Isovaleryl dehydrogenase, MCCase-3-methylcrotonyl Co-A carboxylase.

Respiration was not changed in the transgenics (data not shown). Additionally, decrease in phenylalanine and chlorogenic acid content indicates downregulation of phenylpropanoid pathway. Analysis of T1 generation lines and real-time qPCR analysis is in progress and might give some insights into the metabolic regulation of the pathway. Additionally, analysis of overexpression lines that are developed in MM is also in progress.

3.4.3 SopenCYP450 positively modifies flux for ic5 acyl-sucrose biosynthesis

Several attempts have been made to upregulate or induce levels of secondary metabolites, specially, metabolites of high commercial value such as flavonoids, carotenoids, chlorogenic acid and acyl-sugar (Butelli et al., 2008; Dal Cin et al., 2011; Kochevenko et al., 2012; Wahyuni et al., 2014). Various approaches have been developed to identify TFs and key enzymes involved in tomato secondary metabolism (Lee et al., 2011; Liu et al., 2014; Alseekh et al., 2015). Here, characteristic of induction of expression upon biotic stress as well as presence of specific eQTL and mQTL (Kochevenko and Fernie 2011; Figure 3.8) helped me to identify SopenCYP450, as a key enzyme that positively modulates metabolic flux for ic5 acyl-sucrose biosynthesis. Obtained result regarding exponential increase in chlorogenic acid content in leaf tissues was surprising. Respiration was not changed in the transgenics (data not shown). At this level, mechanism behind upregulation of flux for iC5 acyl-sucrose synthesis is not known. SopenCYP450 (Solyc03g111940) is presently annotated as a CYP450 71A4 like protein. Published studies have implicated CYP450 71A proteins in various biosynthetic pathways. For example, Hallahan et al., (1992 and 1994) found that avocado CYP71A family protein catalyzed 2,3- or 6,7-epoxidation of monoterpenoids (nerol and geraniol) while catmint CYP71A family protein catalyzed their hydroxylation. In arabidopsis, it has been shown that CYP71A13 catalyzes the conversion of indole acetaldoxime to indole acetonitrile in camalexin synthesis (Nafisi et al., 2007).

Using SherLoc2, an online service tool for prediction of protein localization (http//abi.inf.unituebingen.de/Services/SherLoc2), surprisingly I found that *S. lycopersicum* homolog of this CYP450 protein (encoded by Solyc03g111940) is localized to mitochondria (probability 0.26) and endoplasmic reticulum (probability 0.20) while *S. pennellii* protein encoded by Sopen03g031040 is localized to only endoplasmic reticulum (probability 0.64). In the recent review on CYP450 it has been discussed that presence of CYP450 in mitochondria seems to be characteristic of animal cells (Omura and Gotoh 2017). Plants have many microsomal (endoplasmic reticulum) as well as chloroplast localized CYP450s, but no mitochondrial CYP450 has been reported for plants and fungi. Considering this, in my case study, possibility of *S. lycopersicum* CYP450 being inactive or less active is much higher. Here, alteration in few base pairs might have led to differential localization and hence to modified metabolic flux.

In a nutshell, effect of altering just few base pairs on adaptive fitness of the species is witnessed again. In order to decipher function of SopenCYP450, I have cloned this gene into vector pYeDP60 and functional analysis is in progress. Furthermore, volatile analysis for leaf and fruit tissues might give some hints regarding reaction catalyzed by this CYP450 71A4 like protein.

Chapter 4. Mapping of drought stress related mQTLs in 57 *S. pennellii* ILs

4.1 Introduction

Tomato is the second most important vegetable crop in the world in terms of consumption per capita. But abiotic stresses such as drought, heat, UV, salinity and flood limits crop productivity severely. Among them drought stress is most commonly threatening food security, mainly because of climate change led increase in warmer temperatures (Dai, 2011; Shrivastava and Kumar, 2015). As per the report of Intergovernmental Panel on Climate Change (IPCC), an additional 40 to 170 million more people might be undernourished as a direct consequence of climate change (IPCC 2001). Drought is defined as the occurrence of a substantial water deficit in the soil or in the atmosphere (Ceccarelli and Grando 1996). Plants combat drought stress by employing diverse set of strategies such as drought escape, dehydration avoidance and dehydration tolerance (Blum 1988; Kramer 1983). Therefore, understanding drought tolerance at the physiological, molecular and metabolic level is the necessity of time for sustainable agriculture. Wild species of tomato such as *S. chmielewskii* and *S. pennellii* are high altitude drought-tolerant and desert-adapted accessions respectively. These species are the rich source of genetic diversity for developing drought tolerant tomato varieties and therefore have studied at genome, transcriptome and metabolome level very well (Koenig et al., 2013; Bolger et al., 2014; Schauer et al., 2006). Various introgression lines (ILs) have been developed by crossing different wild species with different domesticated tomato varieties (Eshed and Zamir, 1994; Canady et al., 2005; Pertuze et al., 2003; Frary et al., 2003; Monforte and Tanksley, 2000; Fulton et al., 2000; Paran et al., 1995; Robert et al., 2001, Peleman and van der Voort 2003). By using BC1 (back cross 1) population generated by crossing drought-sensitive tomato breeding line and a drought-tolerant *S. pimpinellifolium* accession (LA722), four QTLs on chromosomes 1, 4, 8, 9 and 12 for drought tolerance during tomato seed germination have been detected (Foolad et al. 2003). Various studies have proven that among all the other ILs, panel of 76 *S. pennellii* ILs could be used for unraveling drought responsive mechanism in tomato. For example, three QTLs (IL7–5–5, IL8–3 and IL9–2–5) that contribute to yield under drought condition have been found using this population (Gur and Zamir 2004). Using same population, Gong et. al., (2010) have identified that IL2-5 and IL9-1 can

tolerate drought at seedling stage. Additionally, they have also identified 400 genes that confer this drought tolerance to them. Among these 400 genes, many were TFs, signaling proteins and enzymatic genes involved in organism growth and development. In the recent study, Rigano et al., (2014) had subjected drought tolerant IL9-2-5 and the susceptible M82 genotypes to three different water regimes: irrigation with 100%, 50% and 25% field capacity and found that IL 9-2-5 had higher values of sclerophylly and leaf dry matter content. Here, authors have concluded that leaf morphology and physiology impart drought tolerance to IL9-2-5. These studies have proved that extreme stress tolerance and physiology of *S. pennellii* makes it a vital germplasm that can be exploited for developing new crop varieties. Therefore, international group of scientists have decoded its genome and mapped drought stress related candidate genes as well as QTLs (Bolger et al., 2014). Here, authors have excellently fished out beauty of molecular architecture underlying desiccation tolerance of *S. pennellii* by identifying significant enrichment of *S. pennellii*–specific Copia elements within 5 kb of genes that were more stress responsive in *S. pennellii* than their *S. lycopersicum* orthologs (66 of 293 upregulated genes, $P<0.038$ and 69 of 399 downregulated genes, $P<0.022$). Furthermore, they have also identified 100 candidate genes related to salt and drought stress.

Though strength of 76 *S. pennellii* ILs has been proved, it has also been found that genomic shock might also generate some of the phenotypic variation in the ILs (McClintock, 1984). In order to overcome this hurdle and also have high resolution population, Ofner et al., (2016) have generated BILs composed with 446 lines. In this study, authors have subjected this genetic variation to phenotyping, metabolomics and single nucleotide polymorphism (SNP) analysis and have tracked down possible candidate genes involved in the manipulation of various branches of the wax biosynthesis pathway in tomato. This finding might help in increasing resistance to water flux across the cuticle and development of drought resistance in tomato.

Comparative physiological and molecular study of drought response between *S. lycopersicum* and *S. pennellii* found that *S. pennellii* leaves shows greater ability to avoid water loss and oxidative damage mainly because of upregulation of genes involved in N assimilation, GOGAT/GS cycle and GABA-shunt, JA/ET (Jasmonic acid/Ethylene) biosynthesis and signaling pathways (Egea et al., 2018). Several reports have documented that plant metabolome changes upon drought stress

(Selmar and Kleinwächter 2013). In *A. thaliana*, content of 5 flavonols and 5 anthocyanins were increased (Nakabayashi et al., 2014). In this case, changes in secondary metabolites had also accompanied with elevation in the levels of drought stress marker metabolites such as proline, raffinose and galactinol. In wheat (*Triticum aestivum*), water stress had resulted in increase in proline, phenol and ascorbate content and decrease in total chlorophyll content in the leaves (Chakraborty and Pradhan et al., 2012). Drought stress study in tomato have shown that drought causes stunted growth and elevated levels of proline, abscisic acid (ABA) and late embryogenesis abundant gene transcripts (Tamburino et al., 2017). In this study, chloroplast proteome analysis have helped authors to also discern elevated expression of genes related to photosynthesis, primary and secondary metabolism, transport and protein processing.

In order to dissect major QTLs for fruit quality traits, Albert et al., phenotyped 141 highly diverse small fruit tomato accessions, and with association mapping have identified 31 QTLs under drought condition (Albert et al., 2016). But impact of genetic variation on whole metabolome level under drought condition has not been studied deeply in many of the important crop species. Recent study in barley by Piasecka et al., (2016) have done LC-MS profiling on population of 100 RILs that was subjected to drought stress; and found mQTLs that associates with genes related to the defense response and response to cold, heat and oxidative stress. This study has found most significant changes for ferulic and sinapic acid derivatives as well as acylated glycosides of flavones. This study has also revealed that polyamine derivatives hordatines as well as terpenoid blumenol C derivatives could be drought related metabolites. In this chapter, I represent metabolic profiling of young leaves from drought stress subjected population of 57 *S. pennellii* ILs, aiming to investigate the genetic architecture of leaf metabolic composition under drought stress. In doing so, with GC-MS and LC-MS profiling, I identified 806 and 344 mQTLs respectively at stringent threshold. Furthermore, I found that well known drought responsive marker metabolites were significantly affected in ILs 11-2, 3-1, 8-3-1 and 10-1-1. Next, in these four ILs, I attempted to narrow down drought responsive candidate gens with the help of ortholog annotation and it´s expression under drought stress. These led to identification of potential candidates involved in drought responsive mechanisms such as wax synthesis, sulphur use efficiency and lignification.

4.2. Materials and Methods

4.2.1 Drought stress treatment

Seeds of 57 ILs were surface sterilized for 30 min with sterilization solution. Sterilization solution was prepared by mixing 1.6 ml 37 % hydrochloric acid (HCL) and 0.02 % (v/v) Tween-20 in 100 ml double distilled water. Thereafter, the seeds were rinsed in sterile deionized water three times. Seeds were then germinated in vitro on half strength Murashige and Skoog (MS) basic medium (Murashige and Skoog 1962) containing 1% (w/v) sucrose and kept for 3 days in the dark in a growth chamber with 22°C, 70% humidity and 16/8h light/dark regime. After stratification, light of intensity 150 µmol photons $m^{-2}s^{-1}$ was given. As per the speed of germination, we sorted the M82 and ILs into 3 batches. After 18 days, 5 seedlings for M82 and each of IL were transferred to standard greenhouse soil (Stender AG; Schermbeck, Germany) in plastic pots of a 0.1 L capacity. The trays containing the pots were placed under 8/16h light/dark, 22/16˚C, 60/75% relative humidity and 180 µmol photons $m^{-2}s^{-1}$ light intensity. Plants were then allowed to acclimatize to greenhouse condition for 6 days and then subjected to drought stress for 10 days. Leaf and stem samples were then collected, weighed and processed for GC-MS and LC-MS analysis as per the protocol mentioned in one of the previous section.

4.2.2 mQTL mapping

GC-MS and LC-MS data were normalized by internal standard ribitol and isovitexin respectively. This data were then again normalized by fresh weight and then fold changes were calculated over that of M82. Thereafter average of 5 replicates was taken. This data was then used for mQTL mapping, each IL was compared (by t test, using permissive $p \leq 0.05$ and stringent $p \leq 0.01$ cut offs) to M82. If the particular IL shows the significantly different fold change from the reference genotype M82, then that IL have the introgression that harbours mQTL. Significantly increasing and decreasing mQTLs were identified.

4.2.3. Heat maps

Heat maps were generated based on average of fold change values, using the MultiExperiment Viewer Program (MeV 4.7.3) (Howe et al., 2010). Normalized

metabolite data was log10-transformed and then false colour imaging was performed. I scaled data internally on a column basis to have a mean of 0 and a standard deviation of 1. If the metabolite content was determined in all four replicates of an introgression line, then only metabolite data was considered.

4.2.4. Fine mapping of drought tolerance specific mQTL and candidate genes

ILs 11-2, 3-1, 8-3-1 and 10-1-1 were chosen based on significant changes in drought stress marker metabolites. Genetic markers that define ends of overlapping region between IL11-1 and 11-2 as well as between IL11-2 and 11-3 introgressions on chromosome 11 (Figure 4.3A), were next used to identify the genomic sequence in the assembly of tomato genome available at the Sol genomics network (SGN, assembly ITAG2.3; http://solgenomics.net). Total genes in this region were then filtered by using 3 filters as drought responsive genes, annotable genes and putative function. For this, arabidopsis orthologs of all the annotable genes were searched and then its functional annotation as well as expression pattern under abiotic stress was checked with the help of TAIR website (https://www.arabidopsis.org/) and arabidopsis eFP browser (https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) respectively. Literature was also searched for functionally well characterized drought responsive candidates and regulatory mechanisms. Same protocol was used for narrowing down candidates from ILs 3-1, 8-3-1 and 10-1-1.

4.3. Results

4.3.1 Identification of drought stress related mQTLs

In order to find out the effect of genetic variation on metabolite composition under drought stress, I used the 57 *S. pennellii* ILs. After GC-MS profiling of leaf samples, relative quantification was done for 88 metabolites. These compounds include not only primary metabolites but also some of the secondary metabolites. The full data set from the primary metabolite profiling study are presented in an heat map, wherein red and blue rectangles depict fold change increases and decreases with respect to that of M82 (Figure 4.1). The relative differences in the content of any given metabolite range between a 0 (not present) and 106-fold increase compared with the recurrent parental cultivar M82. This data was further used to identify mQTLs. mQTLs were determined using t tests, at a significance levels of 0.01 (stringent) and 0.05 (permissive), to compare statistically each IL with M82. I identified total 806 and 1440 mQTLs at stringent and permissive threshold, respectively. ILs 1-1-2, 1-1-3, 2-6-5, 5-5 and 8-3-1 showed highest number of mQTLs at both threshold levels as compared to any of the IL.

Thereafter, I went on to check significantly affected metabolites and QTLs thereof (Figure 4.2). I found that total 797 mQTLs were significantly affected across all the ILs at stringent threshold. This includes 173 and 624 mQTLs that were significantly increased (positive mQTL) and decreased (negative mQTL) respectively, at stringent threshold. After having close look on this mQTLs data, I found that ILs 7-5 and 7-4 harbored positive mQTL for 11 metabolites. Twenty ILs did not showed significant increase in any of the metabolite identified. Interestingly, I found that glucose, phosphoric acid and *cis*-quinic acid 3-caffeoyl were the metabolites that showed highest number of positive mQTLs, across all the ILs. Furthermore, I found that ILs 1-1-2 and 1-1-3 showed significant decrease for highest 45 and 30 metabolites respectively. Additionally, highest numbers of negative mQTLs were found for salicylic acid, β-alanine, serine-O-acetyl and citric acid across panel of 57 ILs.

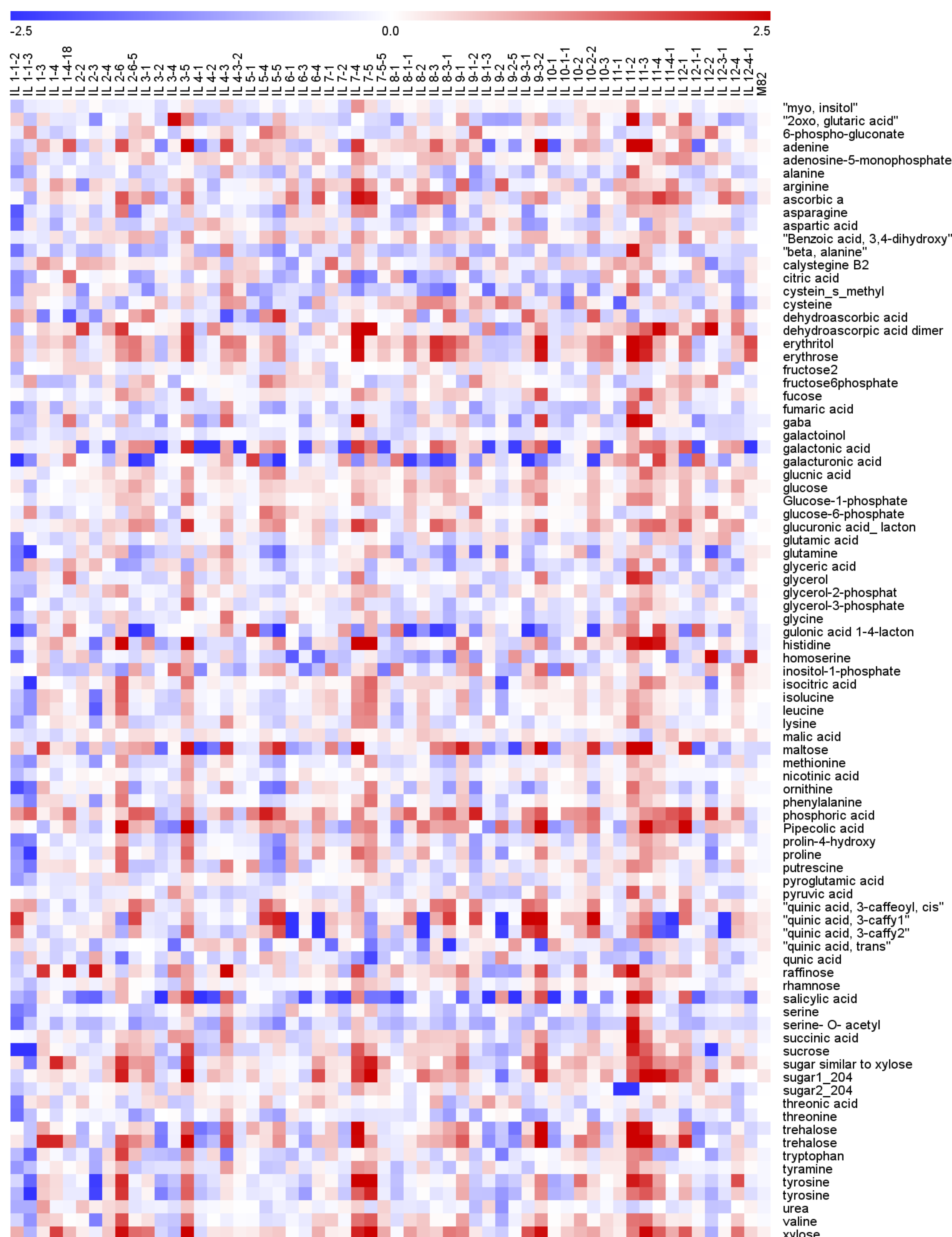


Figure 4.1 Heat map of metabolite profiles of ILs under drought stress. Each square represents the effect of chromosomal segment substitution on the amount of every metabolite using a false-color scale. Regions of red or blue indicate that the metabolite content is increased or decreased, respectively, after the introgression of *S. pennellii* segments.

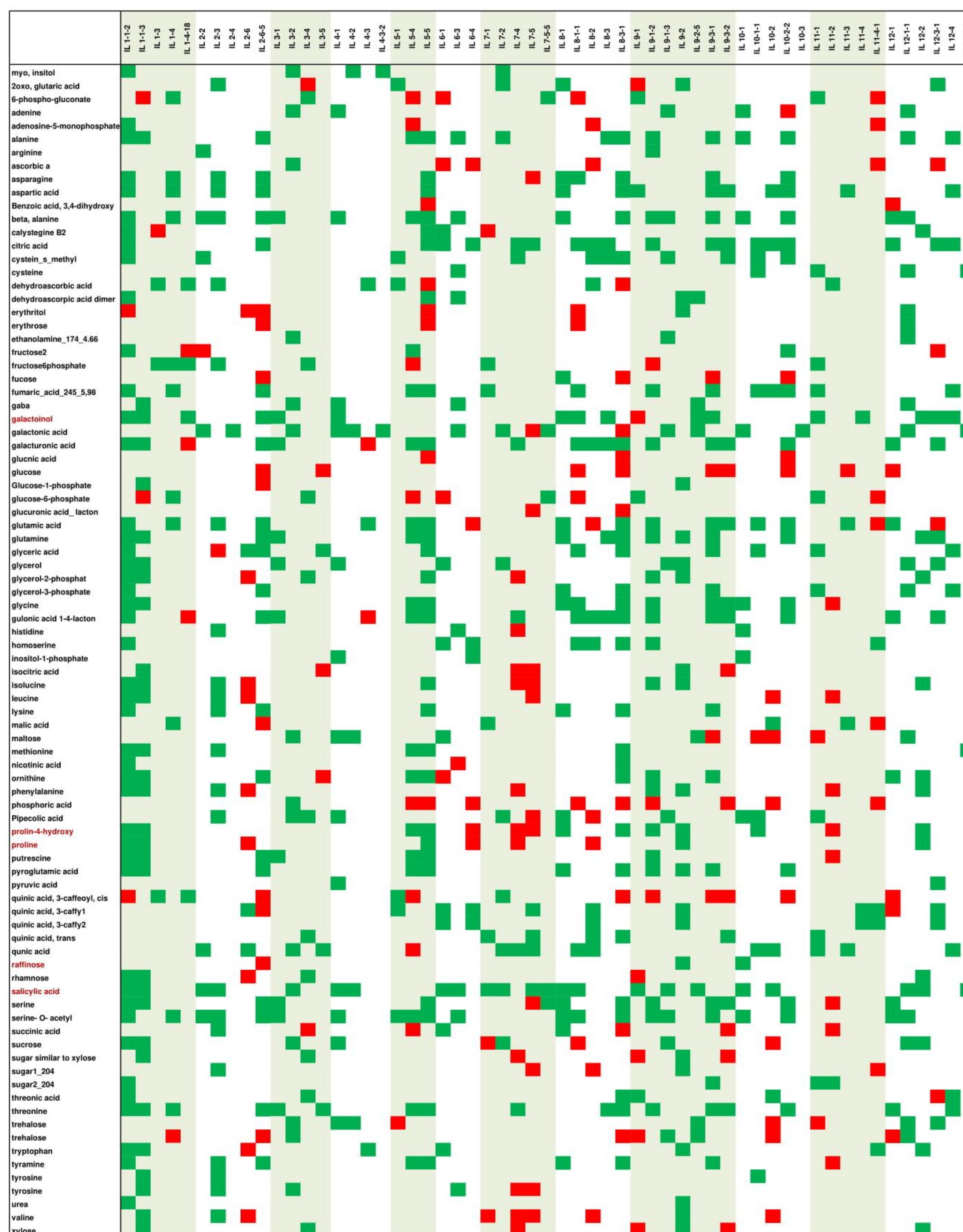


Figure 4.2 Genetic architecture of primary metabolites QTL under drought stress across the 57 *S. pennellii* introgression lines. Shown are the distribution and number of QTL across the tomato genome at the significance level of p≤ 0.01. Regions of red or green indicate that the metabolite content is significantly increased or decreased, respectively compared to M82. X-axis indicates 57 ILs according to their chromosome number. Light green colour is used for differentiating the chromosomes. Y-axis indicates metabolites. Dark red colour is used for some of drought stress marker metabolites.

I went further to check significantly affected drought stress marker metabolites. I found that proline showed positive mQTL only in ILs 2-6, 6-4, 7-4, 8-2 and 11-2. Levels of raffinose and galactinol were significantly elevated in ILs 2-6-5 and 9-1 respectively. Moreover, I found that ILs 1-1-3, 8-1 and 12-2 harbored negative mQTL for all the three drought stress marker metabolites proline, galactinol and salicylic acid.

Published study revealed major differences in accumulation of some of the sugars and TCA cycle metabolites in drought sensitive and drought tolerant wheat genotypes (Guo et al., 2018). So, here I analyzed the effect of water deficit on accumulation of sugars and TCA cycle metabolites in tomato leaf. I found that sugars such as glucose (1.5 fold), sucrose (4.5 fold) and trehalose (14 fold) as well as TCA cycle metabolites such as fumaric acid (3 fold) and succinic acid (5 fold) were significantly accumulated in IL11-2; for which GABA (11 fold), proline (1.6 fold), salicylic acid (6.5 fold) and galactinol (1.6 fold) (Figure 4.3A) were significantly increased at permissive threshold.

4.3.2 Fine mapping of candidate genes from mQTL for primary metabolites

I found that *S. pennellii* genomic region of IL11-2, excluding regions that overlapped with ILs 11-1 and 11-3, contained 198 predictable genes. These genes include 6 genes that were annotated as wax synthases and 48 as unknown proteins (Figure 4.3B). With the help of arabidopsis ortholog search and eFP browser, 27 genes were detected as responsive to only drought stress. Lastly, from those 27 genes, 6 drought responsive candidate genes could be finalized by using the protocol mentioned in the methods section. I found that arabidopsis ortholog of one of those candidate Solyc11g013560 was characterized as KUA1 that modulates leaf cell expansion and final organ size by controlling ROS (reactive oxygen species) homeostasis (Lu et al., 2014). This is interesting because many published reports have shown that regulating ROS levels under drought stress is of high importance in maintaining plant growth and development (Dat et al., 2000).

Additionally, candidates Solyc02g094040, Solyc02g094120 and Solyc02g094180 were narrowed down by analysing *S. pennellii* genomic region of IL2-6-5. Arabidopsis orthologs of these candidates were characterized as myzus persicae induced lipase 1, sulphite oxidase and peroxidase respectively. As per

arabidopsis eFP brawser, these candidates were found to be drought responsive.

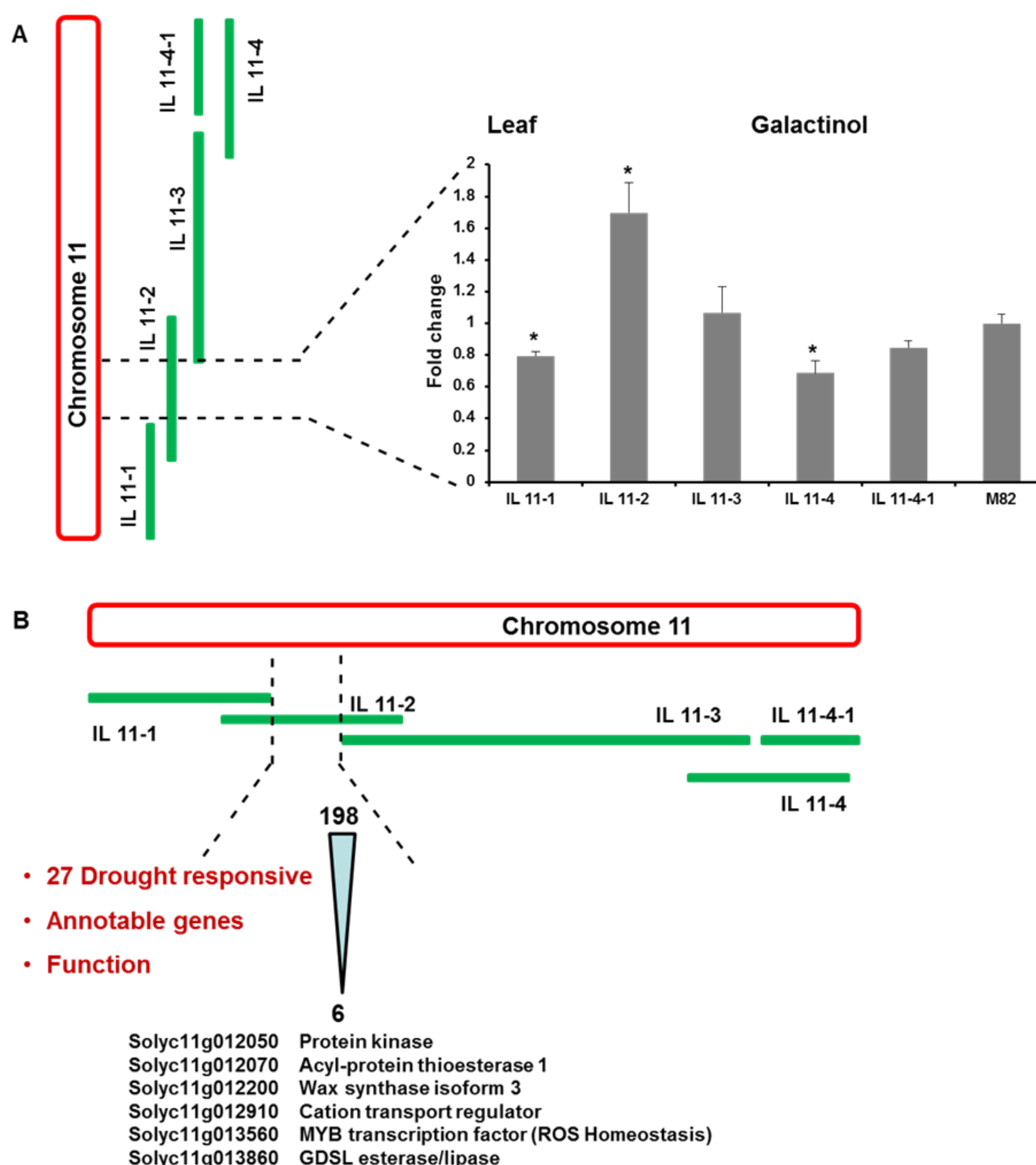


Figure 4.3 Schematic representation of *S. pennellii* ILs for chromosome 11. A) The introgressed fragments in ILs are shown on the right of the chromosomes. Galactinol content was calculated as fold-change of M82. Data represent means of fold change ± SE from five independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test ($P<0.05$). B) Fine mapping of drought stress related mQTL. 6 key drought responsive genes were filtered from total 198 candidates using 3 filters. Filters are shown in dark red.

4.3.3 Identification of drought stress related secondary metabolites mQTLs

Next, I assessed effect of genetic variation on secondary metabolite accumulation under drought stress. For this, methanol extracts of leaf tissues of above mentioned plant samples, were subjected to LC-MS analysis. LC-MS data processing allowed me to distinguish major 45 chromatographic peaks. Relative quantification was done for all the 45 metabolites. The full data set from this analysis are presented in an heat map, wherein red and blue rectangles depict fold change increases and decreases with respect to that of M82 (Figure 4.4). Range for the relative differences in the content of any given metabolite lies between a 0 (not present) and 239-fold increase compared with the recurrent parental cultivar M82. mQTLs were identified as mentioned in previous section. Total 344 and 751 mQTLs were identified at stringent and at the permissive threshold, respectively.

After checking the significantly affected secondary metabolites and QTLs thereof (Figure 4.5), strikingly, I detected 226 positive mQTLs and 117 negative mQTLs at stringent threshold. This result is in line with previous reports and especially with the results from the recent study in barley where the authors found accumulation of several secondary metabolites in the leaf samples of 100 barley RILs population under drought stress (Selmar and Kleinwächter 2013; Piasecka et al., 2016). Next, I observed that 18 and 24 ILs did not show any positive and negative mQTLs respectively. Unknown metabolites with mass 503 and 681 eluting at retention times 8.25 and 13.51 min respectively, showed highest number of positive and negative mQTLs respectively, across all the ILs.

IL7-4 showed decreased shoot weight (data not shown) and highest number of 20 positive mQTLs at stringent threshold. Additionally, an interesting observation was made that unknown secondary metabolite (949 *m/z*) eluting at 6.98 min was significantly increased only in IL11-2 that showed significant accumulation of drought stress marker metabolites (described in previous section).

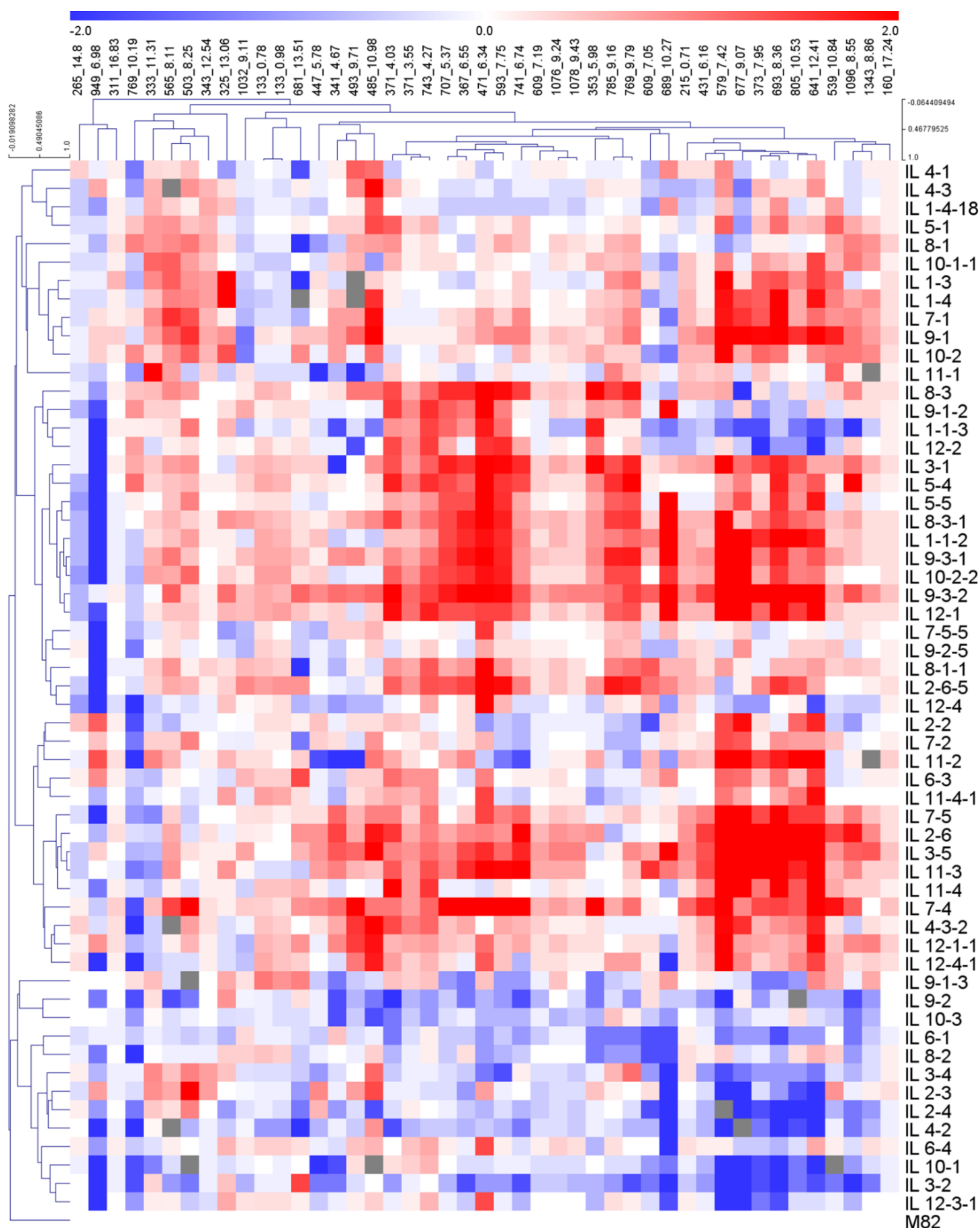


Figure 4.4 Heat map of secondary metabolite profiles of ILs under drought stress. Each square represents the effect of chromosomal segment substitution on the amount of every metabolite using a false-color scale. Regions of red or blue indicate that the metabolite content is increased or decreased, respectively, after the introgression of *S. pennellii* segments. X-axis indicates retention times. Y-axis indicates 57 ILs.

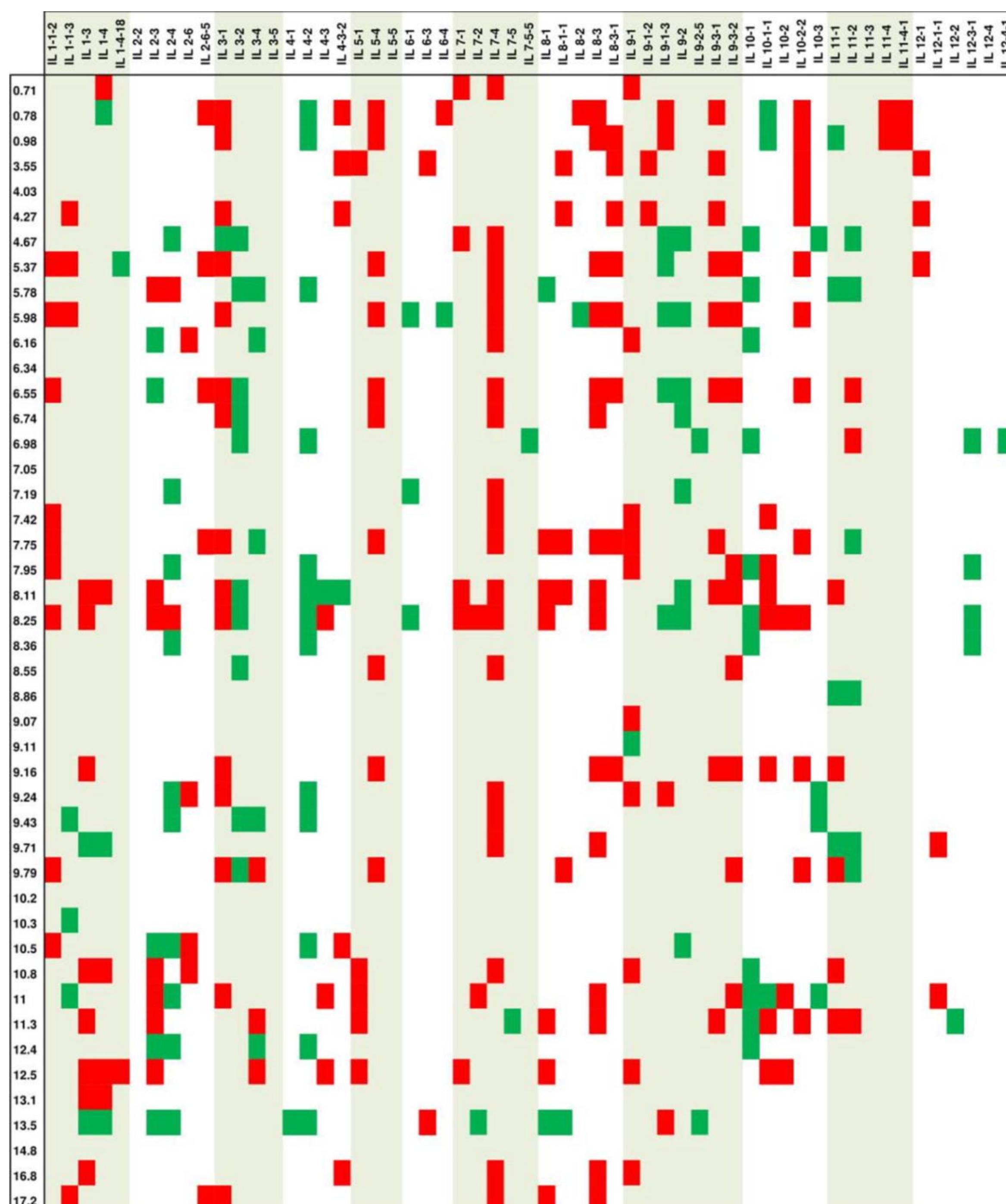


Figure 4.5 Genetic architecture of secondary metabolites QTL under drought stress across the 57 tomato introgression lines. Shown are the distribution and number of QTL across the tomato genome at the significance level of p≤ 0.01. Regions of red or green indicate that the metabolite content is significantly increased or decreased, respectively compared to M82. X-axis indicates 57 ILs according to their chromosome number. Light green colour is used for differentiating the chromosomes. Y-axis indicates retention times.

It has been well documented that polyphenolic and flavonoid content increases under drought stress in many plant species (Savoi et al., 2016; Chaves et al., 2010; Ramakrishna and Ravishankar 2011). After having close look on my mQTLs data, I found levels of chlorogenic acid and rutin were significantly accumulated in IL3-1. Additionally, I found that the mass spectra of unknown compound (*m/z* = 503) was significantly affected in overlapping ILs 3-1 and 3-2; and 10-1 and 10-1-1. Moreover, I found that galactinol was significantly decreased in ILs 3-1 and 10-1-1. Therefore, ILs 3-1 and 10-1-1 were further used for fine mapping of candidate genes related to drought response.

4.3.4 Fine mapping of candidate genes from mQTL for secondary metabolites

I found that *S. pennellii* genomic region of IL3-1, excluding region that overlapped with IL3-2 contained 179 predictable genes. These genes include 26 annotated as unknown proteins (Figure 4.6B). With the help of arabidopsis ortholog search and eFP browser, 31 genes were detected as responsive to only drought stress. Among them 2 were JA responsive, 2 involved in lignification, 3 involved in root development and 2 were lipase. Lastly, 5 candidate genes involved in drought tolerance could be finalized by using the protocol mentioned in the methods section. Arabidoopsis orthologs of candidates Solyc03g005570 (Myb TF) and Solyc03g006700 were characterized as MYB15 and PRX52 respectively, and were shown to be involved in lignification (Chezem et al., 2017; Fernández-Pérez et al., 2014). These candidates are important because of the finding that has shown positive correlation between lignin content in leaves of inbred maize lines and their drought tolerance (Hu et al., 2009).

Same protocol was followed for filtering candidates from IL10-1-1. I could able to select 3 candidates with gene ID Solyc10g005480, Solyc10g005490 and Solyc10g005510. Arabidopsis orthologs of these 3 candidates were characterized as playing role in abiotic stress response, thermotolerance memory and root development respectively.

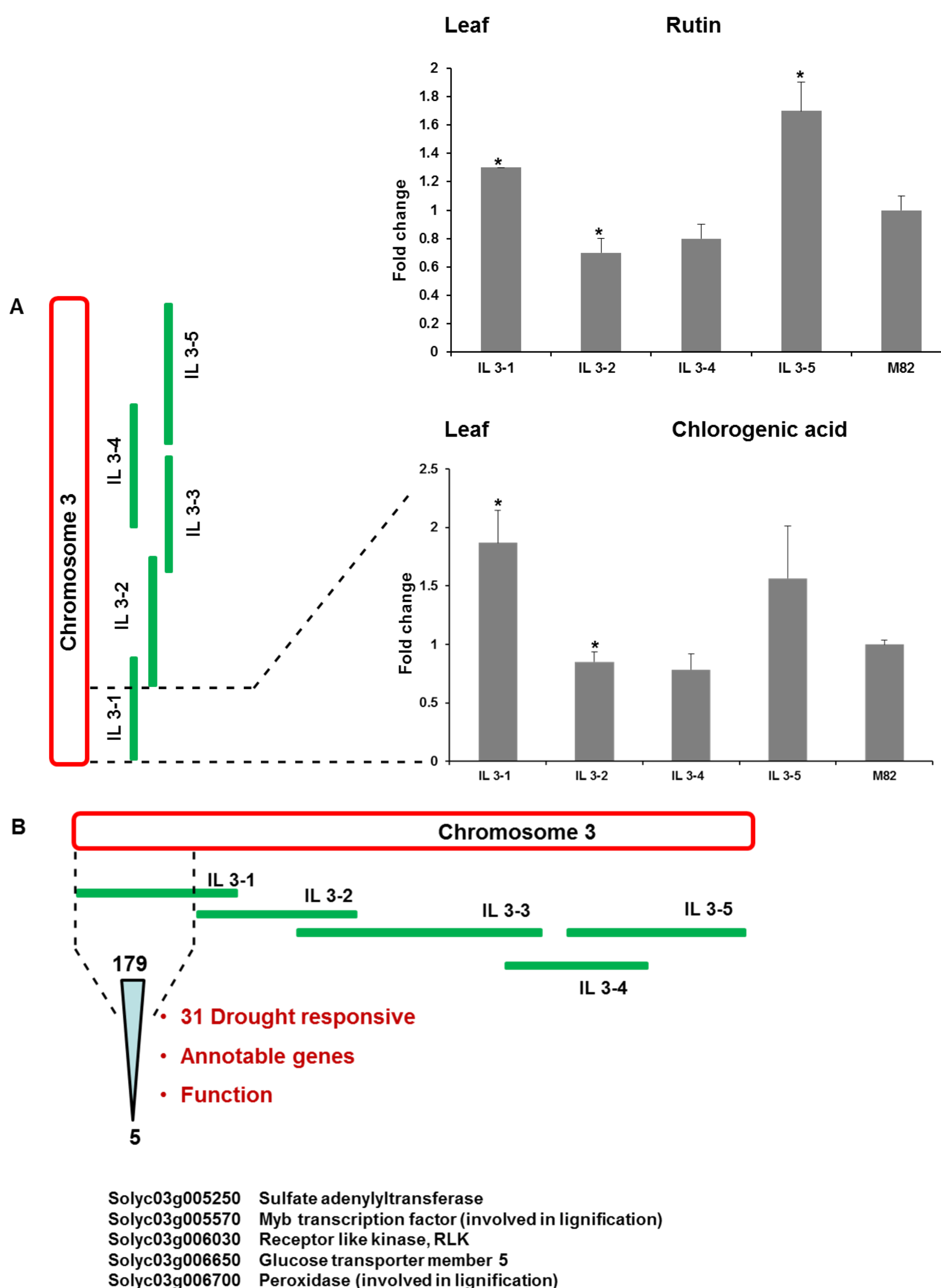


Figure 4.6 Schematic representation of *S. pennellii* ILs for chromosome 3. A) The introgressed fragments in ILs are shown on the right of the chromosomes. Rutin and Chlorogenic acid content was calculated as fold-change of M82. Data represent means of fold change ± SE from five independent biological replicates. Columns marked with an asterisk indicate statistically significant changes, as determined by Student's t-test (P<0.05). B) Fine mapping of drought stress related mQTL. 5 key drought responsive genes were filtered from total 179 candidates using 3 filters. Filters are shown in dark red.

4.4 Discussion

Increasing world population and climate change led increase in drought events poses major threats to food security in this era. By 2050 the world's population will reach 9.1 billion, 34 percent higher than today (FAO 2009). Therefore in order to meet increasing food demand, in recent years, attempts have been increased to develop drought tolerant crop varieties. Studies involving mutants, transgenics as well as genetic variation apparent in wild and some of drought tolerant species, have helped to identify several drought responsive QTLs and unravel molecular mechanism underlying drought tolerance (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al., 1997; Gur and Zamir 2004; Xue and Loveridge 2004; Chen et al., 2011). But nowadays number of studies has taken the advantage of recently developed next generation sequencing techniques and high throughput metabolomics platforms to address key questions of plant biology. For example, eQTL mapping in 76 *S. pennellii* ILs have deciphered several genetic hot spots regulating a large number of transcript level patterns relating to diverse biological processes such as plant defense and photosynthesis (Ranjan et al., 2016). mQTL mapping on the same population have identified 679 mQTLs and candidate genes involved in secondary metabolism in tomato fruit pericarp (Alseekh et al., 2015). These results therefore demonstrate that strength in eQTL and mQTL mapping using *S. pennellii* ILs can be exploited to understand drought tolerance mechanism.

In the current study, I attempted to map drought stress responsive mQTLs in young tomato leaf, by using population of 57 *S. pennellii* ILs. Total 806 and 344 mQTLs were identified for primary (88 traits) and secondary metabolism (45 traits) respectively, at stringent threshold. Interestingly, I find large number of positive mQTLs (226) than negative mQTLs (117) for secondary metabolism. This trend was exactly opposite in case of those for primary metabolism. This result is in line with the fact that *S. pennellii* is a desert adapted species and accumulates diverse secondary metabolites (Ronene et al., 2000; Fernandez-Moreno et al., 2017). On average I observed 10.46 mQTL per primary metabolite family. Maximum fold change in metabolite abundance across all lines was observed for secondary metabolite than that for primary metabolite.

Since random residual variance among ILs can help us to track down the genetic influence of developmental instability (Wu et al., 2007), I next calculated percentage

of coefficient of variation (CV) across the ILs for both primary as well as secondary metabolites. The distribution of the trait values was slightly wide for both the primary as well as secondary metabolites, across all genotypes; with CV ranging between 0.5-0.1 for majority of the metabolites (Figure 4.7).

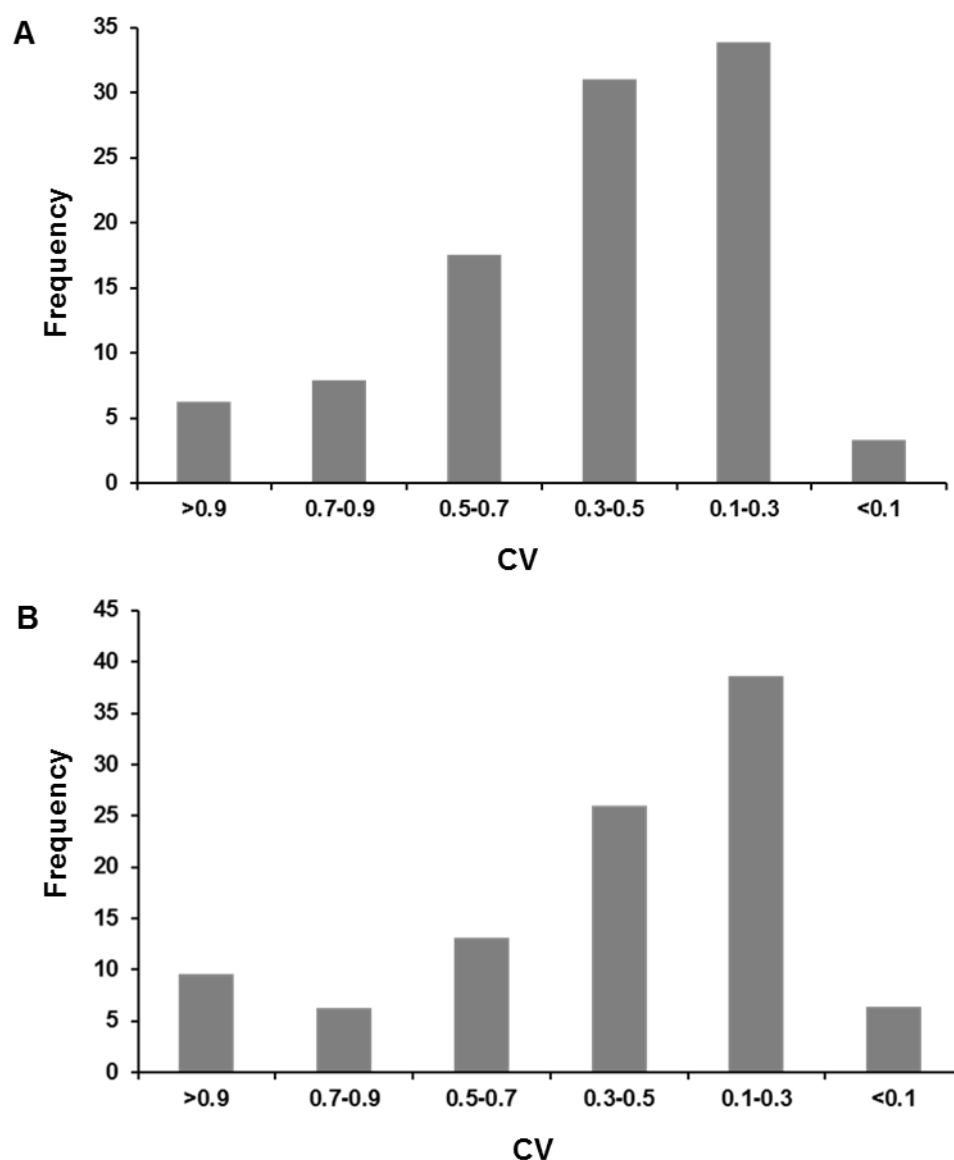


Figure 4.7 Histogram of the distribution of the primary (A) and secondary (B) metabolic variation. Data shown are percentage of CV across the ILs for all identified metabolites.

IL12-2 showed highest CV of 2.17 for homoserine while IL8-3-1 showed lowest CV of 0.02 for drought stress marker metabolite galactinol. Upon checking CV for secondary metabolites data, I found that IL2-2 showed highest CV of 2.23 for rutin while IL1-1-3 showed lowest CV of 0.021 for unknown metabolite (*m/z* 160) eluting at 17.24 min.

4.4.1 Identification of drought tolerance candidate genes

Previous studies have shown that GABA accumulates in high amount not only under abiotic but also biotic stress (Bown and Shelp 1997). Therefore, it is difficult to use GABA as a drought specific marker. Hence, requirement of new drought specific markers has been discussed in the review by Selmar and Kleinwächter (2013). Abiotic stresses such as drought induces ROS production in plant cells (Bowler et al., 1992; Foyer et al., 1994). It has been shown that proline plays a vital role in cell osmotic adjustment as well as during dehydration (Ibarra-caballero et al., 1988). Additionally, with the help of transgenic approach, it has been reported that raffinose and galactinol impart tolerance to drought stress (Taji et al., 2002). Besides these 3 marker metabolites, recent work by Ullah et al., (2017) has found significant accumulation of succinate in drought tolerant cultivar of wheat. Significant accumulation of proline, raffinose, galactinol, putrescine and succinate in IL11-2 allowed me to identify it as an IL that might harbour QTL specific to drought tolerance. Presence of 6 wax synthases and 27 drought responsive genes in IL11-2 strengthened my mQTL data analysis. Furthermore, 6 candidate genes were finalized and will be validated by transgenic approach. Interestingly, arabidopsis ortholog of one of those candidates (Myb TF) is characterized as KUA1 that modulates leaf cell expansion and final organ size by controlling ROS homeostasis.

After checking the shoot weight data, I found that shoot weights for ILs 7-5 and 8-3-1 were not affected. Moreover, I noticed that only these 2 ILs showed highest number of significantly increased primary metabolite mQTLs. Following this interest, total 126 genes were found on *S. pennellii* genomic sequence in IL8-3-1. Arabidopsis ortholog search and efp browser helped me to decipher 16 candidates as drought responsive. Among them, 3 candidates with gene ID Solyc08g083170, Solyc08g082560 and Solyc08g082820 were found to encode bHLH TF, f box protein and heat shock proteins 70 respectively. Next, I constructed phylogenetic tree (not shown here) by using all the *A. thaliana* bHLH TFs and *S. lycopersicum* bHLH TFs. Interestingly, I found that arabidopsis bHLH TF with gene ID AT1G05805 was closest ortholog for candidate Solyc08g083170. AT1G05805 was characterized as AKS2 (ABA-RESPONSIVE KINASE SUBSTRATE 2). Previous study has demonstrated that in order to prevent water loss under drought stress, ABA phosphorylates AKS2 and closes stomata (Takahashi et al., 2013). Therefore, this

TF candidate Solyc08g083170 (SlbHLH) and previously mentioned Myb TF are promising candidates that can be used for further analysis.

Similar observations were found for IL3-1 that has exhibited significant accumulation of chlorogenic acid and rutin. Previous reports have implicated sulfur use efficiency and lignification processes in drought stress tolerance (Lee et al., 2016; Hu et al., 2009). The fact that IL3-1 harbours candidates involved in root development, lignification and sulfur use efficiency strengthens my candidate gene selection method.

4.4.2 Secondary metabolites accumulates under drought stress

Water shortage led stomatal closure significantly decreases uptake of CO_2 by plant and therefore causes over accumulation of reduction equivalents. Previous reports have well proven that this metabolic event in turn results into induction of biosynthetic pathways of reduced compounds, such as isoprenoids, terpenes, phenols or alkaloids (Selmar and Kleinwächter 2013, Gray et al., 2003; de Abreu and Mazzafera 2005; Singh-Sangwan et al., 1994; Jaleel et al. 2007; Schreiner et al., 2009). For example, de Abreu and Mazzafera (2005) observed strong increase in xanthones, betulinic acid and polyphenolic content in *Hypericum brasiliense* under drought stress. Therefore, I attempted to understand the genotypic plasticity of primary and secondary metabolite accumulation.

Interestingly, obtained results showed genotypic plasticity of 57 *S. pennellii* ILs for significantly decreasing the primary metabolite content and significantly increasing the secondary metabolite content under drought stress (Figure 4.8B and C). Such genotypic plasticity had also been evident from recent report that had documented accumulation of several secondary metabolites in 100 barley RILs population under drought stress (Piasecka et al., 2016).

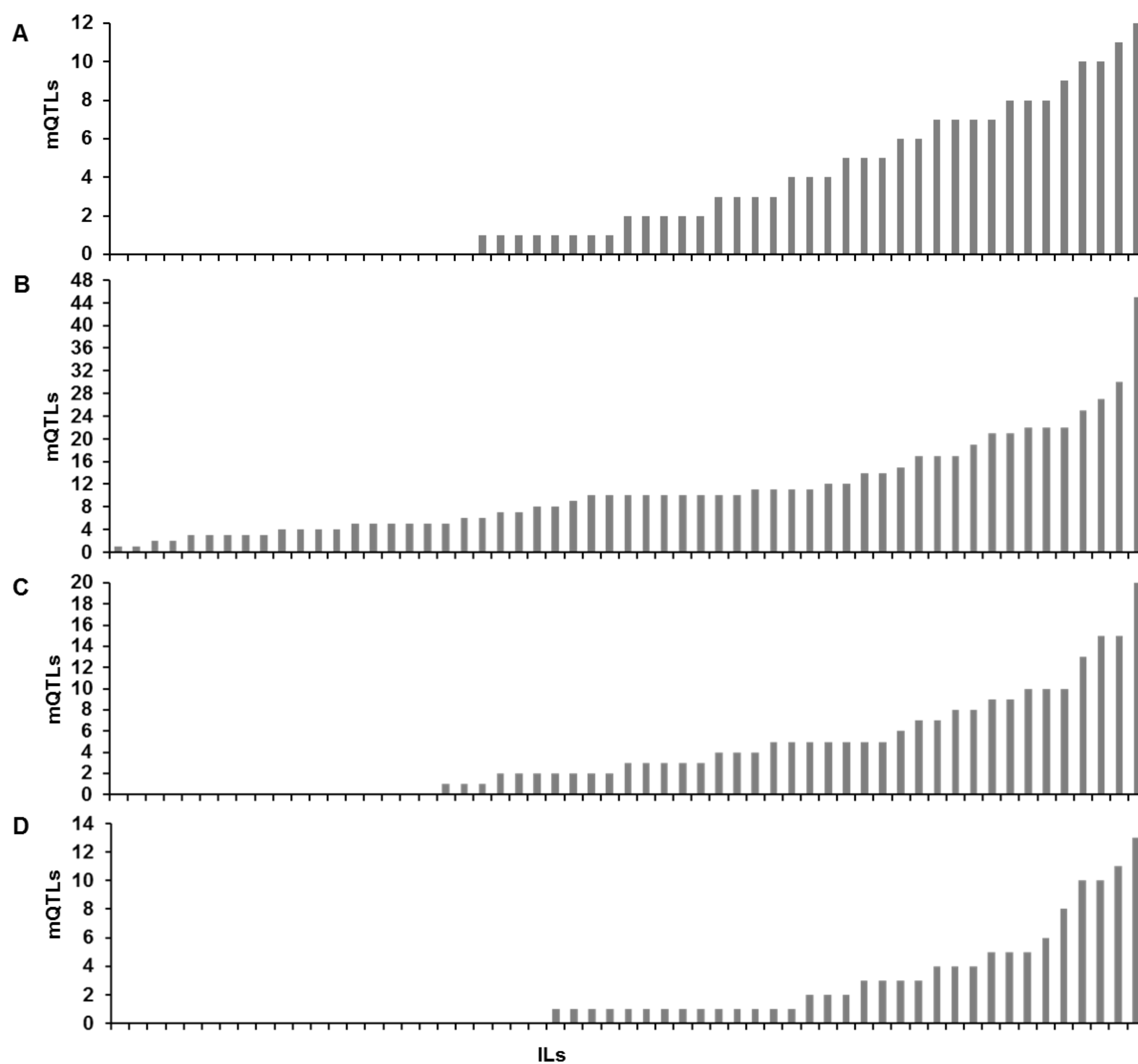


Figure 4.8 Histogram of the distribution of significantly increasing (A) and decreasing (B) primary as well as significantly increasing (C) and decreasing (D) secondary metabolite mQTLs. Data shown are number of mQTLs per IL.

Plants are sessile in nature and therefore constantly face fluctuations in their environment. Among other abiotic stresses, drought stress is the most common adverse environmental condition. In order to survive under drought stress, plants devise various survival mechanisms. Nowadays, new approaches are being developed to understand drought tolerance mechanisms developed by plants. For example, mutant, transgenic, QTL, META QTL, eQTL, mQTL and GWAS approaches have been developed (Baureima et al., 2012; Liang 2016; Lanceras et al., 2004; Wehner et al., 2016; Templer et al., 2017; Wang and Qin 2017). Plant acquisition of water and productivity under drought has been shown to be improved by various root traits (Wasson et al., 2012). Recently it has been found that genotype with higher sulphur use efficiency is more tolerant to polyethylene glycol induced drought stress (Lee et al., 2016). Additionally, lignification has also been implicated in drought stress tolerance (Lee et al., 2007; Yan et al., 2018). In this study, I have shown that candidate genes involved in ROS homeostasis, root development, lignification and sulphur use efficiency are localized to the ILs (11-2 and 3-1) that have been identified by mQTL approach. In summary, these well characterized physiological and biochemical mechanisms underlying drought stress tolerance supports my candidate gene selection method. In future, we will also fine map genes using new *S.pennellii* (LA716) based population of BILs, ortholog coexpression as well as promoter and eQTL analysis. Further experiments involving reverse genetic approaches are required to validate and confirm these results. This might strengthen our confidence on use of mQTL approach in unravelling drought tolerance mechanism.

Chapter 5. General Discussion

As tomato fruit develops, chloroplasts differentiate into chromoplasts. This physiological transition is also accompanied by metabolic transition i.e. from partially photosynthetic to fully heterotrophic metabolism (Klee and Giovannoni, 2011). This transition process as well as processes of tomato fruit softening and accumulation of several secondary metabolites are regulated by primary hormone, ethylene (Seymour et al., 2002, 2013; Uluisik et al., 2016). These characteristics makes tomato fruit a preeminent model for secondary metabolomics and therefore have been studied very deeply at genomic, transcriptomic, metabolomic and proteomic level very well. Previous studies have well characterized various genes involved in tomato fruit development and ripening. For example, TFs TAGL1, FUL1, FUL2, RIN, HB1 and ZFP2 have been shown to regulate tomato fruit ripening under ethylene response. SlMYB12, which regulates flavonoid biosynthesis and SlAREB1 that control primary metabolism in tomato fruit have also been identified (Ballester et al., 2010; Bastias et al., 2014). Moreover, published studies have also shown that leaf tissues of some of wild species of tomato synthesize various secondary metabolites that impart biotic or abiotic stress resistance to those species (Schilmiller et al., 2015; Rigano et al., 2014). Additionally, population of 76 *S. pennellii* ILs has been exploited to identify eQTLs and mQTLs underlying metabolic and developmental regulation. In this thesis, in order to unravel metabolic regulation and key genes of tomato secondary metabolism, I have used 3 different approaches as transgenic, eQTL and TF and lastly mQTL approach. Here, chapter 2, 3 and 4 discuss tomato metabolomics and how it is influenced by developmental, genetical and environmental factors. Obtained results are discussed within a broader context.

5.1 KARI might be a rate limiting enyme for iC5 acyl-sucrose synthesis in young tomato leaf

Most of the enzymes of BCAAs biosynthetic pathway are regulated allosterically (TD, ALS and IPMS) (Grant, 2006; Binder 2010). It has also been shown that metabolic flux through BCAAs catabolism does not result in larger effects on BCAAs pools and volatile synthesis (Kochevenko et al., 2012) but instead support respiration in young tomato fruits. In chapter 2, I attempted to identify metabolic linkage between leucine (primary metabolism) and secondary metabolites. Obtained results show that KARI

might be a rate limiting enzyme for iC5 acyl-sucrose synthesis in young leaf. Interestingly, this is not the case for red ripe fruit tissue because of lower photorespiration activity and more availability of NADPH. This is also supported by levels of glycine that were increased in antisense lines over that of WT, in red ripe fruit tissues but not in young leaf. Besides environmental and genetical factors, plant metabolism is under strict control of developmental factors. Here, developmental regulation of iC5 acyl-sucrose synthesis is evidenced by inability of red ripe fruits to support photorespiration. However, one should be aware of the fact that photorespiration also plays a positive role in plant defense signaling (Wu et al., 1997). Further experiments involving expression profiling of photorespiratory pathway genes in WT and antisense lines by quantitative real-time PCR, will be carried out.

Besides this physiological finding, transcript analysis of 2 isoforms of DHAD suggests that these isoforms might be functionally different; and therefore this characteristic might have resulted in only DHAD lines having significantly changed levels of 3-methylbutanal, iC5 acyl-sucrose and α-tomatine in red ripe tomato fruits. Additionally, results also showed that increased flux for synthesis of upstream phenylpropanoid pathway specific flavor volatiles in red ripe pericarp tissue might have had negative effect on rutin content in those tissues of the antisense lines, as per the prediction by Tieman et al., (2006).

5.2 eQTL approach unravels metabolic regulation in tomato

Day by day number of species, whose genome has been decoded, is increasing. With that speed, understanding or validating genotype-phenotype associations is a challenging task. Simultaneously, several efforts mainly helped by next generation sequencing and metabolomics are also unraveling regulatory complexity underlying development. Published studies have proved that understanding biological networks plays vital role in unraveling such complexity (Persson et al., 2005; Fukushima et al., 2012; Mutwil et al., 2011; Proost and Mutwil 2018). There are several such biological networks in living beings. Some of them are normal and some of them can be induced upon stress. For example, pattern of coexpression of particular gene with specific gene/genes, across different tissues (differential coexpression correlation) (Fukushima et al., 2012) and correlation between gene expression and metabolite abundance in specific genotypes. Furthermore, these bionetworks can also be

studied deeply, by combining correlation and clustering analysis (Urbanczyk-Wochniak et al., 2003; Hoefgen and Nikiforova 2008). One such study attempted to combine transcriptome and metabolome data by principal component analysis and batch-learning self-organizing map (BL-SOM) analysis; and found coordinated modulation of the genes and metabolites involved in glucosinolate metabolism (Hirai et al., 2004). This was the first attempt of its kind in this area of plant secondary metabolomics and remained the inspiration for later studies. Next, transcript to metabolite correlation analysis helped Carrari et al., (2006) to decipher novel associations between ripening-associated transcripts and TCA-cycle metabolites such as organic acids and sugar phosphates, underlining the importance of the TCA cycle during tomato fruit development. In tomato, first attempt of deciphering fruit gene regulatory network was carried out by Pan et al., (2013); by using artificial neural network inference analysis and TF gene expression profiles derived from fruits sampled at various points during development and ripening. This study successfully identified an APRR2-like gene linked to pigment accumulation in tomato and pepper fruits.

Multiple research studies have suggested that eQTLs and mQTLs can also be used as components, to construct or study bionetworks (Keurentjes et al., 2007; Itkin et al., 2013; Alseekh et al., 2015; Ranjan et al., 2016; Wu et al., 2018). One such attempt has been carried out in arabidopsis by combining eQTL mapping and regulator candidate gene selection. This approach has successfully revealed clusters of coregulated genes and their most likely regulators (Keurentjes et al., 2007). One of the recent studies by Alseekh et al., (2015) have taken advantage of mQTLs as well as metabolic networks and successfully identified candidates involved in secondary metabolism in tomato. Here, in this thesis, I have exploited the ability of both of these components (eQTLs and mQTLs) to unravel metabolic regulation underlying tomato secondary metabolism.

Tomato fruit ripening process involves coordinated activation of multiple genetic and biochemical pathways. Specific eQTL, differential coexpression correlation and VIGS analysis helped me to predict that SlWD40 could be regulating lycopene biosynthesis. Phenotyping of fruit specific amiRNA and overexpression lines clearly showed that TF SlWD40 indeed regulates tomato fruit development and ripening. Metabolite profiling on transgenics suggested that SlWD40 TF tightly controls primary metabolism (mainly amino acid metabolism) and have fruit

developmental stage specific effects. For example, synthesis of valine, glutamine and lysine were significantly increased in 2 cm fruits of overexpression lines. Additionally, overexpression of SlWD40 TF negatively affected malate content at red ripe stage. This is a similar metabolic change in fumarase antisense lines obtained by Centeno et al., (2011). Here, authors found that this was due to alterations in the activation state of AGPase, caused by alterations in the redox status of the plastid. Future experiments involving SlWD40 protein sequence, promoter and coexpression analysis might help in finding putative SlWD40 interacting partners.

Based on characteristic of induced expression under biotic stress and correlation between specific eQTL and mQTL for leucine, 2 candidates were selected and successfully tested. SlADH among them was found to be ic5 acyl-sucrose pathway specific and might catalyze 3-methylbutanal conversion into 3-methylbutanoic acid or 3-methylbutanol. Altered 3-methylbutanal production in overexpression lines clearly showed metabolic linkage between leucine catabolism and synthesis of branched chain volatiles and iC5 acyl-sugars (Figure 3.17 and 3.18). Here, SlADH overexpression in tobacco might have some commercial implications with respect to content of acyl-sugars.

Another candidate *S. pennellii* allele SopenCYP450 was found to exponentially upregulate metabolic flux for iC5 acyl-sucrose synthesis in overexpression lines. *S. lycopersicum* homolog of this CYP450 protein is presently annotated as amorpha-4,11-diene 12-monooxygenase. It has also been found that microsomal CYP450 monooxygenases carry out epoxidation and oxygenation reactions in volatiles synthesis pathways (May 1979, Hallahan et al., 1992 and 1994). Therefore, volatiles analysis in these SopenCYP450 overexpression lines might shade light on exact function of this SopenCYP450. Based on results from Sherloc2, a protein localization prediction tool, *S. lycopersicum* homolog of this CYP450 protein might not be localized to endoplasmic reticulum and therefore might be inactive or less active. This also explains the inability of *S. lycopersicum* to synthesize acyl-sugars in large amounts. Future study involving development of plants that constitutively express SopenCYP450 (and also SlCYP450 separately) fused to green fluorescent protein, might confirm the protein localization. Volatile analysis and SopenCYP450 protein production in yeast, might also give some hints regarding mechanisms underlying increased metabolic flux.

Besides above mentioned results, VIGS for candidates Solyc07g052700

(MADS box TF) and Solyc03g044460 (bHLH TF) showed green and yellow phenotypes respectively. Various studies have implicated MADS box TFs as key genes in regulating tomato fruit ripening (Martel et al., 2011; Shima et al., 2014). Moreover, arabidopsis ortholog (AT1G66470) for candidate Solyc03g044460 (bHLH TF) have been shown to be interacting with EIN3/EIL1, a well known ethylene-stabilized TFs (Feng et al., 2017). Therefore, both of these MADS box and bHLH TFs can be a promising candidates and will be validated by transgenic approach.

Collectively, these results demonstrate that transcriptomics combined with metabolomics is able to identify genes that might have clear impact on fruit quality.

5.3 Drought stress responsive mQTLs and potential candidates

Environmental factors influence gene expression and in turn change metabolite profile of cell. In this study, population of 57 *S. pennellii* ILs has been exploited to map drought stress responsive mQTLs in young tomato leaf. Highest number of mQTLs (806) were identified for primary metabolism (88 traits) than secondary metabolism (45 traits, 344 mQTLs), at stringent threshold. Close inspection of significantly affected metabolites data showed plasticity of secondary metabolite accumulation under drought stress. Analysis of CV on this metabolite data revealed that highest and lowest CV among primary metabolites was for homoserine and drought stress marker metabolite galactinol respectively. Highest CV among secondary metabolites was found for rutin.

Additionally, co-localization of drought stress responsive positive mQTLs and well characterized candidates in similiar ILs, led to identification of new potential candidates. For example, IL11-2 that showed positive mQTLs for drought stress marker metabolites harboured 6 wax synthases and 27 drought responsive genes. Additionally, I found that arabidopsis ortholog of one of the potential candidate was characterized as KUA1, involved in ROS homeostasis.

Based on previous studies (Savoi et al., 2016; Chaves et al., 2010; Ramakrishna and Ravishankar 2011), chlorogenic acid and rutin were used as drought responsive marker metabolites in tomato. I found that these marker metabolites were significantly changed in IL3-1. Previous studies have implicated pathways involved in lignification, root development and cuticular wax loading in drought stress tolerance (Fu et al., 2017; Lee et al., 2007; Sánchez et al., 2001). The

fact that IL3-1 harboured candidates related to lignification, root development and lipid metabolism supports candidate gene selection method. In future, experiments involving use of new *S.pennellii* (LA716) based population of BILs, ortholog coexpression as well as promoter and eQTL analysis will be carried out to fine map more drought responsive candidate genes. Validation of these candidates by using reverse genetic approaches might shed light on drought tolerance mechanisms developed by *S. pennellii*.

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Supplementary information - Chapter 2 and 3

Supplementary information is provided on CD.