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Characterization of molecular functions of polyamines in fruit development and ripening in tomato (Solanum lycopersicum)

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CHARACTERIZATION OF MOLECULAR FUNCTIONS OF POLYAMINES IN FRUIT DEVELOPMENT AND RIPENING IN TOMATO (*Solanum lycopersicum*)

A Dissertation

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of

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of

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To my wife

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LIST OF ABBREVIATIONS

ABA, abscisic acid

ADC, arginine decarboxylase

AHC, agglomerative hierarchical clustering

B, color breaker stage

CDK, cyclin-dependent kinase

CuAO, Cu-containing diamine oxidase

CYC, cyclin (involved in cell cycle progression)

DAB, days after breaker stage

DAP, days after pollination

DAR, days after red stage

DBP, days before pollination

G, mature green stage

GABA, γ-aminobutyric acid

ODC, ornithine decarboxylase

P, turning pink stage

PA(s), polyamine(s)

PAO, polyamine oxidase

PCA, principal component analysis

PUT, putrescine

R, red stage

SA, salicylic acid

SAM, *S*-adenosylmethionine

SAMdc, *S*-adenosylmethionine decarboxylase

SPD, spermidine

SpdSyn, spermidine synthase

SPM, spermine

SpmSyn, spermine synthase

TSS, total soluble solids

WT, wild-type

ABSTRACT

Anwar, Raheel. Ph.D., Purdue University, December 2014. Characterization of Molecular Functions of Polyamines in Fruit Development and Ripening in Tomato (*Solanum lycopersicum*). Major Professor: Avtar K. Handa.

Putrescine (PUT), spermidine (SPD) and spermine (SPM) are three major polyamines (PAs) present in all living organisms. These biogenic amines have been implicated in diverse plant growth and development processes, including seed germination, tissue lignification, organogenesis, flowering, pollination, embryogenesis, fruit development, ripening, abscission, senescence, and stress responses. To elucidate molecular roles of PAs in fruit development and ripening, I characterized transgenic tomato plants ectopically expressing yeast spermidine synthase (*ySpdSyn*) or *S*-adenosylmethionine decarboxylase (*ySAMdc*) under constitutive CaMV 35S and/or fruit-specific SlE8 promoters. The *ySpdSyn*-expression enhanced PUT, SPD and SPM level in floral buds and fertilized developing ovaries by 2- to 3-fold compared to WT tissues with majority being sequestered as bound forms. Higher PA levels altered fruit shape of transgenic tomatoes to more obovoid than WT by regulating expression of fruit shape genes (*SUN1* and *OVATE)*, and cell division and expansion genes (*CDKB2, CYCB2, KRP1* and *CCS52B).*

Characterization of PA homeostasis during fruit growth and ripening revealed a strong correlation of conjugated PAs with transcripts abundance of PA biosynthesis (*ODC*, *ADC*, *SAMdc3*) and catabolizing genes (*CuAO-like, PAO4-like*) and the bound PAs to transcript levels of *ySpdSyn* and *SAMdc2* suggesting a significant metabolic inter-conversion among the various forms of PAs. Co-expression of *ySpdSyn* and *ySAMdc*

transgenes showed that SAMdc is the rate limiting step in biosynthesis of higher PAs with potential to alter PA homeostasis in fruit tissues.

Characterization of *ySpdSyn* and *ySAMdc* transgenic and WT fruits showed that expression of transgenes was associated with higher firmness of ripened fruits both on-planta and after harvest up to 17 days after ripe stage. Free SPD/SPM levels were positively correlated with fruit firmness, accumulation of total solids and delay in fruit shriveling and inversely correlated with fresh fruit weight, juice pH and seed number in tomato fruits. Free PUT levels exhibited trends opposite to that seen with SPD/SPM confirming hypothesis that PUT and SPD/SPM ratios play significant roles in the outcome of biological functions of PAs. Evaluation of *ySpdSyn* lines under field conditions showed 50% increase in fruit yield per plant due to continued fruit set until late in the season and up to 60% increase in fruit fresh and dry weight much beyond the fruit breaker stage.

The metabolomic changes in transgenic fruits were determined using the nuclear magnetic resonance spectroscopy (^{1}H NMR) and compared to WT fruit metabolic profile during on-planta fruit ripening and post-ripening stages. Free SPD levels were positively correlated with Ile, Val, Glu, Gln, Trp, malate, citrate and trigonelline. The levels of Ala, Glu, Asp and UDP-NAcGLU were negatively correlated with free SPD levels but positively correlated with free PUT indicating differential function of these two PAs. Levels of fructose and AMP were also negatively correlated with free SPD. Conjugated and bound PAs exhibited a limited correlation with metabolome profiles. The node-edge network analyses among PAs, metabolites and their associated pathways showed that PAs upregulate many anabolic pathways, but negatively affect glycolysis, starch and sugar metabolism, and zeatin biosynthesis. Taken together these results indicate that SPD is associated with enhancing many metabolic pathways and delaying senescence-related processes leading to improved postharvest fruit quality. I have collated transcriptome of transgenic plants and mutants with altered PA levels. Its analyses revealed complex and differential relationships among PUT, SPD and SPM in regard to regulation of plant hormone biosynthesis and signaling.

In summary, the use of transgenic plants with modified PA levels provide an insight into molecular functions of PAs in altering fruit architecture, improving fruit quality attributes, increasing fruit production and delaying ripening-related changes in tomatoes. Limited transcriptome profile suggest a complex crosstalk between PAs and plant growth hormones during fruit ripening. Metabolome profiles of transgenic fruits showed a significant impact of PAs on fruit quality improvement by restoring metabolic pathways during fruit ripening.

CHAPTER 1. INTRODUCTION

Fruit and vegetable crops are the dietary sources of vitamins, antioxidants and minerals and have the potential to ameliorate not only physiological disorders but also decrease incidence of human diseases such as cancer. Consequently, consumption of fruits and vegetables has increased in recent years, further increasing their global demand (FAOSTAT, 2012). Consumers expect good quality fruit to be flavorful, succulent, juicy and nutritional in addition to attractive size and appearance (Shewfelt, 1999). Other consumer-desirable characteristics of fruits include crispness, chewiness and oiliness. But, for the fruit handler, shipper and retailer the desirable fruit quality attributes include less proneness to handling and shipping damages, slow softening during storage and longer shelf life without affecting consumer appeal (Shewfelt, 1999). Fruit processors consider better quality fruit to have high solids, appropriate rheological properties, tolerance to mechanical processing including during peeling or crushing, and prolonged maintenance of the processed products during marketing. A recent trend towards the organic farming adds another desirable parameter to fruit quality (Lind et al., 2003; Reich, 2012). Enhanced phytonutrient levels add to the overall quality of fruit crops (Mattoo, 2014; Mattoo et al., 2010b), although consumers expect fruits at the same time to be free of unfavorable chemicals such as cyanogenic glucosides, oxalates, heavy metals, dioxane and pesticides, and contaminations due to microbes.

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Following domestication of crop plants, the traditional breeding approaches have extensively improved certain qualities of horticultural crops. In the last three decades, several new tools, especially quantitative trait locus (QTL) mapping, have allowed identification of regions of genome associated with particular phenotypic traits (Causse et al., 2007; Grandillo et al., 1999; Seymour et al., 2002). Genomic tools such as chromosome walking, DNA sequencing and bioinformatics have further facilitated isolation, identification and characterization of the genomic regions controlling fruit quality parameters. Also, the understanding of molecular basis of impaired ripening in different *Solanum lycopersicum* (tomato) mutants has added to our knowledge on the regulatory mechanisms underlying fruit ripening process (Giovannoni, 2004). Molecular genetics has provided many additional tool kits that have enhanced the molecular engineering of crop plants. These include plant transformation methods that made candidate gene approach a reality to test phenotypic role of a particular gene by altering its expression during plant growth and development (Fatima et al., 2009). The gain of function (ectopic overexpression) or loss of function (repression by antisense RNA, or RNAi) approaches have made it possible to characterize the phenotypes associated with a single gene and its potential to regulate desirable phenotype in crop plants. This chapter summarizes some of the progress made using these tools to enhance fruit quality attributes.

Fruits being derived from different parts of a flower including inflorescence have enormous diversity in their structure and physiological functions (Handa et al., 2012). Since development, maturation and ripening of diverse classes of fruits differ significantly, it is a challenge to improve quality attributes of a chosen fruit by biotechnology. Nonetheless, many biochemical and regulatory mechanisms impacting quality of fruits during ripening are similar, therefore it is possible to genetically alter ripening and/or slow down deterioration to enhance fruit quality. Ethylene is a gaseous plant hormone decidedly integral to fruit ripening, especially in fruit types that have a burst of respiration during ripening and classified as climacteric fruits (Abeles et al., 1992; Mattoo and Suttle, 1991). The elucidation of its biosynthesis and perception has eased biotechnological

strategies to regulate ripening and senescence processes in plants. Thus, regulation of both production and perception of ethylene in fruit crops via molecular engineering have led to remarkable effects on various aspects of fruit quality (Klee and Giovannoni, 2011; Lin et al., 2009). Molecular engineering of a number of fruit crops including apple, banana, berries, citrus, cucumber, grape, melon, potato, eggplant and tomato is a subject of research in many laboratories world over. Tomato has become a model fruit crop of choice to elucidate role of various genes in fruit quality (Fatima et al., 2009; Giovannoni, 2007; Klee and Giovannoni, 2011). Here, we have primarily focused on the molecular engineering of shape, size, texture, phytonutrient levels and volatiles in tomato fruit and also reference genetic engineering studies in other fruit crops.

1.1 Molecular engineering of fruit appearance

Fruit size and shape are attributes quantitatively inherited and determine yield and consumer appeal in most fruit crops. These attributes were given considerable attention during domestication and selection of new fruit cultivars (Rodríguez et al., 2011c). During domestication, small fruited wild type *S. pimpinellifolium* was developed to larger fruit varieties such as Giant Heirloom, in the process the fruit mass of 1-2 g per fruit was increased to over 1000 g per fruit and locule numbers from 2 to more than 10 (Lippman and Tanksley, 2001). Other fruit species were also bred to similar increase in size during domestication of their wild progenitors (Smartt and Simmonds, 1995). The application of molecular marker and high-resolution fine mapping approaches made it possible to identify quantitative trait loci (QTLs) and genes encoded within these loci affecting fruit size and shape. In tomato alone, over 30 QTLs have been identified, however 10 of them contributing to most of the observed phenotypic variation (Doganlar et al., 2002; Grandillo et al., 1999; Tanksley, 2004; van der Knaap et al., 2002; van der Knaap et al., 2004). Among them, *fruit weight* (*fw2.2*) controls fruit size without affecting fruit shape or seed production (Cong et al., 2002; Frary et al., 2000; Liu et al., 2003), *sun*, *ovate* and *fruit shape chromosome* (*fs8.1*) regulate fruit shape with minimum effect on

fruit size, and *fasciated* (*fas*) and *locule number* (*lc*) determine carpel number and effect both fruit size and shape (Figure 1.1) (Ku et al., 2000; Rodríguez et al., 2011c). *fw2.2,* cloned by high-resolution positional mapping, has been reported to share homology with the cell membrane localized Ras-like G-protein (Frary et al., 2000), and negatively regulates fruit size. A mutation in its 2.7-kilobase upstream promoter region resulted in null expression and large tomato fruit phenotype (Nesbitt and Tanksley, 2002). *fw2.2* has been further shown to suppress the anticlinal, but not periclinal, cell division in placenta and pericarp causing reduction in fruit length to perimeter ratio but not the pericarp thickness (Liu et al., 2003). In pepper (*Capsicum chinense* and *C. frutescens*), *fw2.1,* but not *fw2.2,* is the single major fruit-weight QTL responsible for 62% of the trait variation (Ben Chaim et al., 2006; Zygier et al., 2005).

Fruit size and weight are a function of the number of cells within the ovary prior to fertilization and cell expansion (Bohner and Bangerth, 1988). Additionally, endoreduplication that increases cell expansion contributes to the final fruit size (Cheniclet et al., 2005). Cyclins and CDK complexes regulate progression of cell division while CDK inhibitor such as *WEE1* induces endoreduplication (Sun et al., 1999). Expression of antisense *Slwee1* under CaMV 35S promoter reduced ploidy-levels, fruit mass, plant growth and seed size (Gonzalez et al., 2007). Another gene that promotes endoreduplication is *cell cycle switch* (*CCS52A*) (Cebolla et al., 1999). Overexpression of *CCS52A*, which activates anaphase-promoting complex E3 ubiquitin ligase, led to increased tomato fruit size (Mathieu-Rivet et al., 2010).

A retrotransposon-mediated gene duplication at *sun* locus resulted in morphological variation of tomato fruit (Xiao et al., 2008). Overexpression of *IQD12,* one of the five genes at *sun* locus, significantly increased fruit elongation while impairing its expression by RNAi significantly decreased fruit elongation (Xiao et al., 2008). The molecular function of IQD12 is not yet known but it exhibits homology with a member of the IQ67 protein family containing the calmodulin-binding domain and likely changes the fruit shape by affecting the pattern along the apical–basal axis (Xiao et al., 2008).

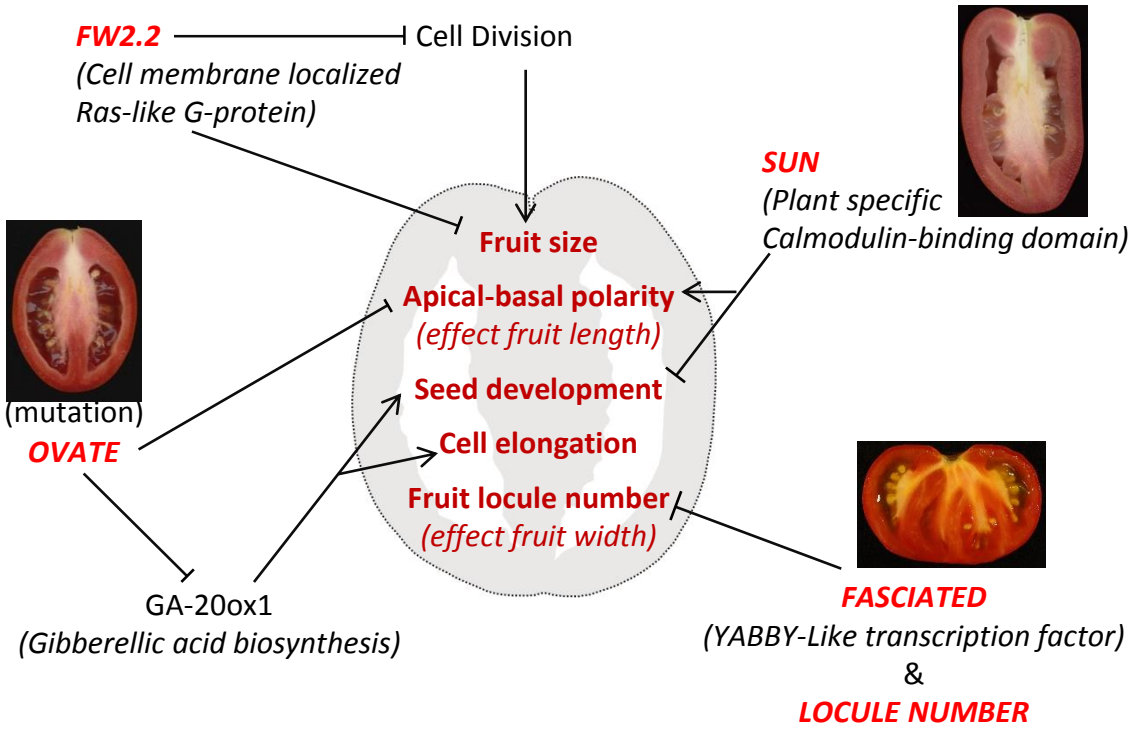


Figure 1.1: A model presenting various tomato fruit appearance factor known to be regulated by QTLs.

The *ovate* locus, another important QTL responsible for the development of a pear-shaped instead of an oval-shaped tomato fruit, encodes a transcription repressor regulating *GA20ox1,* a gibberellic acid (GA) biosynthesis enzyme (Wang et al., 2007). Overexpression of the ovate family protein 1, *AtOFP1,* reduced fruit elongation in tomato (Ku et al., 1999) and pepper (Tsaballa et al., 2011). Complementation of pear-shaped fruit phenotype TA503 by either native *OVATE* or ectopic expression of *OVATE* under the control of CaMV35 promoter (*35S:OVATE*) produced round-shaped fruit (Liu et al., 2002). Silencing of *OVATE* in round-fruited pepper cv. "Mytilini" resulted in increased expression of *GA20ox1* and an oblong-shaped fruit (Tsaballa et al., 2011). Molecular identity of genes present at other QTLs determining fruit shape and size including *fs8.1, fs10.1*, *fs3.1, fas,* and *lc* remains to be determined. Similarly, biochemical signals regulating fruit size and shape genes are also still largely unknown (Handa et al., 2012). It will be interesting to explore downstream and upstream regulators of these QTLs through which these loci impart their effect on fruit quality attributes like size and shape. Although the fruit shape and size genes can alter fruit architecture by molecular genetics approach, they are not yet used to develop fruit with novel architecture for commercial purposes. However, all emerging evidences indicate that these genes would provide a rich resource to develop desirable fruit phenotypes.

1.2 Molecular engineering of fruit texture

Changes in fruit texture are essential for fruit softening and making a fruit edible and desirable for human consumption. Fruit softening is associated with several attributes including crispness, mealiness, grittiness, chewiness, succulence and juiciness, fibrousness, toughness and oiliness. Further, the fruit textural changes are connected with the development of organoleptic characteristics, such as sweetness, sourness, astringency, bitterness and production of volatile compounds that provide the aroma. However, excessive fruit softening can cause some undesirable attributes including development of off flavors and susceptibility to phytopathogens. The fact that excessive

fruit softening makes most fruit unacceptable leading to large economic losses has generated considerable interest among plant biologists to understand the molecular basis of fruit softening and modify this process by recombinant technology (Negi and Handa, 2008). In this chapter, we focused on biotechnological approaches for enhancing textural qualities of fruits.

Based on observed modifications of the polysaccharides in the primary cell wall and dissolution of middle lamella during fruit softening, it had been hypothesized that cell wall depolymerizing enzymes play important roles in fruit textural changes (Brady, 1987). This hypothesis gained further credence when it was shown that expression of several cell wall degrading enzymes was severely reduced in tomato mutants impaired in fruits ripening (Biggs and Handa, 1989; DellaPenna et al., 1989; Tigchelaar et al., 1978). A test of this hypothesis led to the development of the first genetically engineered tomato cultivar designated as 'Flavr Savr'. In 'Flavr Savr' fruit, polygalacturonase gene (*SlPG2*) was silenced by antisense RNA technology (Kramer and Redenbaugh, 1994). The impaired *SlPG2* expression resulted in enhanced juice viscosity, but fruit softening was not significantly affected, thus it failed to meet the market expectation of an extended shelf life fruit (Giovannoni et al., 1989; Thakur et al., 1997). The ectopic expression of *SlPG2* also failed to enhance softening of the ripening mutant, *rin*, suggesting a limited role of *SlPG2* in tomato fruit softening (Giovannoni et al., 1989). In contrast, antisense inhibition *FaPG1* expression resulted in reduced strawberry fruit softening (Quesada et al., 2009). Reduced softening of *FaPG1*-antisense fruit occurred in spite of only a slight reduction in total PG activity, as the most PG activity was contributed by another isozyme, *FaPG2*, whose expression was not impaired by the *FaPG1* antisense gene (Quesada et al., 2009).

Multiple isozymes of pectin methylesterase (PME), an enzyme that demethoxylates pectin, are expressed during fruit development but their roles in fruit texture are not as yet understood (Gaffe et al., 1994; Harriman et al., 1991; Phan et al., 2007; Tieman and Handa, 1994). Over 95% reduction in PME transcripts, protein and enzymatic activity by the antisense expression of *SlPME3* under the CaMV 35S promoter did not affect fruit softening but greatly enhanced juice viscosity and increased TSS

(Thakur et al., 1996a; Thakur et al., 1996b; Tieman and Handa, 1994; Tieman et al., 1992). The fruit integrity, however, was compromised if low PME fruits were stored for extended period (Tieman and Handa, 1994). In another study, silencing of *SlPMU1,* a ubiquitously expressed PME isozyme, enhanced softening of transgenic fruit even when reduction in PME activity was only 25% compared to wild type fruit (Gaffe et al., 1997; Phan et al., 2007). These studies on PG and PME further emphasize that only specific isozymes among cell wall modifying isozymes contribute to fruit textural changes. Interestingly, tomato fruit with reduced *PME* expression also exhibited reduction in fruit blossom end rot, a calcium associated fruit disorder (de Freitas et al., 2012). These authors showed that apoplastic calcium levels increased due to reduced calcium binding to high methoxyl pectin, a consequence of low *PME* activity, and influenced development of blossom end rot symptoms in tomato fruits (de Freitas et al., 2012).

Preferential loss of galactose and/or arabinose from cell walls during early fruit ripening has led to the suggestion that β-galactosidase (β-gal) plays an important role in fruit textural changes (Gross and Sams, 1984). Among the seven β-gal genes (*SLTBG1-7*) expressed in developing fruit, only silencing of *SlTGB4* (about 90% reduction in extractable exo-galactanase activity) led to about 40% increase in fruit firmness compared to the wild type fruits at the comparable stages of ripening (Smith et al., 2002; Smith and Gross, 2000). The total exo-galactanase activity, cell wall galactose content and fruit softening were not affected in transgenic fruits exhibiting about 90% reduction in *SlTBG1* transcripts obtained by homology-dependent gene silencing (Carey et al., 2001). The role of endo-β-mannanase (β-Man), which hydrolyzes mannose in hemicellulose polymers to mannobiose and mannotriose, was tested by developing transgenic plants expressing its antisense RNA or by gene-specific hairpin RNAi gene. These transgenic fruits exhibited reduced β-Man activity but a clear correlation between fruit firmness and β-Man activity was not found (Bewley et al., 2000).

The role of xyloglucan xyloglucosyltransferase/endohydrolase (*XTH*) in fruit textural changes was examined by overexpressing *SlXTH1*, a tomato homolog of *Nicotiana tabacum NtXET-1* gene, under the CaMV 35S promoter (Miedes et al., 2010). *XTHs* have

been suggested to play dual role in cell wall chemistry by integrating newly secreted xyloglucan chains into an existing wall-bound xyloglucan and by catalyzing transglucosylation during restructuring of existing cell wall bound xyloglucan molecules. The transgenic fruits had more than 4-fold increase in *XET* activity associated with reduced xyloglucan depolymerization and reduced fruit softening, suggesting its role in maintaining the structural integrity of cell walls (Miedes et al., 2010). Most fruit species contain multiple genes for pectate lyase (*PL*), an enzyme that hydrolyzes the unesterified galacturonosyl linkages by a β-elimination reaction. Although expression of several *PL* isozymes increases during fruit ripening, understanding their role in pectinolysis and fruit texture changes is still in its early stages. Introduction of an antisense gene of a strawberry *PL* (*njjs25*) under the CaMV 35S promoter inhibited the expression of *PL* and the transgenic strawberry fruit registered a decrease in ripening-associated firmness. These transgenic fruit showed extended postharvest shelf life, reduction in pectin solubility, decreased depolymerization of bound polyuronides, and loss of cell-cell adhesion in the transgenic fruits (Jiménez-Bermúdez et al., 2002; Santiago-Doménech et al., 2008). Transgenic inhibition of *CEL1* and *CEL2,* two endo-*b*-1,4-glucanase (EGases, cellulases) present in many fruits, had little effect on fruit softening (Brummell et al., 1999a; Lashbrook et al., 1998). Down regulation of *Cel1* and *Cel2* in strawberry fruits yielded similar results with little influence on fruit softening but slightly high abundance of the larger hemicellulosic polymers was present in the fruit (Mercado et al., 2010; Pang et al., 2010).

Expansins are family of proteins that induce extension in isolated plant cell walls, expressed during fruit development and ripening, and their roles in fruit textural changes have been examined using molecular genetic techniques (Choi et al., 2006). The antisense RNA inhibition of a ripening-specific expansin, *SlExp1,* caused reduction in polyuronide depolymerization without affecting breakdown of other structurally important hemicelluloses and the transgenic fruit retained firmer texture than wild type fruit (Rose et al., 1997). The constitutive expression of *SlExp1* caused an opposite phenotype and the transgenic fruit was softer and associated with precocious and extensive

depolymerization of structural hemicelluloses without altering polyuronide depolymerization (Brummell et al., 1999b). It was proposed that *Exp1* modulates relaxation of the cell walls and regulates polyuronide depolymerization by controlling access of a pectinase to its substrate, whereas the depolymerization of hemicellulose occurs independently or requires only very small amounts of Exp1 protein (Brummell et al., 1999b). Firmer fruit texture and high cellular integrity during longer storage was observed in the fruits in which *Exp1* and *PG* were simultaneously down-regulated (Powell et al., 2003).

After an initial demonstration that a protein glycosylation inhibitor, tunicamycin impaired fruit ripening (Handa et al., 1985), the role of protein glycosylation in fruit ripening and textural changes has begun to emerge using transgenic technologies. Tunicamycin inhibits the UDP-HexNAc:polyprenol-P HexNAc-1-P family of enzymes and blocks the synthesis of all N-linked glycoproteins (N-glycans). Suppression by antisense RNA technology of two N-glycosylating enzymes, β-mannosidase (β-Man) and β-D-N-acetylhexosaminidase (β-Hex), led to reduction in ripening-associated softening and improved fruit shelf life (Meli et al., 2010) whereas their ectopic expression caused excessive softening of the transgenic fruit. These studies provided a novel way to alter fruit ripening and extend their shelf life.

1.3 Molecular engineering of carotenoids

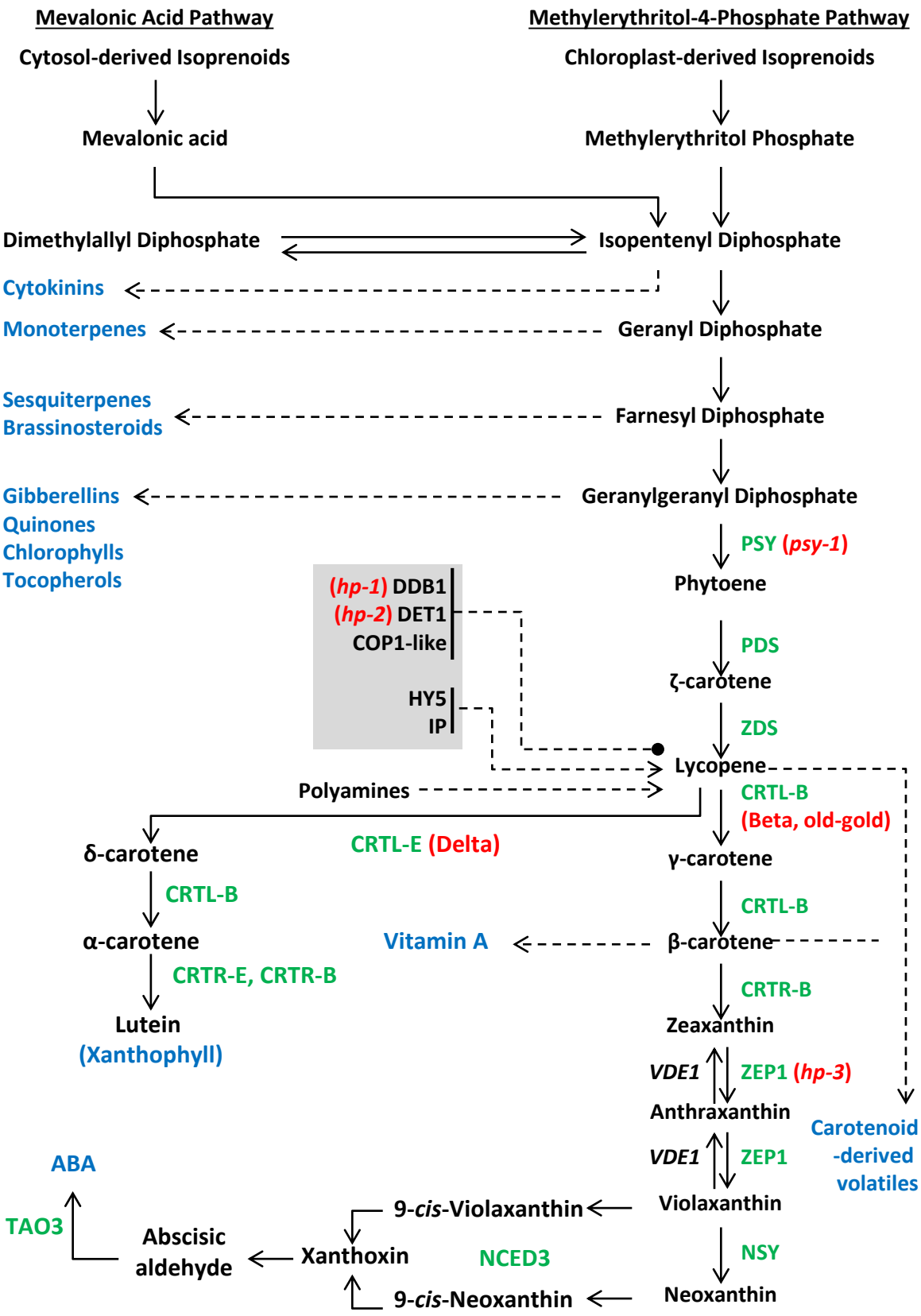
Fruits are naturally rich in carotenoids, one of the most abundant groups of plant pigments. Over 600 carotenoids have been structurally identified and this list continues to increase as new compounds are added. Carotenoids play several roles in plants including photosystem assembly, light harvesting, free radical detoxification, photomorphogenesis, non-photochemical quenching, lipid peroxidation and a substrate for phytohormone ABA (Namitha and Negi, 2010). However, it is the human health benefit of carotenoids that has attracted significant attention in recent years (Dixon, 2005; Mattoo et al., 2010b). The role of vitamin A (retinal) in preserving eyesight, especially

preventing night blindness, is one of the best known functions of carotenoids in human health (Cook, 2010). Due to their high antioxidant activity, carotenoids are implicated in protection against cataract and macular degeneration of eye; and cervical, lung, prostate, colorectal, stomach pancreatic, and esophagus cancers. Carotenoids may also reduce low-density lipoprotein (LDL) implicated in cardiovascular disease and boost the immune system to provide protection against many other diseases such as osteoporosis, hypertension and neurodegenerative diseases like Alzheimer's, Parkinsons and vascular dementia (Mattoo et al., 2010b; Namitha and Negi, 2010). The emerging consensus in favor of the beneficial role of carotenoids has led to significant research activity to raise their cellular levels in fruit and vegetable crops using novel approaches.

Genes encoding carotenoid biosynthetic pathway enzymes have been identified and cloned from several species but regulations of their accumulation in plants is complicated and poorly understood (Klee and Giovannoni, 2011). A detailed carotenoid biosynthesis pathway is illustrated in Figure 1.2. A series of additions and condensation reactions convert isopentenyl diphosphate (IPP) to the formation of geranylgeranyl diphosphate (GGPP). Two different pathways, mevalonate (MVA) dependent (cytosolic) and MVA independent (plastid), generate IPP (Rodríguez-Concepción, 2010). Phytoene synthase (*psy*) is the first committed step in carotenoid biosynthesis and catalyzes the condensation of two GGPPs to form phytoene which is converted into ζ-carotene by phytoene desaturase (PDS). The ζ-carotene desaturase (ZDS) converts ζ-carotene to lycopene, which in turn is converted into either β-carotene by lycopene β-cyclase (CRTL-B), precursor of vitamin A, or α-carotene by lycopene α-cyclase (CRTL-E) and CRTL-B. Lutein, a major xanthophyll involved in light-harvesting and preventing macular degeneration of eyes in older people, is synthesized from α-carotene (Ronen et al., 1999).

Figure 1.2: Carotenoid biosynthesis pathway in plants.

Transgenic intervention in enzyme characterization is shown in green color and their mutants are shown in red. Broken arrows indicate involvement of multiple steps. Phytohormones, aroma volatiles and other compounds indicated in blue show direct (solid arrows) or indirect (with broken arrows) connections with carotenoids biosynthesis pathway. Photomorphogenic signal transduction factors are shown in the grey box. A step in PA action on lycopene accumulation is highlighted. Inhibitory (blunt-end line) or stimulatory (with arrow head) effects are shown. ABA, abscisic acid; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTL-B, lycopene β-cyclase; CRTR-B, carotenoid β-hydroxylase; CRTR-E, carotenoid ε-hydroxylase; ZEP1, zeaxanthin epoxidase; VDE1, violaxanthin de-epoxidase; NSY, neoxanthin synthase; CRTL-E, lycopene ε-cyclase; NCED3, 9-*cis*-epoxycarotenoid dioxygenase 3; TAO3, abscisic-aldehyde oxidase.


Mevalonic Acid Pathway
Cytosol-derived Isoprenoids
Mevalonic acid
Methylerythritol-4-Phosphate Pathway
Chloroplast-derived Isoprenoids
Methylerythritol Phosphate
Dimethylallyl Diphosphate
Isopentenyl Diphosphate
Cytokinins
Monoterpenes
Geranyl Diphosphate
Sesquiterpenes
Brassinosteroids
Farnesyl Diphosphate
Gibberellins
Quinones
Chlorophylls
Tocopherols
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PSY (psy-1)
Phytoene
(hp-1) DDB1
(hp-2) DET1
COP1-like
HY5
IP
PDS
ζ-carotene
ZDS
Lycopene
Polyamines
CRTL-B
(Beta, old-gold)
CRTL-E (Delta)
δ-carotene
γ-carotene
CRTL-B
CRTL-B
α-carotene
Vitamin A
β-carotene
CRTR-E, CRTR-B
CRTR-B
Lutein
(Xanthophyll)
Zeaxanthin
VDE1
ZEP1 (hp-3)
Anthraxanthin
Carotenoid
-derived
volatiles
VDE1
ZEP1
ABA
Violaxanthin
9-cis-Violaxanthin
TAO3
Abscisic
aldehyde
Xanthoxin
NCED3
NSY
9-cis-Neoxanthin
Neoxanthin

Figure 1.2

Transgenic approaches have been widely used to enhance levels of carotenoids in many crop species by expressing various genes of the carotenoid pathways (Table 1.1). Ectopic expression of a bacterial phytoene synthase (*crtB*) under the control of a fruit-specific promoter increased phytoene (2.4-fold), lycopene (1.8-fold), β-carotene (2.2-fold) and lutein levels in tomato fruit (Fraser et al., 2002). The constitutive expression of citrus lycopene β-cyclase (*CRTL-B*) increased β-carotene 4.1-fold with a 30% increase in total carotenoids while suppressing fluxes downstream into β-carotene pathway and concomitant increase in α-carotene (Guo et al., 2012). A mutation in lycopene ε-cyclase (*CRTL-E*) caused accumulation of δ-carotene at the expense of lycopene in *Delta* (*Del*), a fruit-color mutant (Ronen et al., 1999). Two other genes, *CYP97A29* and *CYP97C11*, have been functionally characterized by expressing them in tomato under CaMV 35S promoter. *CYP97A29* and *CYP97C11* encode P450 carotenoid β-hydroxylase (*CRTR-B*) and carotenoid ε-hydroxylase (*CRTR-E*), respectively. *CRTR-E* converts α-carotene into lutein, and *CRTR-B* converts β-carotene into zeaxanthin and α-carotene into lutein (Stigliani et al., 2011). Zeaxanthin is further converted to violaxanthin by zeaxanthin epoxidase (*ZEP1*). A mutation in *zep1* caused ABA-deficiency in tomato plants with concomitant accumulation of 30% more carotenoids in mature red tomato fruit (Galpaz et al., 2008). RNAi-mediated fruit-specific suppression of 9-*cis*-epoxycarotenoid dioxygenase 3 (*NCED3*), an enzyme that catalyzes first step of ABA biosynthesis converting 9-*cis*-violaxanthin to 2-*cis*,4-trans-xanthoxin, not only suppressed ABA synthesis but also stimulated accumulation of upstream compounds such as β-carotene and lycopene in transgenic tomato fruits (Sun et al., 2012b).

Table 1.1: Engineering of carotenoid pathway in tomato fruit to alter carotenoid levels

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
3-Hydroxy-3-methyl-glutaryl CoA reductase (*HMGR-1)* (*Arabidopsis thaliana*)	3-Hydroxy-3-methylglutaryl CoA → mevalonic acid	CaMV 35S (OE)	2.4-fold ↑total phytosterol No change in lycopene or β-carotene (Enfissi et al., 2005)
1-Deoxy-D-xyluose-5-phosphate synthase *(DXS)* (*Escherichia coli*)	Pyruvate and D-glyceraldehyde-3-phosphate → 1-deoxy-D-xylulose-5-phosphate	CaMV 35S or fibrillin (OE)	1.6-fold ↑ carotenoids 2.4-fold ↑ phytoene 2.2-fold ↑ β-carotene (Enfissi et al., 2005)
Phytoene synthase *(crtB)* (*Erwinia uredovora*)	Geranyldiphosphate → phytoene	*SlPG* (OE)	2.4-fold ↑ phytoene 1.8-fold ↑ lycopene 2.2-fold ↑ β-carotene (Fraser et al., 2002)
Phytoene synthase (*psy-1*) (*S. lycopersicum*)	As above	CaMV 35S (OE)	1.2-fold ↑ total carotenoids 1.3- fold ↑ β-carotene 2.3-fold ↑ phytoene 1.8-fold phytofluene (Fraser et al., 2007)
Phytoene synthase (*psy-1)* (*S. lycopersicum*)	As above	CaMV 35S (OE)	↑ lycopene (386 μg g^{-1} DW) ↓ plant height 30-fold ↓ GA_1 (Fray et al., 1995)
Phytoene desaturase (*crtI)* (*E. uredovora*)	Phytoene → ζ-carotene	CaMV 35S (OE)	3-fold ↑ β-carotene. No effect on total carotenoids: reduction in lycopene and

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
			phytoene. No effect on plant growth and development (Römer et al., 2000)
Lycopene β-cyclase (*SpB*) (*S. pennellii*)	Lycopene → β-carotene δ-carotene → α-carotene	CaMV 35S (OE)	>6-fold ↑ β-carotene 1.8-fold ↓ lycopene (Ronen et al., 2000)
Lycopene β-cyclase (*SpB*) (*S. pennellii*)	As above	CaMV 35S (AS)	>6-fold ↓ β-carotene Slight ↑ lycopene (Ronen et al., 2000)
Lycopene β-cyclase (*Lyc-b*) (*S. lycopersicum*)	As above	CaMV 35S (OE)	31.7-fold ↑ β-carotene at the expense of lycopene No morphological and developmental defects (D'Ambrosio et al., 2004)
Lycopene β-cyclase (*ϐ-Lcy*) (*A. thaliana*)	As above	*Sl*Pds (OE)	>6-fold ↑ β-carotene No change in lycopene (Rosati et al., 2000)
Lycopene β-cyclase (*ϐ-Lcy*) (*S. lycopersicum*)	As above	*Sl*Pds (AS)	1.3-fold ↑ lycopene 1.7-fold ↑ lutein 50% ↓ *ϐ-Lcy* expression (Rosati et al., 2000)
Lycopene β-cyclase (*Lycb-1*) (Citrus)	As above	CaMV 35S (OE)	4.1-fold ↑ β-carotene 30% ↑ total carotenoids (Guo et al., 2012)
Lycopene β-cyclase (*crtY*) (*E. herbicola*) or (*carRA*) (*Phycomyces blakesleeanus*)	As above	atpI (OE)	4-fold ↑ β-carotene Slight ↓ lycopene & total carotenoids (Wurbs et al., 2007)

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
Lycopene β-cyclase (*b-Lcy*) (*A. thaliana*) + Carotene β-hydroxylase (*b-Chy*) (*C. annuum*)	Lycopene → β-carotene δ-carotene → α-carotene + β-carotene → zeaxanthin α-carotene → lutein	*Sl*Pds (OE)	12-fold ↑ β-carotene 10-fold ↑ total xanthophyll (Dharmapuri et al., 2002)
9-cis-Epoxycarotenoid dioxygenase (*NCED*) (*S. lycopersicum*)	9-*cis*-Violaxanthin → xanthoxin 9-*cis*-Neoxanthin → xanthoxin	E8 (RNAi)	↑ β-carotene and lycopene 20-50% ↓ ABA (Sun et al., 2012b)
DE-ETIOLATED (*DET1*) (*S. lycopersicum*)	TFs negatively regulate photomorphogenic responses	P119, 2A11, TFM7 (RNAi)	2-fold ↑ lycopene 4-fold ↑ β-carotene 3.5-fold ↑ flavonoids No change in fruit weight and TSS in red-ripe fruit (Davuluri et al., 2005)
Cryptochrome 2 (*CRY2*) (*S. lycopersicum*)	Blue light photoreceptor	CaMV 35S (OE)	1.5-fold ↑ lutein 1.7-fold ↑ carotenoids 2.9-fold ↑flavonoids (Giliberto et al., 2005)
ELONGATED HYPOCOTYL 5 (*HY5*) (*S. lycopersicum*)	Positive regulator of phytochrome signal transduction	CaMV 35S (RNAi)	↓ carotenoid (Liu et al., 2004)
CONSTITUTIVELY PHOTOMORPHOGENIC 1 (*COP1-like*) (*S. lycopersicum*)	TFs negatively regulate photomorphogenic responses	CaMV 35S (RNAi)	2-fold ↑ carotenoids (Liu et al., 2004)

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
Spermidine synthase (*SPE3*) (*S. cerevisiae*)	PUT → SPD	CaMV 35S (OE)	40% ↑ lycopene (Nambeesan et al., 2010)
Spermidine synthase (*Md-SPDS1*) (Malus x domestica)	As above	CaMV 35S (OE)	↑ *PSY* and *PDS* and ↓ *CRTL-B* and *CRTL-E* transcripts 1.3-2.2-fold ↑ lycopene (Neily et al., 2010)
SAM decarboxylase (*SPE2*) (*S. cerevisiae*)	SAM → decarboxylated SAM	E8 (OE)	2-3-fold ↑ lycopene (Mehta et al., 2002)

Abbreviations: CaMV 35S, Cauliflower mosaic virus 35S promoter; E8, Tomato fruit-specific E8 promoter; Pds, Fruit-specific phytoene desaturase promoter; PG, Fruit-ripening specific polygalacturonase 2 promoter; atpI (ATPase IV subunit), tobacco plastid-specific promoter; P119, 2A11, TFM7, Fruit-specific promoters; Fibrillin, Ripening-enhanced promoter of fibrillin; RNAi, RNAi-mediated repression of target gene; OE, Overexpression of the introduced gene; AS, anti-sense–mediated down-regulation, TF, Transcription factor; SAM, *S*-adenosylmethionine; ↑, increased levels; ↓, decreased levels; →, substrate to product conversion.

Characterization of several tomato mutants that accumulate high levels of carotenoids than wild type fruits have helped to discover factors regulating flux through carotenoid pathway. *UV-DAMAGED DNA BINDING PROTEIN 1* (*DDB1*) and *DE-ETIOLATED 1 (DET1)* are transcription factors that negatively regulate photomorphogenic responses (Azari et al., 2010b). Mutations in *DDB1* and *DET1* exhibit recessive *high-pigment 1* (*hp-1*) and *hp-2* phenotypes with severe developmental defects (Azari et al., 2010a; Davuluri et al., 2004; Levin et al., 2003; Mustilli et al., 1999). However, organ-specific silencing of *DET1* by RNAi under a fruit-specific promoter resulted in 2-fold increase in lycopene, 4-fold increase in β-carotene, and up to 3.5-fold increase in flavonoids without significant changes in fruit weight and TSS in red-ripe fruit (Davuluri et al., 2005). The transgenic expression of cryptochrome 2 (*35S:CRY2*) resulted in 1.7-fold increase in carotenoids and 2.9-fold increase in flavonoids (Giliberto et al., 2005). RNAi-mediated repression of *ELONGATED HYPOCOTYL 5* (*HY5*) reduced carotenoid accumulation and repression of *CONSTITUTIVELY PHOTOMORPHOGENIC 1* (*COP1*)-like showed elevation in tomato fruit carotenoids suggesting involvements of light signaling factors in carotenoid biosynthesis (Liu et al., 2004).

Deficiency of vitamin A is a major issue affecting child health, especially in developing nations. To increase its synthesis in staple foods, transgenic technologies have successfully been used to develop rice varieties (Golden Rice) engineered to accumulate high levels of Provitamin A "β-carotene". Introduction of maize *PSY* in combination with carotene desaturase from *E. uredovora* resulted in 23-fold increase in total carotenoids in rice (Paine et al., 2005). During rice processing, an aleurone layer is removed to avoid rancidity of rice grains during storage while rice endosperm lacks β-carotene. Using DNA recombination technology, three transgene constructs were co-transformed and transformants containing all three transgenes were selected and characterized. The three transgenes introduced were daffodil *PSY* under the control of endosperm-specific gluten promoter, *E. uredovora PDS* under the control of CaMV 35S promoter and *Narcissus pseudonarcissus CRTL-B* under the control of a rice gluten promoter. Expression of these transgenes in rice endosperm led to high accumulation of β-carotene (Ye et al., 2000).

Transgenic approaches have also been used to enhance levels of carotenoids in flaxseed (Fujisawa and Misawa, 2010), corn (Naqvi et al., 2011), kumquat citrus (Zhang et al., 2009a), wheat (Cong et al., 2009), Brassica (Fujisawa et al., 2009; Wei et al., 2009; Yu et al., 2008), rice (Burkhardt et al., 1997; Rai et al., 2007), tobacco (Frey et al., 2006; Qin and Zeevaart, 2002) and canola (Ravanello et al., 2003).

1.4 Molecular engineering of flavonoids

Flavonoids are aromatic, low-molecular weight secondary metabolites and classified as plant phenolics (Robards and Antolovich, 1997). Their hydrophilic properties (Rice-Evans et al., 1997) complement the hydrophobic nature of carotenoids. More than 6,000 naturally occurring flavonoids have been identified (Harborne and Herbert, 1999) and classified based on degree of unsaturation and oxidation of a three-carbon bridge in flavone skeleton between phenyl groups of flavonoids.

Antioxidant and free radical scavenging properties of flavonoids have been associated with reducing the risks of heart and age-related diseases and cancers (Ross and Kasum, 2002). Fruit juice is a major source of flavonoids in human diet and total fruit juice consumption seems to account for 20-30% of dietary intake of flavonoids (Robards and Antolovich, 1997). Apart from emerging therapeutic role in alternative medicinal science, flavonoids are also known to provide protection to plants against UV-B light and microbial interaction (Harborne and Williams, 2000). This attribute is important for fruits to maintain their resistance against fungi during storage. Flavonoids also contribute towards various fruit quality attributes including color (red, violet, blue), flavor and texture. On the other hand, undesirable brown pigmentation (bruises) on fruit surface has been attributed to oxidation of phenols to quinones that then polymerize into brown pigments, for example, flavan-3-ols in apples (Amiot et al., 1992; Goupy et al., 1995; Robards and Antolovich, 1997). Different classes of flavonoids also combine with proteins and cause sedimentation in fruit juices and wines (Amiot et al., 1992). Flavonoid compounds found in different fruits and vegetables have also been summarized

elsewhere (Nicoletti et al., 2007; Robards and Antolovich, 1997; Slimestad and Verheul, 2009; USDA, 2007).

The genetic regulation of the flavonoid biosynthesis pathway was primarily investigated by the inheritance pattern of flower colors and radio-labeling. However, the genetic engineering technology has added a new dimension to the understanding of flavonoid biosynthetic enzymes and substrates and their diversity among various plant species (Table 1.2). Mutants and transgenic plants have provided direct evidence on the function of various genes involved in flavonoid biosynthesis pathway (reviewed elsewhere, Ververidis et al., 2007b). Flavonoids are mainly synthesized from phenylalanine via the phenylpropanoid pathway. Following cinnamate hydroxylation by cinnamate 4-hydroxylase and 4-coumarate:CoA ligase step in phenylpropanoid pathway, flavonoid biosynthesis pathway branches out into phenolics (chlorogenic acid) and favonols (naringenin, quercetin, and their derivatives) (Anterola and Lewis, 2002; Ververidis et al., 2007a).

Table 1.2: Studies on tomato engineered to alter fruit flavonoids

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
Stilbene synthase (*StSy*) (*Vitis vinifera*)	Malonyl-CoA and *p*-coumaroyl-CoA → resveratrol	CaMV 35S (OE)	↑ trans-resveratrol (48.48 mg kg^{-1} FW) ↑ trans-piceid (126.58 mg kg^{-1} FW) 2-fold ↓ rutin, 2.4-fold ↓ naringenin Seedless fruit (Giovinazzo et al., 2005; Nicoletti et al., 2007)
Stilbene synthase (*StSy*) (*V. vinifera*)	As above	TomLoxB (OE)	↑ resveratrol, *trans*-resveratrol and piceid (D'Introno et al., 2009)
Stilbene synthase (*STS*) (*V. vinifera*)	As above	CaMVd35S (OE)	↑ stilbenes (resveratrol and piceid) ↑ naringenin chalcone and rutin (Schijlen et al., 2006)
Chalcone synthase (*Chs1*) (*S. lycopersicum*)	Phenylpropanoids → chalcones	CaMVd35S (RNAi)	↓ total flavonoid Parthenocarpic fruit (Schijlen et al., 2007)
Chalcone isomersae (*Chi-A*) (*Petunia hybrida*)	Chalcones → flavanones	CaMVd35S (OE)	78-fold ↑ peel flavonols, mainly due to ↑ rutin (Muir et al., 2001)
Chalcone synthase (*Chs1*) (*P. hybrida*) + Chalcone reductase (*CHR*) (*Medicago sativa*)	Phenylpropanoids → chalcones + Phenylpropanoids → deoxy-chalcones	CaMV 35S (OE)	↑butein & isoliquiritigenin ↑ naringenin chalcone & rutin (Schijlen et al., 2006)

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
Chalcone isomerase (*CHI*) (*P. hybrida*) + Flavone synthase (*CYP93B2*) (*Gerbera hybrida*)	Chalcones → flavanones + Flavanones → flavones	CaMV 35S (OE)	16-fold ↑ rutin flavonol ↑ luteolin-7-glucoside, luteolin aglycon, quercetin glycosides, naringenin chalcone and rutin (Schijlen et al., 2006)
Isoflavone synthase (*IFS2*) (*Glycine max*)	Naringenin → genistein	CaMV 35S (OE)	↑ genistin in leaves Only marginal increase in fruit peel ↑ naringenin chalcone in fruit peel (Shih et al., 2008)
LC (*LC*) (*Zea mays*)	LC - a member of maize R gene family of MYC-type TFs and determines the tissue-specific expression of anthocyanin in maize	CaMV 35S (OE)	↑ anthocyanins in all vegetative tissues but to lesser extent in green fruit (Goldsbrough et al., 1996)
C1 (*C1*) + LC (*LC*) (*Zea mays*)	MYB-type C1 and MYC-type LC are TFs required for production of anthocyanin in plants	E8 or CaMVd35S (OE)	Induced flavonoid synthesis in fruit flesh 10-fold ↑ total flavonoids 20-fold ↑ total flavonol, mainly due to ↑ kaempferol (Bovy et al., 2002)
RP (*Myc-rp*) (*Perilla frutescens*)	Myc-like TF regulate anthocyanin biosynthesis	CaMV 35S (OE)	↑ anthocyanin in vegetative tissues and flowers (Gong et al., 1999)
Delila (*Del*) (*Antirrhinum majus*)	Myc TFs that activate biosynthesis of anthocyanin	CaMV 35S (OE)	↑ anthocyanins in mature leaves (23-fold), corolla (40-fold) & stamen (50-fold) but none in fruit (Mooney et al., 1995)
Rosea1 (*AmRos1*) + Delila (*Del*) (A. *majus*)	TFs that activate biosynthesis of anthocyanin	E8 (OE)	↑ anthocyanin in pericarp comparable to blackberries and blueberries (Butelli et al., 2008)
MYB12 (*MYB12*) (*A. thaliana*)	R2R3-MYB TF mediates the accumulation of flavonoids in tomato peel	CaMV 35S (OE)	27-fold ↑ chlorogenic acid 26-fold ↑ dicaffeoyl quinic acid 42-fold ↑ tricaffeoyl quinic acid

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
			67-fold ↑ quercetin rutinoside 593-fold ↑ kaempferol rutinoside (Luo et al., 2008)
MYB12 (*MYB12*) (*S. lycopersicum*)	As above	CaMV 35S (RNAi)	↓ flavonoid pigment naringenin chalcone Exhibited a *y*-like phenotype (Adato et al., 2009)
MYB12 (*MYB12*) (*S. lycopersicum*)	As above	CaMV 35S (OE)	Rescued colorless-peel 'y' tomato mutant phenotype (Adato et al., 2009)
ANTHOCYANIN 1 (*ANT1*) (*S. lycopersicum*)	Flavonoid-related R2R3-MYB TF	CVM (OE)	500-fold ↑ anthocyanin (Mathews et al., 2003)
ANTHOCYANIN 1 (*ANT1*) (*S. chilense*)	As above	CaMV 35S (OE)	↑ anthocyanadins (petunidin, malvidin, delphinidin) in tomato (*S. lycopersicum*) fruit (Schreiber et al., 2012)
TOMATO AGAMOUS-LIKE 1 (*TAGL1*) (*S. lycopersicum*)	MADS-box TF	E8 (*TAGL1-SRDX*)	↓ lycopene and isoprenoids (Itkin et al., 2009)
TOMATO AGAMOUS-LIKE 1 (*TAGL1*) (*S. lycopersicum*)	As above	CaMV 35S (OE)	↑ lycopene and naringenin chalcone (Itkin et al., 2009)
Cullin 4 (*CUL4*) (*S. lycopersicum*)	DDB1a and DET1 form a complex with CUL4, an ubiquitin-conjugating E3 ligase, and target proteins for proteolysis	CaMV 35S (RNAi)	pleiotropic phenotype ↑anthocyanins and carotenoids 2-fold ↑ lycopene (Wang et al., 2008)

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
UV-DAMAGED DNA BINDING PROTEIN 1 (*DDB1*) (*S. lycopersicum*)	TF negatively regulates photomorphogenic responses	E8 (RNAi)	↑ pigment accumulation due to ↑ plastid compartment space (Wang et al., 2008)
SAM decarboxylase (*SPE2*) (*Saccharomyces cerevisiae*)	As above	E8 (OE)	↑ transcripts related to flavonoid biosynthesis genes (Mattoo et al., 2007; Mehta et al., 2002)
Phytoene synthase (*Psy-1*) (*S. lycopersicum*)	As above	CaMV 35S (OE)	↓ phenylpropanoids and flavonoids (Fraser et al., 2007)

Abbreviations: CaMVd35S: Cauliflower mosaic virus double 35S promoter; TomLoxB, Tomato fruit-specific promoter; CVM, Constitutive cassava vein mosaic promoter; TAGL1-SRDX, Chimeric TAGL1 fused to EAR (ERF-associated amphiphilic repression) motif that functions as dominant repressor, SAM, *S*-adenosylmethionine; Other detail as in Table 1.1.

Tomato fruits synthesize significant amount of carotenoids but are poor in production of flavonoids in fruit flesh. Flavonoid production in fruits is mainly restricted to peel with accumulation of naringenin-chalcone, flavonol rutin and kaempferol 3-*O*-rutinoside (Bovy et al., 2002; Crozier et al., 1997; Muir et al., 2001). The major focus of flavonoid biotechnology research is to increase flavonoid accumulation in fruit flesh, and determine the potential to induce production of new flavonoids (Table 1.2). Tomato fruit do not have stilbene synthase gene (*StSy*) (Giovinazzo et al., 2005) and cannot normally produce resveratrol, a stilbenoid flavonoid. However, expression of a grape *StSy* induced the production of not only resveratrol in transgenic fruits, but also led to accumulation of *trans*-resveratrol and *trans*-resveratrol-glucopyranosides (piceid), further elevating the antioxidant capacity in tomato fruit (D'Introno et al., 2009). Transgenic tomato lines were developed which constitutively expressed flavonoid genes from different plant species (Schijlen et al., 2006). It was shown that the expression of grape *StSy* produced high amount of resveratrol and piceid (a stilbenoid glucoside), while combined expression of petunia chalcone synthase and alfalfa chalcone reductase induced high levels of butein and isoliquiritigenin (deoxychalcones). Combined expression of petunia chalcone isomerase and gerbera flavone synthase resulted in elevated production of luteolin-7-glucoside, luteolin aglycon (flavones) and quercetin glycosides (flavonols). Although the constitutive expression of *StSy* produced up to 10-fold high resveratrol it resulted in complete male sterility likely due to lack of coumaric and ferulic acid production (Ingrosso et al., 2011). The seedless parthenocarpic fruit phenotype resulting from male sterility is of much interest because of its desirability by both the consumer and food industry (Ficcadenti et al., 1999; Pandolfini et al., 2002; Rotino et al., 1997).

The ectopic expression of petunia chalcone isomersae (*CHI*) in tomato resulted in 78-fold increase in peel flavonols, which was mainly due to accumulation of rutin, a quercetin glycoside. After processing tomato fruits, paste still retained 65% of the total flavonols present in fresh fruit (Muir et al., 2001). Although isoflavones are legume-specific flavonoids, tomato plants engineered to constitutively express soybean isoflavone synthase (*35S:GmIFS2*) showed significant accumulation of genistin (a major

isoflavone metabolite) in leaves with a marginal increase in fruit peel. Naringenin chalcone biosynthesis was also upregulated in these transgenic fruit indicating naringenin as a limiting factor (substrate) for isoflavone biosynthesis in fruit peel (Shih et al., 2008).

In addition to the candidate gene approach to enhance flavonoid content, transcription factors have also been tested to achieve similar objectives. Coordinated expression of maize MYB-type *C1* and MYC-type *LC*, transcription factors implicated in anthocyanin production, in tomato induced flavonoid biosynthesis in fruit flesh the tissues where flavonoids are very poorly synthesized (Bovy et al., 2002). Overall, 10-fold increase in total flavonoids and 20-fold increase in total flavonols in ripe tomato fruit was achieved mainly due to increased production of kaempferol in transgenic fruit (Bovy et al., 2002; Le Gall et al., 2003). Expression of *Rosea1* and *Delila,* transcription factors that activate biosynthesis of anthocyanin, driven by E8 promoter resulted in enhanced anthocyanin production in tomato pericarp at concentrations comparable to blackberries and blueberries (Butelli et al., 2008). Transgenic tomato fruits constitutively expressing *ANTHOCYANIN1* (*ANT1*), a flavonoid-related R2R3-MYB transcription factor, had high levels of anthocyanadins including that of petunidin, malvidin and delphinidin (Schreiber et al., 2012). Down regulation of *TOMATO AGAMOUS-LIKE 1 (TAGL1),* a MADS-box transcription factor resulted in lowering the levels of lycopene and isoprenoids whereas its overexpression caused high accumulation of lycopene and naringenin chalcone (Itkin et al., 2009).

Altering expression of transcriptional regulators of photomorphogenic responses enhanced production of flavonoids. Fruit-specific RNAi-mediated silencing of *DET1*, transcriptional repressor of photomorphogenic responses, not only increased carotenoids but also increased flavonoids by 3.5-fold (Davuluri et al., 2005). Constitutive overexpression of Cryptochrome 2 resulted in about 3-fold increase in flavonoids (Giliberto et al., 2005). Fruit-color tomato mutant *hp-1*, carrying mutation in *DDB1* increased levels of both carotenoids and flavonoids (chlorogenic acid and rutin) (Long et al., 2006). Likewise, RNAi-mediated repression of DDB1-interacting protein CUL4 in

tomato lines (*35S:CUL4-RNAi*) resulted in elevated levels of anthocyanins and carotenoids (Wang et al., 2008).

A mutation in phytoene synthase *PSY-1 (*tomato mutant *rr)* did not increase levels of phenylpropanoids and flavonoids (chlorogenic acid, caffeic acid, *p*-coumaric acid and ferulic acid) in pericarp tissues (Long et al., 2006), but constitutive overexpression of *PSY-1* showed increase in phenylpropanoids and flavonoids including 3-caffeoylquinic, naringenin-chalcone and quercetin derivatives in red ripe tissues (Fraser et al., 2007).

Other studies have highlighted the importance of the afore-mentioned strategies in either enhancing the biological activity of endogenous flavonoids or achieving fruit quality attributes rather than just enhancement of flavonoids. For example, prenylated flavonoids, derived from addition of hydrophobic molecules to flavonoids, are biologically more active than their native forms possibly because of lipophilicity of prenyl moiety which makes flavonoids more membrane permeable (Maitrejean et al., 2000; Murakami et al., 2000). Fruit-specific expression of *Streptomyces* prenyltransferase *HypSc* in tomato fruits resulted in accumulation of 3'-dimethylallyl naringenin, a prenylated form of native naringenin flavonoid (Koeduka et al., 2011). The other example of achieving an industry-driven objective is to induce parthenocarpy (Ficcadenti et al., 1999; Pandolfini et al., 2002; Rotino et al., 1997).

Together, these results provided strong evidence in favor of biotechnological interventions for not only enhancing the levels and composition of health-related polyphenols in fruits, but also to produce novel compounds by the engineering flavonoid and other pathways (Schijlen et al., 2006). Studies on tomato model system mentioned above clearly support the significance of transgenic approaches in enhancing sensory fruit quality attributes. Similar approaches have also been adopted to manipulate flavonoid biosynthetic pathway in strawberry (Lunkenbein et al., 2006), maize (Li et al., 2007; Sidorenko et al., 2000), grapes (Bogs et al., 2007; Boss et al., 1996), rice (Furukawa et al., 2007; Shin et al., 2006), *Medicago truncatula* (Pang et al., 2007), citrus (Frydman et al., 2004; Koca et al., 2009; Moriguchi et al., 2001), brassica (Auger et al., 2009; Hüsken et al., 2005; Nesi et al., 2009; Wei et al., 2009), flax seed (Lorenc-Kukula et al., 2005; Zuk et al.,

2011), apple (Ban et al., 2007; Flachowsky et al., 2012; Flachowsky et al., 2010; Han et al., 2012; Rühmann et al., 2006), soybean (Nagamatsu et al., 2007) and tobacco (Aharoni et al., 2001).

1.5 Molecular engineering of flavor volatiles

Flavor, an important quality attributes of a fruit, is the sum of specific interactions of fruit constituents among which sugars, acids and a number of volatile molecules are significant components (Mathieu et al., 2009). Preference for a specific flavor (sugar:acid ratio) and perception of volatiles by olfactory receptors in human nose are partly a social/cultural science that vary with diversity in ethnicity, age, and personal likes and dislikes. In general, components concentration and odor threshold are important variables in determining contribution of various volatiles to fruit flavor (Baldwin et al., 2000). Most fruits and vegetables produce aromatic volatiles as has been revealed by studies on mango (Andrade et al., 2000; MacLeod et al., 1988; MacLeod and Snyder, 1985), guava (Porat et al., 2011; Wilson et al., 1982), water melon (Lewinsohn et al., 2005), apple (Dixon and Hewett, 2000), strawberry (Song et al., 1998) and tomato (Buttery et al., 1988; Buttery Ron and Ling Louisa, 1993; Christiansen et al., 2011; Krumbein and Auerswald, 1998; Marković et al., 2007; Maul et al., 1997; Mayer et al., 2008). Over 400 aroma volatiles have been detected in tomato, but less than 30 have been proposed to impact organoleptic properties (Baldwin et al., 2000; Tieman et al., 2006b). These aroma and flavor volatiles include *cis*-3-hexenal, β-ionone, hexanal, β-damascenone, 1-penten-3-one, 2-methylbutanal, 3-methylbutanal, trans-2-hexenal, isobutylthiozole, and trans-2-heptenal (Goff and Klee, 2006; Klee, 2010; Mathieu et al., 2009; Zeigler, 2007).

The fruit breeding programs that focused on developing larger and firmer fruits with extended shelf life have largely ignored organoleptic attributes with unintended consequence of loss of flavor components (Mathieu et al., 2009). The manipulation of flavor components in fruits via biotechnology has been limited particularly because biosynthetic pathways are complex and known only for limited volatile compounds. Thus

the nature and biosynthetic pathways of many volatile compounds still remain to be discovered (Tieman et al., 2006b). The availability of new molecular genetics tools has begun to change this inactivity and efforts to improve fruit flavor components by genetic engineering have a good future. The QTLs regulating production and accumulation of several volatiles compounds in tomato have been identified and functional characterization of genes present on these loci has begun (Mathieu et al., 2009; Tieman et al., 2006b). Transgenic studies on tomato engineered to alter fruit flavor volatiles are listed in Table 1.3.

Most of the flavor volatiles are synthesized during fruit ripening, reaching a maximum at or before full ripening (Klee and Giovannoni, 2011). This temporal regulation of volatile compounds is maintained through the production of their precursors including lipid, carotenoids, amino acids (Iijima et al., 2004) and keto acids (Kochevenko et al., 2012). Aromatic volatiles, 2-phenylacetaldehdye and 2-phenylethanol are derived from phenylalanine and significantly contribute to tomato fruit flavor. A family of aromatic amino acid decarboxylases (*SlAADC1A, SlAADC1B, SlAADC2*) was characterized (Tieman et al., 2006a). Constitutive overexpression of either *SlAADC1A* or *SlAADC2* was found to increase emission of 2-phenylacetaldehyde, 2-phenylethanol and 1-nitro-2-phenylethane greater that 10-fold in transgenic tomato fruit compared to wild type fruit. Also the antisense inhibition of *SlAADC2* resulted in reduced emission of these volatiles. Expression of tomato phenylaldehyde reductase (*SlPAR1, SlPAR2*) in transgenic petunia accelerated the emission of 2-phenylethanol at the expense of 2-phenylacetaldehyde (Tieman et al., 2007). However, how expression of this gene affects quality or quantity of volatiles in fruits has not as yet been evaluated.

Table 1.3: Studies on tomato engineered to alter fruit flavor volatiles

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
Amino acid aromatic decarboxylase (*AADC1A*) (*S. lycopersicum*)	Phenylalanine → phenylethylamine	FMV35S (OE)	10-fold ↑ 1-nitro-2-phenylethane, 2-phenylethanol, 2-phenylacetaldehyde (Tieman et al., 2006a)
SA methyltransferase (*SAMT*) (*S. lycopersicum*)	SA → methyl salicylate	FMV35S (OE)	123-fold ↑ methyl salicylate (Tieman et al., 2010)
ω-3 fatty acid desaturase (*FAD3*) *(Brassica napus*) or/and (*FAD7*) (*S. tuberosum*)	Linoleic acid (18:2) → linolenic acid (18:3)	CaMV 35S (OE)	↑ 18:3/18:2 ratio (Domínguez et al., 2010)
α-Zingiberene synthase (*ZIS*) (*Ocimum basilicum*)	Farnesyl diphosphate → α-zingiberene	PG (OE)	↑ α-zingiberene, other sesquiterpenes & monoterpenes (Davidovich-Rikanati et al., 2008)
Geraniol synthase (*GES*) (*Ocimum basilicum*)	Geranyl diphosphate → geraniol	PG (OE)	↑ carotenoid-derived aroma volatiles ↓ phytoene, lycopene and β-carotene (Davidovich-Rikanati et al., 2007)
Carotenoid cleavage dioxygenase	Carotenoids → volatile terpenoid compounds	FMV35S (AS)	50% ↓ β-ionone (50%) ≥60% ↓ geranylacetone

Transgene	Metabolic reaction or function	Promoter Expression	Metabolic phenotype
(*CCDIB*) (*S. lycopersicum*)			No morphological alterations or changes in carotenoids (Simkin et al., 2004)
Lipoxygenase (*TomLoxC*) (*S. lycopersicum*)	Chloroplast-targeted lipoxygenase isoform, C_6 volatiles made at the expense of linoleic and linolenic acids	CaMV 35S (AS)	1.5% ↓ hexanal, hexenal, hexanol (Chen et al., 2004)
S-linalool synthase (*LIS*) (*Clarkia breweri*)	Geranyl diphosphate → *S*-linalool	E8 (OE)	↑ *S*-linalool and 8-hydroxylinalool No change in phenotype or in terpenoids (Lewinsohn et al., 2001)
Fibrillin (*FIB1, FIB2*) (*C. annuum*)	Involved in synthesis of lipoproteins in certain chromoplast types	Native (OE)	2-fold ↑ carotenoids i.e. 118% ↑ lycopene 64% ↑ β-carotene, 36% ↑ β-ionone, 74% ↑ β-cyclocitral, 50% ↑ citral, 122% ↑ 6-methyl-5-hepten-2-one, 223% ↑ geranylacetone (Simkin et al., 2007)
ODORANT 1 (*ODO1*) (*P. hybrida*)	R2R3-type MYB transcription factor that positively regulates volatile benzoid levels, synthesizing precursors from shikimate pathway	E8 (OE)	No increase in phenylalanine-derived volatile compounds (Dal Cin et al., 2011)

Abbreviations are given in Table 1.1 and Table 1.2.

Hexanals and (*Z*)-hex-3-enal are derived from lipoxygenase pathway and high (*Z*)-hex-3-enal/hexanal ratio correlates with high consumer appreciation of tomato varieties (Carbonell-Barrachina et al., 2006). Omega-3 fatty acid desaturase converts linoleic acid (18:2) to linolenic acid (18:3), the precursor of hexanal and its derivatives. Expression of ω-3 fatty acid desaturase (*BnFAD3*) from *B. napus* in transgenic tomato increased the ratios of 18:3/18:2 and (*Z*)-hex-3-enal/hexanal (Domínguez et al., 2010). The constitutive expression of an antisense gene of chloroplast-targeted lipoxygenase *TomloxC* greatly reduced the production of hexanal, hexenal and hexanol compared to WT (Chen et al., 2004).

Monoterpenes and sesquiterpenes are other important contributors to fruit aroma and volatile components and are connected with the early steps of carotenoid biosynthesis pathway (Figure 1.2). Alpha-zingiberene synthase (*ZIS*) catalyzes the formation of α-zingiberene and other sesquiterpenes from farnesyl diphosphate while geraniol synthase (*GES*) catalyzes the conversion of geranyl diphosphate to geraniol (Iijima et al., 2004). Geraniol is an acyclic monoterpene and precursor of geranial, nerol, citronellol and geraniol and citronellol acetate esters (Davidovich-Rikanati et al., 2007) the compounds that are produced in very minute amounts in ripe tomato fruit (Baldwin et al., 2000). Over expression of lemon basil geraniol synthase under the control of tomato PG promoter resulted in many-fold enrichment of endogenous carotenoid-derived aroma volatiles at the expense of phytoene, lycopene and β-carotene and induced biosynthesis of geraniol and its derivatives and monoterpenes which were not detected in WT fruit (Davidovich-Rikanati et al., 2007). Linalool, another monoterpene, is directly synthesized from geranyl diphosphate by linalool synthase. Tomato fruit does not contain any linalool synthase activity. However, tomato transformed with *Clarkia breweri S*-linalool synthase gene under E8 promoter exhibited greatly induced production of *S*-linalool and 8-hydorxylinalool with no alteration in the phenotype or in the level of terpenoids (Lewinsohn et al., 2001). Transgenic tomato fruit expressing lemon basil α-zingiberene synthase under fruit ripening-specific PG promoter produced high levels of α-

zingiberene and other sesquiterpenes and monoterpenes which were otherwise undetectable in the WT fruit (Davidovich-Rikanati et al., 2008).

Apocarotenoid volatiles, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, β-ionone, β-cyclocitral and geranylacetone, are derived from carotenoid degradation. Thus, production of apocarotenoid volatiles depends on the type and amount of carotenoids being synthesized and the stage of ripening. Constitutive overexpression of native carotenoid cleavage dioxygenases antisense gene did not alter plant morphology or carotenoid accumulation in fruit tissues, but reduced β-ionone by 50% and geranylacetone by ≥60% (Simkin et al., 2004). Since carotenoids are synthesized in plastoglobules, lipid bodies within plastids, and none of the carotenoid cleavage dioxygenase genes is upregulated during ripening, the ripening-associated increase in these volatiles were attributed to physical change in plastids, *e.g.*, chromoplast differentiation (Klee and Giovannoni, 2011). Several studies suggest that carotenoid-derived synthesis of aroma volatiles is ethylene-dependent. However, the pericarp discs of *ACS*-suppressed transgenic tomato fruit deficient in ethylene production converted the exogenously applied lycopene into carotenoid-related volatiles. These results suggest that carotenoids biosynthesis is ethylene-dependent but degradation into volatile compounds ethylene-independent (Gao et al., 2008). More *in vivo* experiments are needed to separate out the role of ethylene in carotenoid production and their catabolism. Carotenoids are also synthesized from chloroplast-derived isoprenoids and their levels increase with total chromoplast area per cell in ripe fruit pericarp of *hp-1* tomato mutant (Cookson et al., 2003; Wang et al., 2008). This led to test the hypothesis that elevating biosynthesis of structural chromoplasts proteins would increase the emission of carotenoid-derived volatile compounds. Transgenic tomato lines over expressing pepper fibrillin, a protein involved in the synthesis of lipoprotein in chromoplast, exhibited increased lycopene (118%) and β-carotene (64%) (Simkin et al., 2007). Elevations in the emission of β-ionone (36%), β-cyclocitral (74%), citral (50%), 6-methyl-5-hepten-2-one (122%) and geranylacetone (223%) were also recorded in these transgenic fruit as a

consequence of increase in the availability of carotenoids for cleavage activity (Simkin et al., 2007).

In addition to the production of volatile compounds from phenylalanine, carotenoid or lipoxygenase-mediated pathways, other sources of important volatile compounds are now known and include guaiacol, synthesized by methylation of catechol that contributes smoky aroma to tomato flavor. Tomato lines silenced for or overexpressing a catechol-*O*-methyltransferase (*CTOMT1*) provided evidence that this gene was responsible for the production of guaiacol in tomato (Mageroy et al., 2012). Transgenic tomato lines constitutively over or under expressing SA methyl transferase (*SlSAMT*) due to sense or antisense chimeric gene construct confirmed functional role of *SlSAMT* in the production and emission of methyl salicylate (Tieman et al., 2010).

The availability of high quality genome sequence of tomato has led to its potential as a fruit-bearing model system to understand and improve fruit quality attributes. Thus, similar transgenic approaches applied to apple (Brown, 2009; Dandekar et al., 2004; Defilippi et al., 2004, 2005a; Defilippi et al., 2005b; Schaffer et al., 2007), cucumber (Zawirska-Wojtasiak et al., 2009), grape (Battilana et al., 2011), berries (Malowicki et al., 2008), strawberry and banana (Beekwilder et al., 2004), potato (Di, 2009), basil (Dudai and Belanger, 2009), melon (Flores et al., 2002) and oranges (Rodríguez et al., 2011a; Rodríguez et al., 2011b) have identified various enzymes involved in volatile biosynthesis pathway and their interaction with genetic and environmental factors such as ethylene and pathogen responsiveness.

1.6 Future perspective

The first edible transgenic crop, ‘Flavr Savr’ tomato, was released for human consumption in 1992, some 20 years ago (Kramer and Redenbaugh, 1994; USDA-APHIS, 1991, 1992). ‘Flavr Savr’ was produced by antisense RNA technology to have reduced *PG* expression and a promise to maintain texture of the ripened tomato fruit after harvest and during long distance transportation (Kramer and Redenbaugh, 1994). It was a big leap

but was not sufficient enough to meet market expectation (Giovannoni et al., 1989; Thakur et al., 1997). However, it provided the impetus and a path to genetically modified crops for enhancing various desirable traits some of which have been discussed in this chapter. Most of the first generation genetically-engineered agronomical crops were developed based on manipulation of simple monogenic traits such as herbicide or insect resistance. Examples of successful genetic engineering of fruit crops, discussed in this chapter, are a testament to an approach that is robust and powerful. Thus, rational strategies have resulted in enhancing several desirable qualities attributes in fruit crops and produced novel phenotypes by employing the gain- or loss-of-function of a candidate gene.

Many desirable crop traits are, however, multigenic in nature, the final outcome being a function of a group of genes. Therefore, enhancing a multigenic trait became a focus of the second-generation genetically engineered crops. These basic strategies were used to accomplish this objective. Because transcription factors could control a number of downstream genes, using them to engineer crops to introduce complex polygenic traits such as tolerance against abiotic stresses and enhancing production of secondary metabolites was another means to co-express multiple genes. Some success using such an approach has been achieved. However, most metabolic pathways have rate limiting step(s) and the simultaneous expression of a number of genes may not always help boost the intended trait(s). Also, such a strategy may introduce a negative over-ride of metabolism and result in lowering the desired attribute(s). Nonetheless, simultaneous introduction of several genes helped develop high β-carotene-rice which is designated as Golden Rice (Paine et al., 2005). This study also demonstrated that the source of gene(s) plays a significant role in increasing a preferred molecule/nutrient, for instance, the use of carotene desaturase from *E. uredovora* resulted in a 23-fold increase in total carotenoids in rice (Paine et al., 2005). It is implicit from such studies that a clear understanding of the complex gene expression and the process of the production of a desired metabolite is needed to enable targeted expression of a transgene at a desired stage of development of a specific tissue. In this context and to solve such complex

hurdles, significant new knowledge is desired to develop chimeric promoters and accomplish targeted expression of the introduced genes at a specific stage of fruit development.

In the near future, we see a need for complementary interaction of practitioners of biotechnology and conventional breeding methods to accelerate development of novel fruit varieties with enhanced and much desired attributes. Molecular genetic tools such as QTL mapping, chromosome walking, genome sequencing, and bioinformatics are powerful catalysts whose use can help bring these approaches together. Whereas, the recombinant DNA approach via transformation provides a direct path to introduce new traits in elite germplasm, more work and effort are required to get rid of undesirable traits introduced by QTL-based approach. The availability of molecular markers associated with known traits should, however, facilitate the use of this approach to introduce desirable attributes in fruit crops. We see a bright and exciting future for precision-based engineering of quality attributes in fruit crops

The biogenic amines, SPD and SPM, which belong to the group of ubiquitous polycations called PAs have also been implicated in increasing carotenoid content in tomato. Constitutive expression of *Saccharomyces cerevisiae SpdSyn* (*35S:ySpdSyn*) or fruit-specific expression of *SAMdc* (*E8:ySAMdc*) in tomato lead to 40% or 200-300% increase in lycopene content, respectively (Mehta et al., 2002; Nambeesan et al., 2010). Transcriptome analysis of high PA-accumulating *E8:ySAMdc* tomato fruits showed an upregulation in the transcription profiles related to carotenoid and flavonoid biosynthesis pathways (Mattoo et al., 2007). These fruit also exhibited increase in carotenoids (70% at 27 DAB), *cis*-10-heptadecanoic acid (50%), linolenic acid (20%) and nervonic acids (28%) (Kolotilin et al., 2011). Transgenic expression of apple *SpdSyn* in tomato fruit not only upregulated *PSY* and *PDS* but also downregulated catabolic enzymes lycopene β- and ε-cyclases resulting in an overall 1.3- to 2.2-fold increase in lycopene content (Neily et al., 2010). These findings indicate a positive correlation between PAs and carotenoids levels during fruit ripening and microarray-based transcriptional profiling of *E8:ySAMdc* tomato fruits (Kolotilin et al., 2011).

In addition to enhance fruit nutrients, PAs have also been implicated in delaying fruit ripening and extending vine/shelf life (Kolotilin et al., 2011; Mehta et al., 2002; Nambeesan et al., 2010; Neily et al., 2010). However, the full impact of PAs, especially different forms of PAs (free, conjugated and bound) on fruit metabolic processes leading to fruit quality has not yet been fully understood. So, during this study, I have characterized molecular functions of PAs in tomato fruit development and ripening.

CHAPTER 2. POLYAMINES REGULATE FRUIT ARCHITECTURE BY MODIFYING CELL CYCLE, CELL EXPANSION AND FRUIT SHAPE GENES

2.1 Introduction

Significant progress has been made in cloning, partially characterizing and genomic analyses of genetic components regulating fruit shape and size (Lin et al., 2014). Six out of nine loci initially identified via QTL mapping to control fruit shape and size have been cloned and characterized (Monforte et al., 2014; Tanksley, 2004; van der Knaap et al., 2014) and include *CNR/FW2.2* (Frary et al., 2000) *KLUH*/*FW3.2* (Chakrabarti et al., 2013), *SUN1* (Xiao et al., 2008), *OVATE* (Liu et al., 2002), *LC* (Muños et al., 2011), *FAS* (Cong et al., 2008), *fw11.3* (Huang and van der Knaap, 2011) and *fs8.1* (Clevenger, 2012). Out of these genes the role of *FW2.2*, *SUN1* and *OVATE* have been characterized at more detail. The *FW2.2* negatively regulates cell division during flower development (Frary et al., 2000) and controls up to 30% and 47% of the total variation in fruit mass in *L. pimpinellifolium* and *L. pennellii*, respectively (Alpert et al., 1995). Mutation in the 2.7 kb upstream in promoter region of the *FW2.2* gene impaired its expression resulting in the larger fruit phenotype during domestication of tomato (Nesbitt and Tanksley, 2002). *SUN1* gene arose from a retrotransposon-mediated gene duplication event during tomato genome evolution (Jiang et al., 2009; Xiao et al., 2008). It encodes a IQ67 domain-containing protein (van der Knaap and Tanksley, 2001) that induces fruit elongation by changing cell division pattern in tomato fruits (Wu et al., 2011). *OVATE* belongs to ovate family proteins (OFPs) and is a negative regulator of plant growth and fruit elongation (Ku et al., 1999; Tsaballa et al., 2011). A mutation in its carboxyl-terminal domain resulted in transformation of round fruit into pear-shaped (Liu et al., 2002). In summary, at least three QTLs impact fruit size and shape by regulating cell division (*FW2.2*), symmetry

(*OVATE*) and cell division pattern (*SUN1*), but the molecular regulators of these genes expression remain to be determine.

PAs are ubiquitous biogenic amines that are required for cell proliferation in mammals, bacteria and yeast (Chattopadhyay et al., 2003; Igarashi and Kashiwagi, 2010; Pegg, 2009; Theiss et al., 2002; Wallace, 2009). PUT, SPD and SPM are three major PAs abundantly found in eukaryotes including plants (Nambeesan et al., 2008). Multifaceted role of PAs in promoting cell cycle progression has been demonstrated in fibroblasts and intestinal epithelia cells where accumulated PAs stimulated cell division by promoting *CYCD1* and *CDK4* and reduced $p27^{Kip1}$ activity, a homolog of *KRP1* in plants (Ravanko et al., 2000; Xiao et al., 2011). Cell cycle progression is regulated by complexes of cyclins and CDKs (Blomme et al., 2013). CDKs can either be activated by CDK-activating kinases (Umeda et al., 2005) or inhibited by CDK inhibitors like *KRPs* and *WEE1* gene products (Gonzalez et al., 2007; Nafati et al., 2010). Exogenous applications of PUT, SPD and SPM have been reported to elevate expression levels of both *CYCA* and *CYCB* to simulate cell division in tobacco BY-2 cells (Jang et al., 2006). Collectively, these studies implicate a role of PAs in regulating cell division and expansion in mammalian cells, yeast, bacteria and in suspension cultures of plant cells. However, functions of PAs in regulating various factors involved in cell cycle and endoreduplication leading to plant and fruit development and architecture are still not understood.

The biogenic amines play crucial roles in growth and development of plants especially during cell division and biotic and abiotic stresses (Tiburcio et al., 2014). Altered levels of PAs, either by mutation or by transgenic expression of PA biosynthetic genes, are associated with a number of phenotypes in plant including dwarfism, leaf twisting, branched stems, small and chlorotic leaves, small and elongated tubers, increased or poor lateral root branching and root growth, altered floral organ formation leading to reduced seed formation, male sterility, parthenocarpy and altered tolerance to biotic and abiotic stress (Nambeesan et al., 2008 and references therein). We have analyzed and collated the information on changes in transcriptome of plant cells with altered PA levels to determine the cross-talk of PAs with plant hormones. Our results showed a complex

network relationship among the three PAs and the biosynthesis and signaling pathways of plant hormones (Anwar et al., 2015).

We have previously developed transgenic tomato plants expressing yeast *SpdSyn* (*ySpdSyn*) under the control of a constitutive (CaMV 35S) or a fruit specific (SlE8) promoter (Nambeesan et al., 2010). Senescence in these transgenic lines was inhibited and their fruits exhibited longer shelf life. Additionally, fruits from these transgenic lines exhibited more obovoid phenotype compared to WT fruit. Herein we show that *ySpdSyn* transgene had a profound effect on PA levels, especially conjugated forms of PAs, and their levels were correlated with the fruit shape, cell division and cell expansion regulating genes. These results provide first evidence of molecular regulation of these genes by PAs leading to determination of fruit shape.

2.2 Material and methods

2.2.1 Plant material and growth conditions

Generation of the transgenic tomato lines homozygous for *ySpdSyn* gene driven by either constitutive CaMV 35S promoter (lines C4 and C15) or fruit/ethylene-specific SlE8 promoter (line E8-8) have been previously described (Nambeesan et al., 2010). Transgenic and parental WT plants were grown in high porosity potting mix (52Mix, Conard Fafard Inc., MA USA) under glasshouse environment with 16h day/8h night photoperiod and 23°C day/18°C night temperature. Tomato flowers and fruit developmental stages were tagged and samples were collected at 10 and 5 days before pollination (DBP) and 2, 5, 10 and 20 days after pollination (DAP) (Figure 2.1) and immediately frozen in liquid N_2 and stored at -80°C until further use.

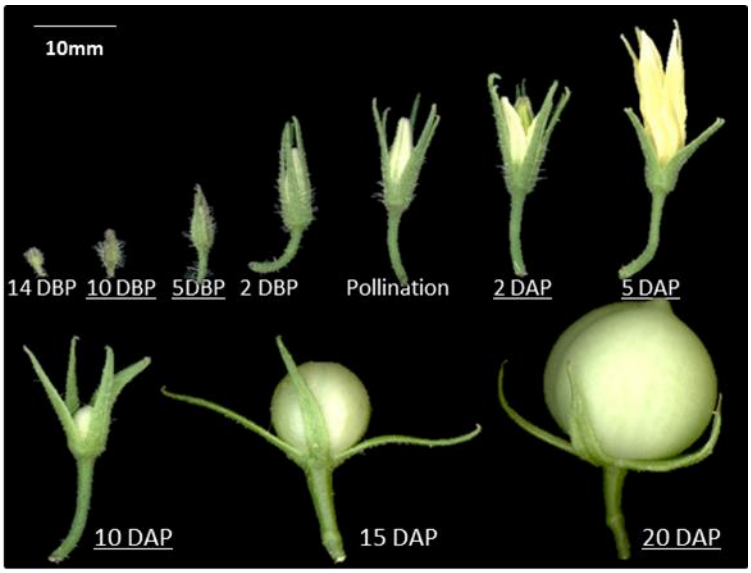

Figure 2.1**:** Representative flower and fruit developmental stages registered in tomato cv. Ohio8245.

Underlined flower and fruit samples at 10 and 5 days before pollination (DBP) and 2, 5, 10 and 20 days after pollination (DAP) were used in cytological, transcriptional and PA analyses.

2.2.2 Cytological analysis

Proximal distal axis slices of fresh tissues were fixed in 10% formalin (pH 6.8-7.2) and processed in Tissue-Tek VIP® (Sakura Finetek USA, Inc.) using following sequential treatments: 70% ethanol for 50 sec; 95% ethanol for 50 sec (2 cycles); 100% ethanol for 33 sec (3 cycles); toluene for 60 sec (2 cycles); paraffin for 45 sec at 63°C (4 cycles). For deparaffinization, tissues were dipped in xylene for 5 min (2 cycles), 100% ethanol for 2 min, 95% ethanol for 2 min, 70% ethanol for 2 min followed by rehydrated in deionized water. Tissues were embedded in paraffin using Cryo-therm (Lipshaw, USA) and 5-7µm thick sections were obtained using microtome (Finesse ME, Thermo Electron, USA). Tissues were stained with 1% toluidine blue–O, sections, dehydrated by serial quick dips in 70%, 95% and 100% ethanol and xylene (Sheehan and Hrapchak, 1987). Slides were scanned with ScanScope CS (Aperio Technologies, Inc. USA) at different magnifications, 40x being the maximum. The digital images were analyzed for cell number in exocarp, mesocarp and endocarp (van der Knaap et al., 2014) and vertical cell layers from exocarp to endocarp (Cheniclet et al., 2005) using ImageScope 11 (Aperio Technologies, Inc. USA), and cell size was using ImageJ (Schneider et al., 2012). At least three independent biological replicates were analyzed at each stage from each genotype.

2.2.3 Transcript analysis by quantitative real-time PCR

For qRT-PCR, the liquid N_2 frozen tissues were ground to powder, total RNA extracted from 100 mg tissue powder with QIAzol® Lysis reagent (Qiagen Sciences, USA) and purified using RNeasy® Mini Kit (Qiagen Sciences, USA). The RNA samples were treated with RQ1 RNase-free DNase (Promega Corporation, USA) and first-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, USA). GoTaq® qPCR Master Mix (Promega, USA) was used in qRT-PCR reaction mixture. All primers and cDNA templates were optimized for gene expression analysis according to $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) was used with program sequence as follows: 95°C for 10 min; 95°C for 15 sec and

60°C for 60 sec (40 cycles); 95°C for 15 sec and 60°C for 60 sec. Comparative C_T values of gene expressions were quantified using StepOne™ 2.0 software (Applied Biosystems, USA). *Actin* was used as standard housekeeping gene to normalize the expression of target genes. Accession numbers of all genes with their primer sequences used in this study are listed in Table 2.1. All reported data represent average ± standard error of at least three independent biological replicates.

2.2.4 Quantification of PAs by high pressure liquid chromatography

PAs in floral buds and fruit tissues of tomato plants were extracted and dansylated as described by Torrigiani et al. (2012) with some modifications. Briefly, 200 mg of finely ground sample from whole floral buds or ovaries was homogenized in 800 µl of 5% ice-cold perchloric acid (PCA) using a hand held homogenizer. The homogenate was centrifuged at 20,000 g for 30 min at 4°C after 60 min incubation at 4°C. Supernatant (100µl) was either used directly or first hydrolyzed in equal volume of 6N HCl for 18 h at 110°C to dansylate and quantify free or PCA-soluble conjugated PAs, respectively. To quantify PCA-insoluble bound PAs, the pellet was washed twice with 5% PCA, re-suspended in 800 µl of 5% cold PCA, and hydrolyzed in equal volume of 6N HCl for 18 h at 110°C. Saturated sodium carbonate (200µl) and 1,7-heptanediamine (400µl, as an internal standard) were added to the 100µl supernatant or the hydrolysates and dansylated with dansyl chloride for 60 min at 60°C under dark conditions. Dansylation was terminated by adding 100µl proline and incubating the reaction mixture for 30 min at 60°C. Dansylated PAs were extracted in 500µl toluene, air dried, dissolved in 250µl acetonitrile. Samples were diluted four times with acetonitrile, filtered through 0.45µm syringe filter (National Scientific, USA) and separated on a reversed-phase Nova-Pak C18 column (3.9 x 150mm, 4.0µm pore size) on Waters 2695 Separation Module equipped with Waters 2475 Multi λ fluorescence detector (excitation 340 nm, emission 510 nm) using a binary gradient composed of solvent A (100% Water) and solvent B (100% acetonitrile) at 1 ml/min. Initial conditions were set at 60:40 (A:B) and then linear gradient

was proceeded with conditions set at 30:70 (A:B) at 3 min; 0:100 (A:B) at 10 min and 60:40 (A:B) at 12 min. Column was flushed with 60:40 (A:B) for 3 min after before next sample injection. To determine PAs recovery and generate calibration curves, standard PAs (Sigma-Aldrich, USA) were used as control. PAs were integrated and quantified using Millennium^{32} 4.0 from Waters Corporation. PCA-soluble, PCA-soluble but detectable after hydrolysis with HCl, and PCA-insoluble but quantified after hydrolysis were designated as free, conjugated and bound forms of various PAs, respectively, throughout the manuscript.

2.2.5 Statistical analyses

A Microsoft Excel add-in statistical package XLStat (2014.3.05) was used for ANOVA, pair-wise comparison, AHC and PCA. Ward's method (Ward, 1963) was used for the AHC analysis. The proximities among dissimilar variables was based on euclidean distance. Correlation matrix was generated using pearson (n) method. Fisher's least significant difference with confidence interval of 95% was used for pair-wise comparison analysis within genotypes at each sample stage.

Table 2.1**:** List of genes and their primer sequences used for quantitative real-time PCR analyses.

Gene ID	**Abbreviated Name**	**Forward Primer (5' to 3')** **Reverse Primer (5' to 3')**
Solyc02g085500.2.1	*OVATE*	GAGCTACCGGCAAGGTTATCG CACTATCGCGAAACTCTCCTTCA
Solyc10g079240.1.1	*SUN1*	CAAACAGCACAGCGAAGCAA TGGCGCTGTCATACATTTCAC
Solyc02g090730.2.1	*FW2.2*	TTTGCTGGGATTGACAGGATT CAAGGTGCCTCTTCCAGATCA
Solyc08g066330.1.1	*CDKA1*	ACTGCTTGGATCACGCCATT AACAGAGGCGGCTGATTCAC
Solyc04g082840.2.1	*CDKB2*	AGTGACAAACCAAGCCCTCTTC CCCAGGCCAGAGTTCTTCATT
Solyc06g065680.2.1	*CYCA2*	CCAAAAGACCAGCCCCAGAT CGCTTAGGCTGTTGAGAAGCA
Solyc02g082820.2.1	*CYCB2*	AAGGCAGCAACAGGGAAACTAA GGCTCACACTTGGCTGCATA
Solyc02g092980.2.1	*CYCD3*	AACATGATGAGCTTGCCACACT CCCCCATTAAAGACCCATCTG
Solyc09g091780.2.1	*KRP1*	GGAGAGCACACCTTGCAGTTT TACTCTGCCGTTGGCCTCAT
Solyc09g074830.2.1	*WEE1*	GCCTCTTCTTCCGGGTCACT TGCAGAAGGACGACGTGTTG
Solyc05g005710.2.1	*SlSpdSyn*	GGAGGAGGAGATGGTGGTGTCC GCAACTCCGTCACCAATGTGGAGAT
Solyc10g052470.1.1	*FSM1*	GGGATGTTTTCTTTATTGACAATGG CAGAGGTGGAATTATGGGATCCT
Solyc08g080080.2.1	*CCS52A*	CTCTGACAGGTCATACATATAGA ACAATTGTCTGTCCATCTGGAG
Solyc06g043150.2.1	*CCS52B*	TCCTGCAGCAGTGAAGGAC TCCTGCGTCTTCCTTGATTT
Solyc04g082030.1.1	*ODC*	TGCGAGCTTTTGCTTCGAAT GGTAATGCGCCGTATTTTGG
Solyc10g054440.1.1	*ADC*	CTCGGCGGACTCCATAACC GCCCAGGGACTGCATAGGT
Solyc08g079430.2.1	*CuAO*	CGATTTCCCCAATCATCCTTT CCGCAATTGAATGAACGATTT
Solyc05g013440.2.1	*CuAO-like*	CAATCGCACTGGGCAGTTAA CTCCTCAAGAATTTTGCCTCTGA
Solyc02g081390.2.1	*PAO4-like*	CCACTTCATATGCTTGCGGTTA TCGAGGTCACAAGCAAGTCTTC
YPR069C	*ySpdSyn*	AGCCACCGAAAGGGATGAATTTGC ACATAACCAGGCTTCCTCAACGGA
Solyc04g011500.2.1	*Actin*	TGG TCG TAC CAC CGGTAT TGTG AATGGCATGTGGAAGGGCATA C

References: *OVATE* (Liu et al., 2002); *CCS52A* and *CCS52B* – (Mathieu-Rivet et al., 2010)

2.3 Result

2.3.1 Expression of *ySpdSyn* altered fruit architecture

We have previously developed transgenic tomato plants expressing *ySpdSyn* under the control of CaMV 35S or fruit/ethylene-specific (SlE8) promoters (Nambeesan et al., 2010). As shown in Figure 2.2, the transgenic fruits exhibited significant alterations in fruit architecture compared to WT fruits. Three independent lines, two expressing *ySpdSyn* under the CaMV 35S promoter and another under the fruit specific E8 promoter, exhibited more obovoid fruits (lower proximal blockiness ratio), higher height-to-width ratios (fruit shape index) and lower fruit perimeter and pericarp thickness compared to WT fruits (Figure 2.3). Transgenic fruits manifested alternation in fruit shape at very young stage and maintained throughout maturation and ripening process (Figure 2.3). Similar, phenotypic alterations were observed in transgenic tomato fruits expressing yeast *SAMdc* (*ySAMdc*) under SlE8 promoter (Figure 2.2) but here we have focused only on *ySpdSyn*-expressing fruits.

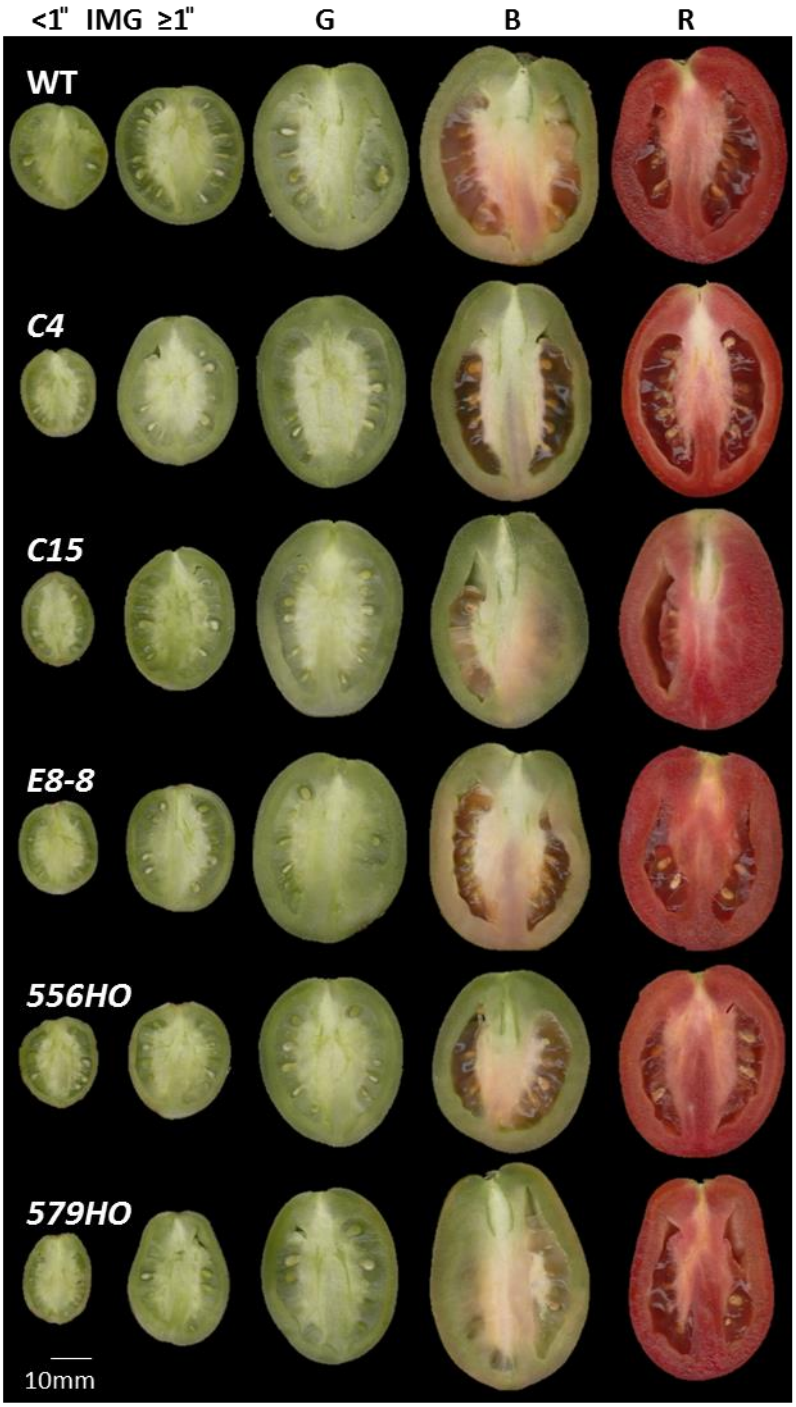

Figure 2.2**:** Phenotype of field grown WT and transgenic fruits transgenically expressing *ySpdSyn* or *ySAMdc*.

Fruits shown represent average growth and development stage of indicated genotype. The white line at bottom left corner representing 10 mm on original scale. IMG, immature green fruits with < 1 or ≥1 inch diameter; B, breaker stage; G, mature green fruits; R, red stage.

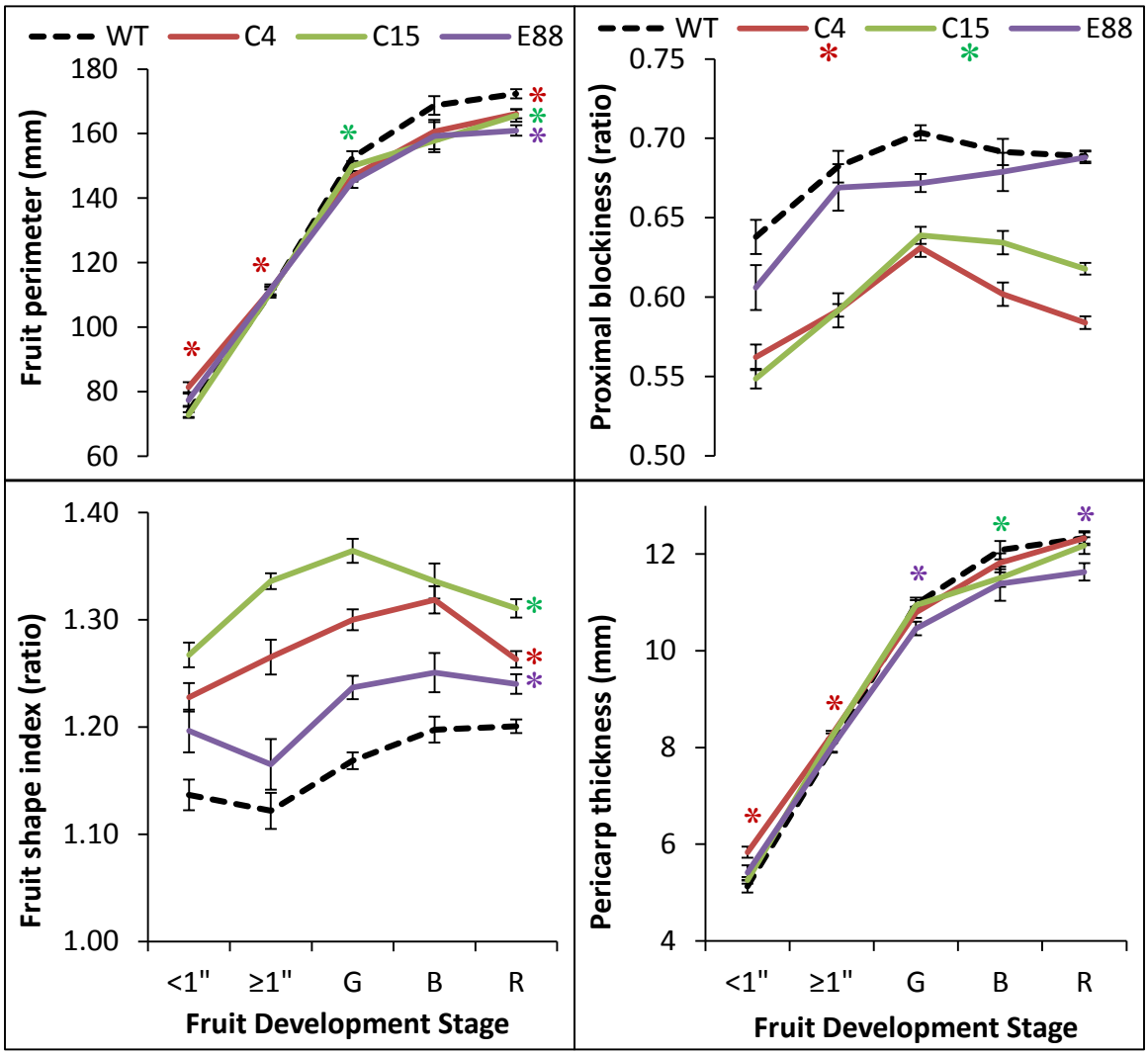

Figure 2.3: Morphometric properties of field grown WT and transgenic fruits expressing *ySpdSyn*.

Vertically cut tomato fruits were scanned and analyzed with Tomato analyzer 3.0 software. Fruit shape index is length to width ratio of fruit. Vertically cut fruits were scanned and analyzed with Tomato Analyzer 3.0 to quantify morphometric attributes. Vertical bars represent ±SE (n≥50 biological replicates). *, statistically different ($p \leq 0.05$) from WT.

2.3.2 Expression of *ySpdSyn* decreased pericarp cell layers, cell size and thickness

To examine the phenotypic basis of altered fruit architecture of transgenic fruits, cytological analyses of developing ovaries from transgenic and WT fruit were performed at 5DBP and 5, 10 and 20 DAP after staining with Toluidine Blue O. Figure 2.4a,b show the stained medial-lateral section of pericarp tissue from the WT and transgenic fruits at 10 and 20 DAP. Quantification of the microscopic pericarp images showed that thickness of pericarp in all three independent transgenic lines was significantly reduced compared to WT fruit, especially C15 and E8-8 fruit (Figure 2.4c). This decrease in transgenic pericarp thickness compared to WT fruits was associated with decrease in number of cell layers (Figure 2.4d). On average, the WT pericarp showed 38 cell layers in 20DAP whereas the C4, C15 and E8-8 pericarp had on average 32, 28 and 28 cell layers, respectively (Figure 2.4d). Analyses of cell sizes from endocarp, mesocarp and exocarp of the transgenic and WT fruits showed significant reduction in the cell size of the 10DAP mesocarp from C4 and C15 compared to WT (Figure 2.4e). However, at 20DAP the mesocarp cell size remained unchanged in C4 and C15 and significantly decreased in E8-8 fruit compared to WT fruit. Endocarp and exocarp from C4 fruit also exhibited significant increase in cell sizes than WT fruit, but this increase was much smaller than the C4 mesocarp (Figure 2.4f). Figure 2.4g shows the ratio of cell sizes in 20DAP and 10DAP fruit. There was 2 to 11-fold increase in endocarp, mesocarp and exocarp cell sizes as fruit shifted from cell division to cell expansion modes. However, this increase was much larger in endocarp and mesocarp of C4 and C15 fruits at 20DAP, suggesting a role of increased PAs in the cell expansion phase of fruit development. To determine the phenotypic basis of reduced pericarp thickness of the transgenic fruits, we evaluated the distribution of small to larger cells in pericarp tissue. As shown in Figure 2.4h, the transgenic pericarp from C4, C15 and E8-8 fruits had lower number of cells per unit area than WT pericarp indicating that higher PAs reduced the periclinal cell division leading to reduction in cell layers. Reduction in cell size was in all cell types, small to large. However, percent total distribution of small and large cells ranging from < 500 to > 5000 remained similar in all genotypes (Data not shown).

Taken together, data suggest that it is not the cell size but the cell number in medial-lateral direction of pericarp is responsible for the reduced pericarp thickness of transgenic fruits.

2.3.3 *SlSpdSyn* and *ySpdSyn* genes are differentially regulated in floral buds and fertilized ovaries

Figure 2.5 shows the qRT-PCR quantification of the expression patterns of *SlSpdSyn* and *ySpdSyn* in floral buds and fertilized ovaries from transgenic and WT plant. The transgene *ySpdSyn* under CaMV-promoter was expressed in both C4 and C15 lines at 5 DBP as well as all fruit ovaries stages (2 to 20 DAP) examined, but was differentially regulated during floral bud development to fruit. Transgene was expressed about 2-fold higher in 5 DAP ovaries than 5 DBP floral buds (Figure 2.5). The 5 DAP fruit also exhibited much higher levels of *CaMV 35:ySpdSyn* transcripts than the other stages of early fruit development examined (Figure 2.5). Transcripts of *E8:ySpdSyn* transgene were also detectable, especially at 5 DBP and 5 DAP, but levels were much lower than *35S:ySpdSyn* transcripts at any stage of fruit development examined (Figure 2.5).

The endogenous *SlSpdSyn* gene exhibited an interesting pattern. In WT fruit, *SlSpdSyn* transcripts were present at very low levels at 5 DBP and 2 and 10 DAP, but accumulated to a higher level at 5 DAP (Figure 2.5). *SlSpdSyn* transcript declined to undetectable levels in 10 DAP fruits before exhibiting another accumulation in 20 DAP fruit which was about 3-fold lower than 5 DAP fruit. The transgenic fruit exhibited similar pattern of *SlSpdSyn* transcript accumulation but their levels were significantly higher in 5 DAP in C4 and C15 and in 20 DAP C15 fruits (Figure 2.5). The pattern of *SlSpdSyn* transcript in the *E8:ySpdSyn* transgenic fruit was similar to that of WT fruit suggesting that *E8:ySpdSyn* transgene did not alter expression of endogenous *SlSpdSyn* gene (Figure 2.5).

Figure 2.4**:** Histological analysis of WT and transgenic fruitlets at 5 days before pollination and 5, 10 and 20 days after pollination.

Toluidine blue O staining of WT and transgenic fruitlets at 10 days after pollination (DAP) (a) and 20DAP (b). Changes in pericarp thickness (c), number of anticlinal cell layers in pericarp (d), cell size at 10DAP (e) and 20DAP (f), cell size ratio of 20DAP/10DAP (g) in endocarp (single innermost cell layer), mesocarp (middle 50% of the pericarp) and exocarp (2 outer cell layers) of tomato ovaries. Number of cells in each category of cell area within each genotype (h). Flowers were tagged and ovaries from flowers at 5 d before pollination and 5, 10 and 20 d after pollination were fixed in 100% methanol, vertically sectioned and stained with 0.04% toluidine blue O. Digital images of pericarp sections were acquired using AperioScan and analyzed using ImageScope 11. Average cell size (e, f) was calculated by dividing total number of cells with the area of endocarp, mesocarp or exocarp. Shown are average $\pm$ standard error ($n \geq 3$ biological replicates). Similar letters above standard error bars indicate non-significant difference (at 95% confidence interval) among genotypes within pericarp section.

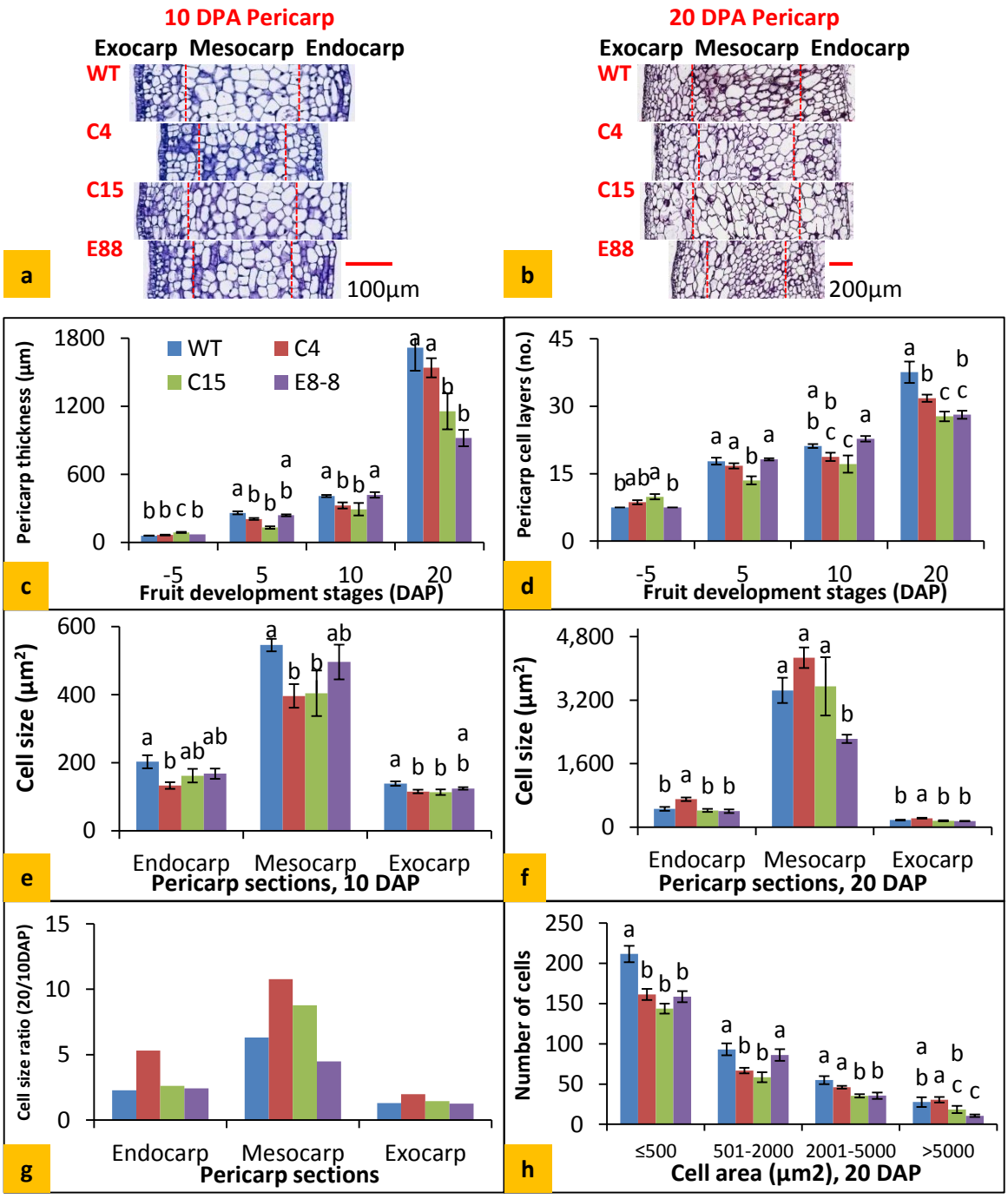
10 DPA Pericarp
Exocarp Mesocarp Endocarp
WT
C4
C15
E88
100µm
a
20 DPA Pericarp
Exocarp Mesocarp Endocarp
WT
C4
C15
E88
200µm
b
Pericarp thickness (µm)
WT
C4
C15
E8-8
Fruit development stages (DAP)
c
Pericarp cell layers (no.)
Fruit development stages (DAP)
d
Cell size (µm²)
Endocarp Mesocarp Exocarp
Pericarp sections, 10 DAP
e
Cell size (µm²)
Endocarp Mesocarp Exocarp
Pericarp sections, 20 DAP
f
Cell size ratio (20/10DAP)
Endocarp Mesocarp Exocarp
Pericarp sections
g
Number of cells
≤500 501-2000 2001-5000 >5000
Cell area (µm2), 20 DAP
h

Figure 2.4

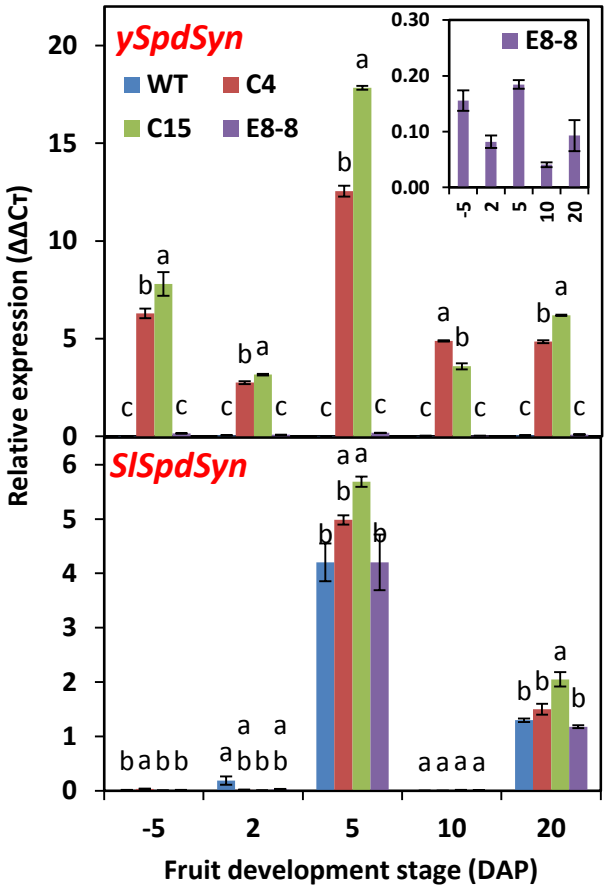

Figure 2.5**:** Changes in steady state levels of *ySpdSyn* and *SlSpdSyn* transcripts during early development of WT and *ySpdSyn*-expressing fruits.

Total RNA from ovaries from flowers 5 DBP and 5, 10 and 20 DAP was independently extracted, reverse transcribed and levels of *ySpdSyn* and SlSpdSyn transcripts were determined using qRT-PCR with gene specific primers (Table 2.1). The inset show the transcript levels of *E8:ySpdSyn* transgene in E8-8 tissues at a higher magnification. Relative expression was calculated by the ΔΔCT method using *SlACTIN* (Solyc04g011500.2.1) as housekeeping gene. Other details were same as in Figure 2.4.

2.3.4 Changes in the levels of free, conjugated and bound PAs in floral and developing ovaries

Levels of free, conjugated, and bound PAs were quantified in 10 DBP, 5 DBP, 2 DAP, 5 DAP, 10 DAP and 20 DAP tissues by HPLC analyses (Figure 2.6). In WT flower and fruit tissues, levels of free PUT and SPD remained similar in 10 DBP to 5 DAP stages before declining at 10 DAP for PUT and 20 DAP for SPD. The levels of free SPM showed about 2-fold increase in 2 DAP and 5 DAP WT fruit before declining in 10 DAP and 20 DAP WT fruits. The conjugated PA levels exhibited variable pattern (Figure 2.6). The conjugated PUT was present in 10 DBP WT flower, declined perceptibly at 5 DBP and then increased until 5 DAP before dramatically declining again in the 20 DAP in WT pericarp. The conjugated SPD was detectable only in 5 DAP WT fruit tissue. In WT, the conjugated SPM exhibited a pattern similar to conjugated PUT as it was present at higher levels in 10 DBP ovaries, peaked in 5 DAP fruit before declining to undetectable levels in 20 DAP developing fruit (Figure 2.6). The bound PUT in WT tissues exhibited a pattern similar to free PUT with the highest amounts present in 5 DAP. The highest levels of bound SPD in WT tissues were present at 5 DBP and declined steadily thereafter, whereas the highest levels of bound SPM in WT tissues were present at 5 DAP before declining to a barely detectable level by 20 DAP (Figure 2.6). The levels of bound PUT, SPD and SPM in WT tissues ranged from 7 to 30 %, 8 to 28 % and 49 to 75 % of that of free PUT, SPD and SPM, respectively, during flower and ovaries development (Figure 2.6). Levels of total PUT, SPD and SPM did not change much during the early fruit development but dropped by 10 DAP in WT tissues (Figure 2.7). The levels of total free, conjugated and bound PAs, all exhibited decline in 20 DAP fruit except that conjugated total PAs exhibited a peak in 5 DAP fruits (Figure 2.6). Collectively, data indicate that developing WT ovaries maintain higher levels of total PAs, during early fruit development (10DBP to 10DAP) before declining in the expansion phase (20 DAP) of fruit development.

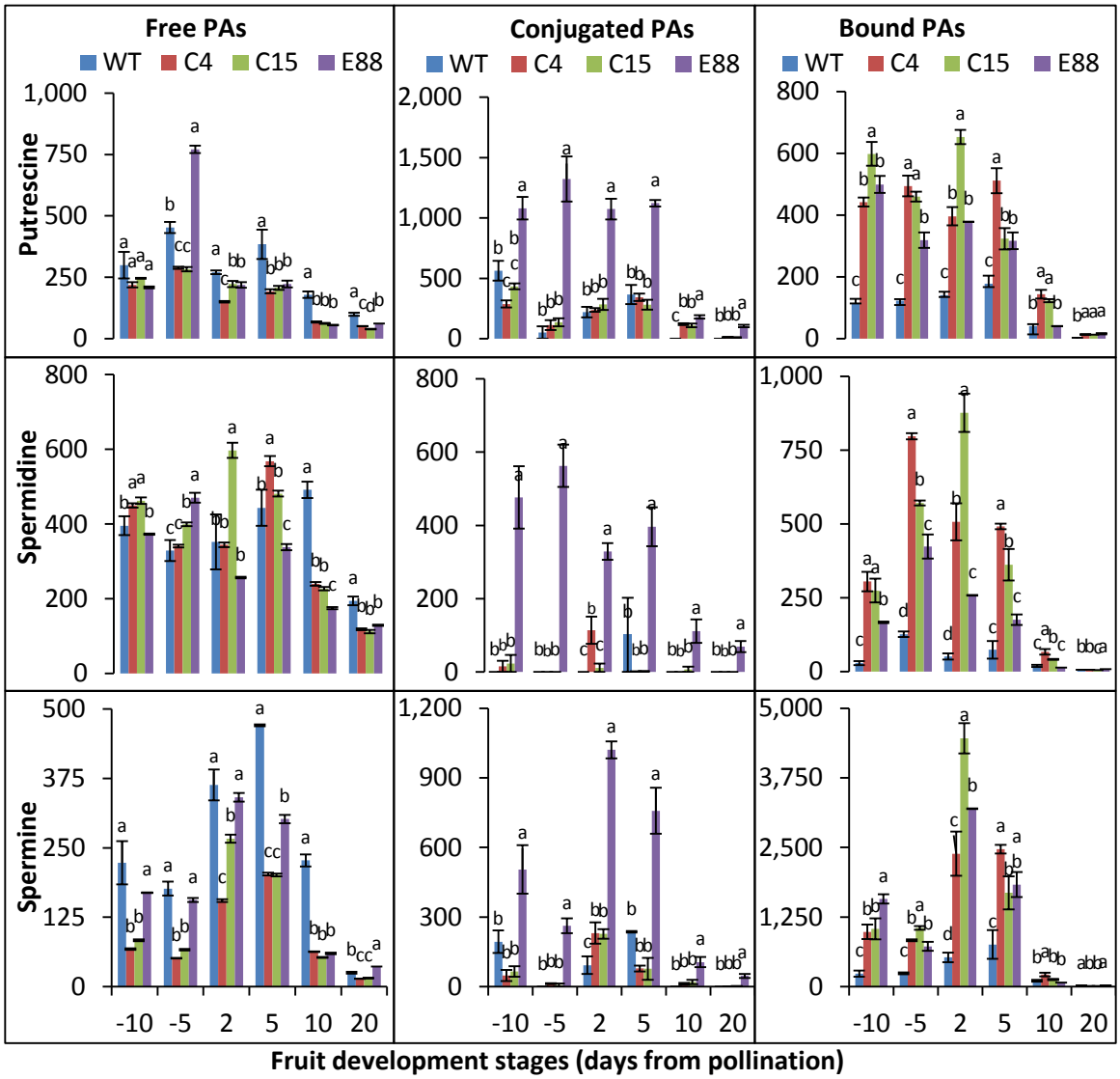

Figure 2.6: Free, conjugated and bound PUT, SPD and SPM levels in floral buds and fertilized ovaries of WT and *ySpdSyn*-expressing transgenic tomato plants.

Free, conjugated (PCA-soluble but hydrolyzed by HCl) and bound (PCA-insoluble but solubilized after hydrolysis with HCl) fractions of PUT, SPD and SPM were extracted and HPLC quantified (ηmol/g FW) as described in the Material and methods section 2.2.4. Other details were same as in Figure 2.4.

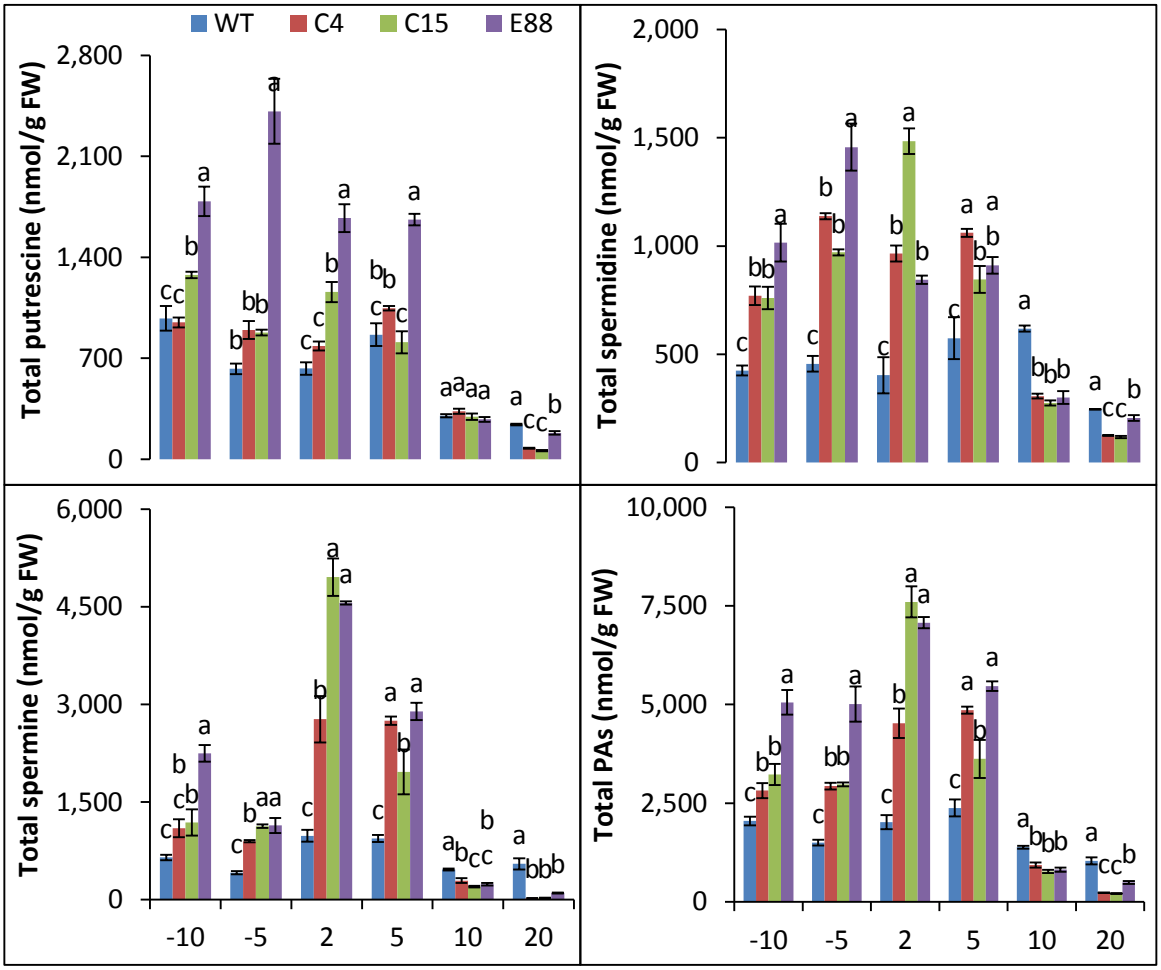

Figure 2.7**:** Changes in total amounts of PUT, SPD, SPM and total PAs in floral buds and fertilized ovaries of WT and *ySpdSyn*-expressing tomato plants.

The total amounts of PUT, SPD and SPM (ηmol/g FW) were determined by adding up levels of free, conjugated and bound fractions of each PA. The total PUT, SPD and SPM at the given stage were added together to determine the total PAs. Other details were same as in Figure 2.4.

2.3.5 Ectopic expression of *ySpdSyn* under a constitutive and a fruit ripening promoter altered accumulation of total PUT, SPD and SPM in floral buds and fertilized ovaries

Figure 2.6 shows the levels of free, conjugated and bound PUT, SPD, SPM and total PAs in three independent transgenic lines expressing *ySpdSyn* under CaMV35 or E8 (a fruit specific) promoters in floral buds and their developing ovaries at 10 DBP, 5 DBP, 2 DAP, 5 DAP, 10 DAP and 20 DAP. All transgenic lines showed consistently lower levels of free PUT except 2-fold increase in E8-8 at 5 DBP compared to WT. Free SPD levels were sporadically higher in transgenic floral buds and fertilized ovaries until 5DAP but declined thereafter at 10 DAP and 20 DAP. Free SPM levels were ubiquitously lower in C4 and C15 tissues, but generally unchanged in E8-8 tissues compared to WT developing ovaries.

Much higher levels of conjugated PAs were present in E8-8 tissues compared to WT and C4 and C15 tissues (Figure 2.6). The conjugated PUT levels were 2 to 26-fold higher in E8-8 compared to WT in various stages of flower and ovaries examined, while C4 and C15 exhibited higher amounts of conjugated PUT at 10 DAP. E8-8 tissues showed very high accumulation of conjugated SPD (up to 476 nmol/g) compared to WT (about 100 nmol/g) at 10 DBP (Figure 2.6). Levels of conjugated SPD at various developmental stages of flower and ovaries tissues from C4 and C15 lines were similar to WT except slight increase in C4 at 2 DAP (Figure 2.6). Tissues from E8-8 line also exhibited 3 to 11-fold increase in conjugated SPM compared to WT, while C4 and C15 tissues followed the same pattern as in WT except slight increase at 2 DAP and decrease at 5 DAP (Figure 2.6). Overall, E8-8 line exhibited 3 to 42-fold increase while C4 and C15 showed 2-fold increase in conjugated PAs at 2 DAP. All transgenic lines exhibited increase in bound PUT (2 to 5-fold), SPD (2 to 10-fold) and SPM (2 to 8-fold) which accounts for 2 to 8-fold increase in total bound PAs (Figure 2.6). The higher level of bound-conjugated PUT resulted in modest increase in total PUT in C4 and C15 but 2- to 4-fold increase in E8-8. The total SPD and SPM levels were also generally > 2-fold higher in transgenic lines compared to WT Figure 2.7.

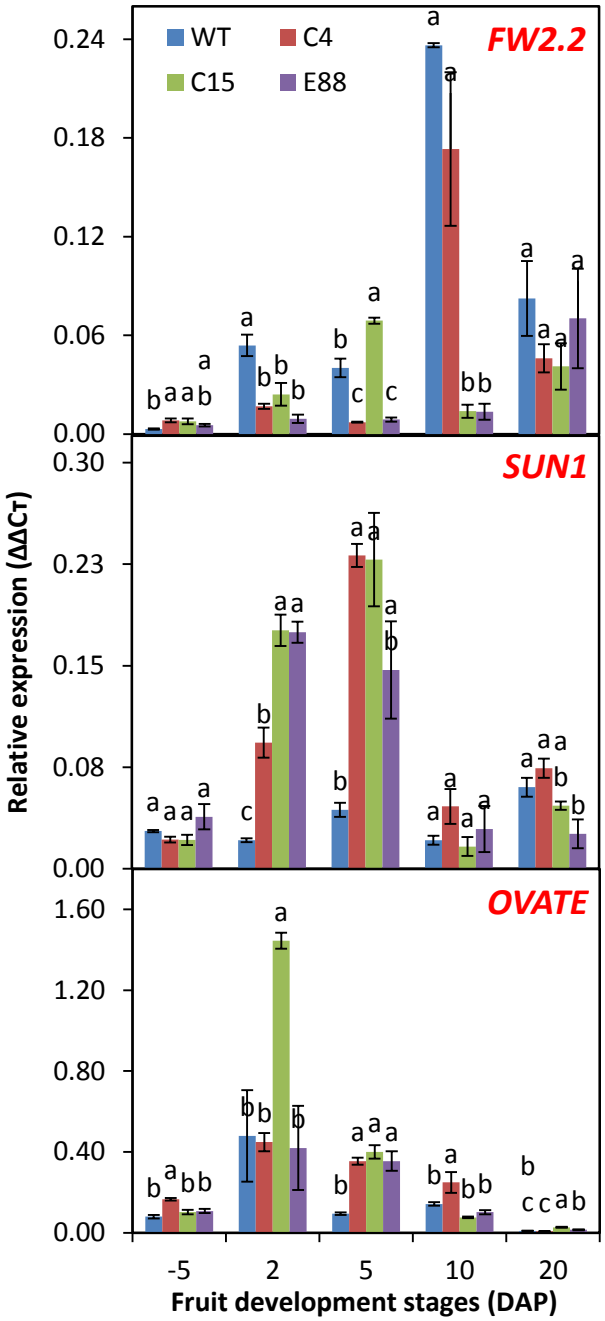

Figure 2.8**:** Changes in steady state transcript levels of fruit shape-related genes in WT and *ySpdSyn*-expressing transgenic tomato floral buds and flower ovaries.

Transcripts were quantified using qRT-PCR as described in Figure 2.5. Other details were same as in Figure 2.4.

Collectively data indicated that expression of *ySpdSyn* under a fruit specific promoter E8 is significantly different from that obtained under a constitutive CaMV 35 promoter. The E8-8 fruit exhibit a very large increase in the conjugated and bound PUT, SPD and SPM. The molecular basis of this observation is not clear, but it could be due to differential activation of a gene(s) encoding an enzyme regulating PA conjugation. We are presently evaluating RNAseq data with the hope to identify such a gene(s).

2.3.6 Effect of higher engineered PAs on expression of genes implicated in fruit size and shape

We determined the expression patterns *FW2.2*, *SUN1* and *OVATE,* the tomato genes that are implicated in fruit shape, using qRT-PCR (Figure 2.8), and correlated with levels of free, conjugated and bound PUT, SPD and SPM (Figure 2.9a) to evaluate if the *ySpdSyn* transgene expression-associated PA changes were responsible for the observed architecture modifications in the transgenic fruits. In WT fruit, expression of *FW2.2* was very low in 5DBP, increased several fold by 2 DAP and reaching a maximum in 10DAP fruit before declining in the 20DAP fruit. As shown in Figure 2.8, the expression of *FW2.2* was generally impaired in 2 to 20 DAP in fruits from all three transgenic lines, except in 5 DAP fruit from C15 line. The expression of *SUN1* in the developing WT tomato fruit remained similar from 5 DBP to 10DAP with slight but significant increase in 20 DAP fruit. In transgenic fruits, however, the expression of *SUN1* was 5-10 fold upregulated in 2 DAP to 5 DAP fruits in all three independent transgenic lines compared to WT fruits, before showing decline at 10 and 20 DAP fruit (Figure 2.8). Expression of *OVATE* in WT fruit was about 5-fold upregulated in 2 DAP fruit compared to 5 DBP ovaries, but remained low thereafter (Figure 2.8). Transcript levels of *OVATE* gene were upregulated only in 2 DAP fruit from C15 line, but exhibited 2 to 3-fold increase in 5 DAP fruit from all three transgenic lines (Figure 2.8). Transcript levels of *OVATE* gene were positively correlated (≥0.5) with free, bound and total SPD and with bound and total SPM and PUT (Figure 2.9a). In addition to positive correlation between *SUN1* transcripts and conjugated PUT,

correlation pattern of *SUN1* gene with PA levels was similar to *OVATE* (Figure 2.9a). High expression of *OVATE* gene would lead to more round than oval fruit, a result in contrast with the one observed in the present study. To confirm if the observed pattern was not due to a mutation in the *OVATE* gene, we determine the sequence of *OVATE* transcripts from WT and transgenic Ohio 8245 fruits. We did not find any mutation or the stop codon (TAA) in transcripts of *OVATE* genes in WT and transgenic genotypes (Figure 2.10).

2.3.7 Transgenically enhanced PAs influenced expression of genes regulating cell division and expansion during fruit development

We examined expression patterns of cell division and expansion genes *CYCs*, *CDKs* and interacting partners, *KPR1* and *WEE1* (CDK inhibitors), *FSM1* (inhibitor of cell expansion), and *CCS52A* and *CCS52B* (promoter of cell expansion) to determine if their expression was modulated by transgenes associated changes in various forms PAs. The levels *CDKA1* transcripts in WT flower and ovary tissues were similar from 5 DBP to 5 DAP with slight increase at 10 DAP before registering a decline in 20 DAP (Figure 2.11). The expression patterns of *CDKA1* in transgenic fruits were variable as its transcripts continued to increase from 2 DAP to 20 DAP in C4, increased in 5 DAP in C15 and remained similar to WT fruit in E8-8 fruits at all stages examined (Figure 2.11). The transcript levels of *CDKB2*, during the WT fruit development, decreased about 2-fold from 5 DBP to 2 DAP and increased several-fold thereafter until 10 DAP before sharply declining in 20 DAP fruit. Variable patterns for *CDKB2* transcript levels were observed during the development of transgenic fruits from the three lines examined. *CDKB2* transcripts in C4 and C15 fruit at 5 DBP and 2 and 5 DAP were unchanged but remained lower in 10 DAP transgenic than the WT fruits (Figure 2.11). A correlation for *CDKA1* transcript levels and various forms of PAs was not obtained, but *CDKB2* transcript levels exhibited a positive correlation with free SPD (Figure 2.9a).

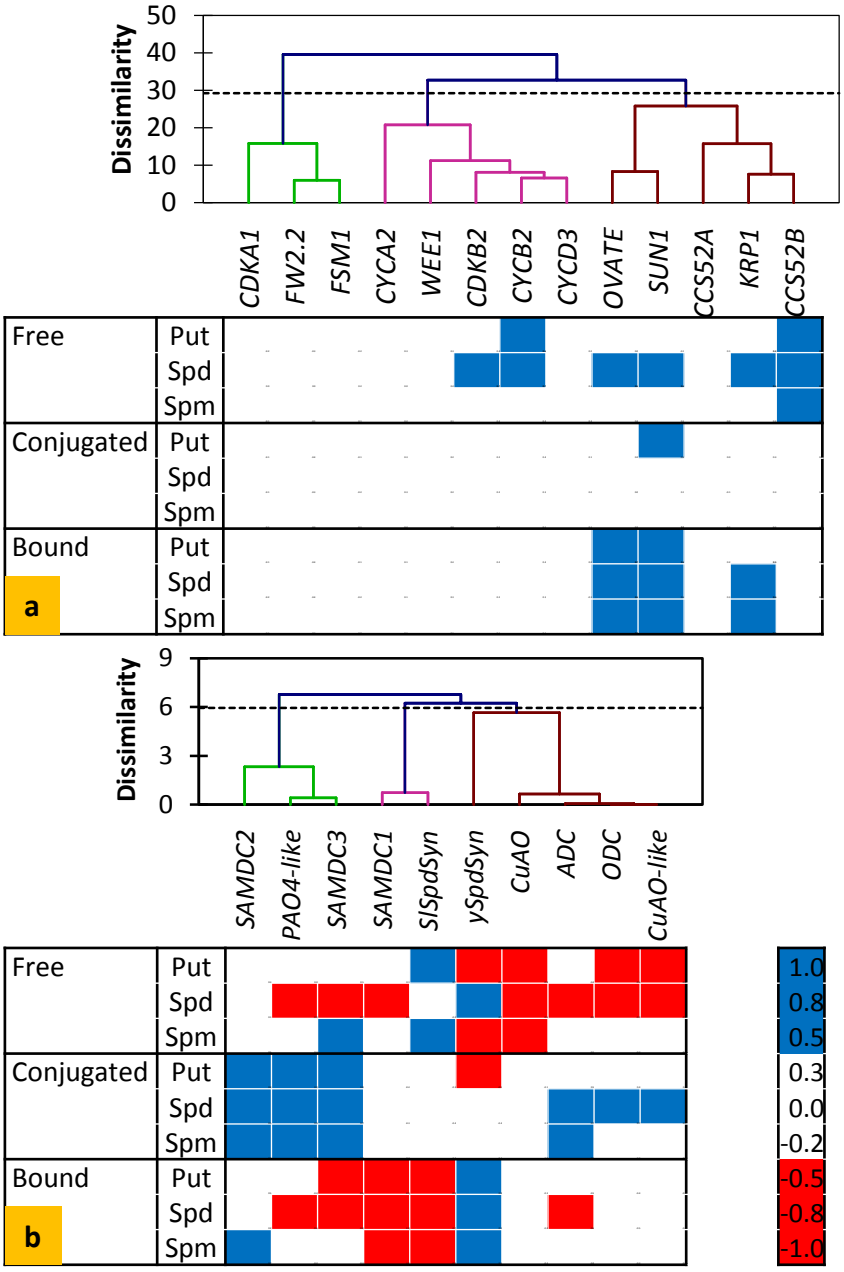

Figure 2.9: Correlation coefficient analysis of PA levels and transcripts of genes involved in fruit shape, cell cycle progression, cell expansion and PA biosynthesis and catabolism.

Correlation coefficient analysis between PA levels and transcripts of cell cycle and fruit shape genes (a) and among PA levels and transcripts of PA biosynthetic and catabolic genes (b) in WT and transgenic tomato ovaries at 2 DAP. Changes in gene expression and levels of different forms of various PAs were analyzed using XLSTAT version 2014.4.06 to determine the correlation and dendrogram (arrangement of the clusters among genes).

```
WT                  AAGAA--GCTGATACCGTGTAGT-GTGGA-TGGGAAAGTGAAGGAGAGTTTCGCGATAGT
C4                  AAGAA--GCTGATACCGTGTAGT-GTGGA-TGGGAAAGTGAAGGAGAGTTTCGCGATAGT
C15                 AAGAAAGCTTGATACCGTGTAGTTGTGGATTGGGAAAGTGAAGGAGAGTTTCGCGATAGT
E8-8                AAGAA--GCTGATACCGTGTAGT-GTGGA-TGGGAAAGTGAAGGAGAGTTTCGCGATAGT
Solyc02g085500.2.1AAGAA--GCTGATACCGTGTAGT-GTGGA-TGGGAAAGTGAAGGAGAGTTTCGCGATAGT
                    *****    ************** ***** ******************************

WT                  GAAGAAATCTCAGGACCCGTACGAAGATTTCAAGAGATCGATGATGGAAATGATTTTAGA
C4                  GAAGAAATCTCAGGACCCGTACGAAGATTTCAAGAGATCGATGATGGAAATGATTTTAGA
C15                 GAAGAAATCTCAGGACCCGTACGAAGATTTCAAGAGATCGATGATGGAAATGATTTTAGA
E8-8                GAAGAAATCTCAGGACCCGTACGAAGATTTCAAGAGATCGATGATGGAAATGATTTTAGA
Solyc02g085500.2.1GAAGAAATCTCAGGACCCGTACTAAGATTTCAAGAGATCGATGATGGAAATGATTTTAGA
                    ********************* ************************************

WT                  GAAGGAAATGTTTGAGAAGAATGAGCTGGAACAGCTTTTACAATGTTTTCTGTCGTTGAA
C4                  GAAGGAAATGTTTGAGAAGAATGAGCTGGAACAGCTTTTACAATGTTTTCTGTCGTTGAA
C15                 GAAGGAAATGTTTGAGAAGAATGAGCTGGAACAGCTTTTACAATGTTTTCTGTCGTTGAA
E8-8                GAAGGAAATGTTTGAGAAGAATGAGCTGGAACAGCTTTTACAATGTTTTCTGTCGTTGAA
Solyc02g085500.2.1GAAGGAAATGTTTGAGAAGAATGAGCTGGAACAGCTTTTACAATGTTTTCTGTCGTTGAA
                    ************************************************************

WT                  CGGAAAGCATTATCATGGAGTGATAGTTGAGGCGTTCTCAGACATTTGGGAGACTTTGTT
C4                  CGGAAAGCATTATCATGGAGTGATAGTTGAGGCGTTCTCAGACATTTGGGAGACTTTGTT
C15                 CGGAAAGCATTATCATGGAGTGATAGTTGAGGCGTTCTCAGACATTTGGGAGACTTTGTT
E8-8                CGGAAAGCATTATCATGGAGTGATAGTTGAGGCGTTCTCAGACATTTGGGAGACTTTGTT
Solyc02g085500.2.1CGGAAAGCATTATCATGGAGTGATAGTTGAGGCGTTCTCAGACATTTGGGAGACTTTGTT
                    ************************************************************

WT                  TTTAGGTAATAATGATAGAGTAAGGAGGATGTCAATTCATGATCCCA------------
C4                  TTTAGGTAATAATGATAGAGTAAGGAGGATGTCAATTCATGATCCCACACCCACCT----
C15                 TTTAGGTAATAATGATAGAGTAAGGAGGATGTCAATTCATGAT----------------
E8-8                TTTAGGTAATAATGATAGAGTAAGGAGGATGTCAATTCATGATCCCACACC---------
Solyc02g085500.2.1TTTAGGTAATAATGATAGAGTAAGGAGGATGTCAATTCATGATCCCACACCCACCTACTG
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Figure 2.10**:** Sequence alignment of OVATE gene in WT and transgenic fruits with its mutated version (ovate) containing stop codon.

Multiple sequence alignment was generated using Clustal Omega (1.2.0). Three base pair mutation in sequence in indicated in red box.

Accumulation of transcripts of *KRP1,* a CDK inhibitor, was high in 5 DBP WT flower, decreased in 2 DAP ovaries before increasing until 10 DAP and exhibiting steep decline in 20 DAP fruit (Figure 2.11). In transgenic tissues, transcripts levels *KRP1* increased dramatically in 2 DAP in C4 and C15 lines, but declined to low levels in 20 DAP fruit in all genotypes including WT (Figure 2.11). The expression of *WEE1*, another CDK inhibitor, in WT tissues declined greatly at 2 DAP compared to 5 DBP, but increased to a peak in 5 DAP, a pattern similar to that reported previously (Gonzalez et al., 2004) (Figure 2.11). *KRP1* transcript levels also exhibited positive correlations with free, bound and total SPD and bound and total SPM, but no significant correlation (≥0.5) was obtained for *WEE1* (Figure 2.9a)*,* suggesting involvement of *KRP1* but not *WEE1* in transgene-associated changes in fruit shape.

Mitotic cyclins is a family of proteins that regulate the cell cycle progression during cell division by activating CDK enzymes. The six cyclin genes are identified in tomato and all are expressed in tomato pericarp (Joubès et al., 2000). Steady-state transcript levels of one cyclin gene from each of the three families (A, B and D) were determined during the development of WT and transgenic fruit (Figure 2.11). Higher expression *CYCB2* and *CYCD3* than *CYCA2* was obtained in the WT fruit, with *CYCD3* expressing at much higher level in 5 DAP fruit as indicated by $\Delta\Delta C_T$. Expression of these cyclins gene decreased greatly in 20 DAP fruit, a pattern similar to reported previously (Joubès et al., 2000). The transcripts of *CYCA2* were down in 5 DAP fruit in all transgenic lines but significantly increased at 10 DAP in C15 and E8-8 lines (Figure 2.11). Transcript levels of *CYCB2* were upregulated at 2 and 5 DAP before exhibiting steady decline in 10 DAP and 20 DAP fruit in all transgenic lines. Transcript levels of *CYCD3* showed a mixed pattern with decrease in 20 DAP fruit. Transcripts of *CYCB2* were positively correlated with free SPD and PUT and with total SPD, whereas *CYCA2* and *CYCD3* did not show significant correlation with any form of PAs (Figure 2.9a). Taken together results suggest a role of *CDKB2* in activating *CYCB2* at 2 DAP and 5 DAP ovaries, the active cell division phase of tomato pollinated ovaries. The precipitated decrease in the expression of all cyclin in 20 DAP tomato fruit in

consistent with end of cell division phase at this stage of fruit development (Gillaspy et al., 1993).

Higher cell expansion was seen in C4 and C15 lines compared to WT in 20 DAP fruits (Figure 2.4g). *FSM1* is implicated in inhibition of cell expansion, whereas *CCS52A* and *CCS52B* have been suggested to promote cell expansion (Machemer et al., 2011; Mathieu-Rivet et al., 2010). Levels of *FSM1* transcripts were barely detectable in WT flower bud but increased steadily in developing fruit reaching a maximum at 10 DAP before declining dramatically in WT 20 DAP fruit, a pattern similar to that reported in the facultative parthenocarpic line L-179 (*pat-2/pat-2*) (Barg et al., 2005). The transcript levels of *CCS52A* showed dual peaks in 2 DAP and 10 DAP fruits. The *CCS52B* transcripts were higher at 5 DBP and peaked at 10 DAP with present at barely detectable levels in 20 DAP fruit (Figure 2.11). The *FSM1* transcript levels were slightly upregulated in C4 floral buds, highly upregulated at 5 DAP in C15 and 10 DAP in C4 lines, respectively. Although, *FSM1* transcript levels significantly dropped in 20 DAP fruit in all genotypes, they remained significantly higher in C15 and E8-8 compared to WT 20 DAP fruit. The transcript levels of both *CCS52A* and *CCS52B* were higher in transgenic flowers but were generally decreased in developing fruit with some exceptions. *CCS52B* transcript levels exhibited positive correlations with free PUT, SPD and SPM and with total SPD, whereas a significant correlations between *FSM1* and *CCS52A* transcript levels and any form of PAs were not obtained (Figure 2.9a).

Figure 2.11**:** Steady-state transcript levels of cell cycle progression and cell expansion regulating genes in WT and *ySpdSyn*-expressing transgenic tomato floral buds and flower ovaries.

Transcripts of cyclin-dependent kinases (*CDKA1* and *CDKB2*), cyclins (*CYCA2*, *CYCB2* and *CYCD3*), CDK1-inhibitors (*WEE1* and *KRP1*) and cell expansion-regulating genes (*FSM1*, *CCS52A* and *CCS52B*) were quantified using qRT-PCR as described in Figure 2.5. Other details were same as in Figure 2.4.

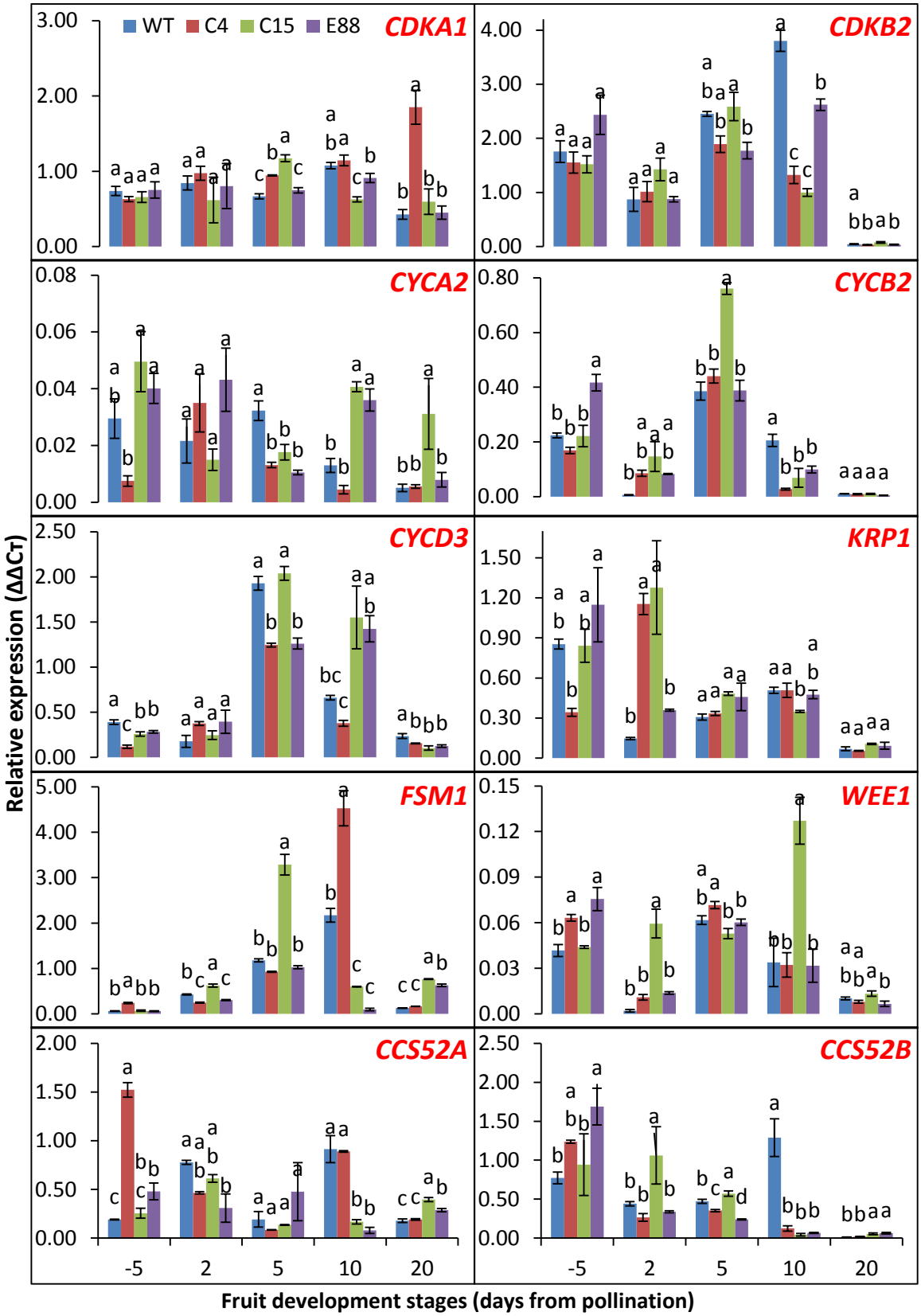

WT
C4
C15
E88
CDKA1
CDKB2
CYCA2
CYCB2
CYCD3
KRP1
FSM1
WEE1
CCS52A
CCS52B
Relative expression (ΔΔCT)
Fruit development stages (days from pollination)
-5
2
5
10
20

Figure 2.11

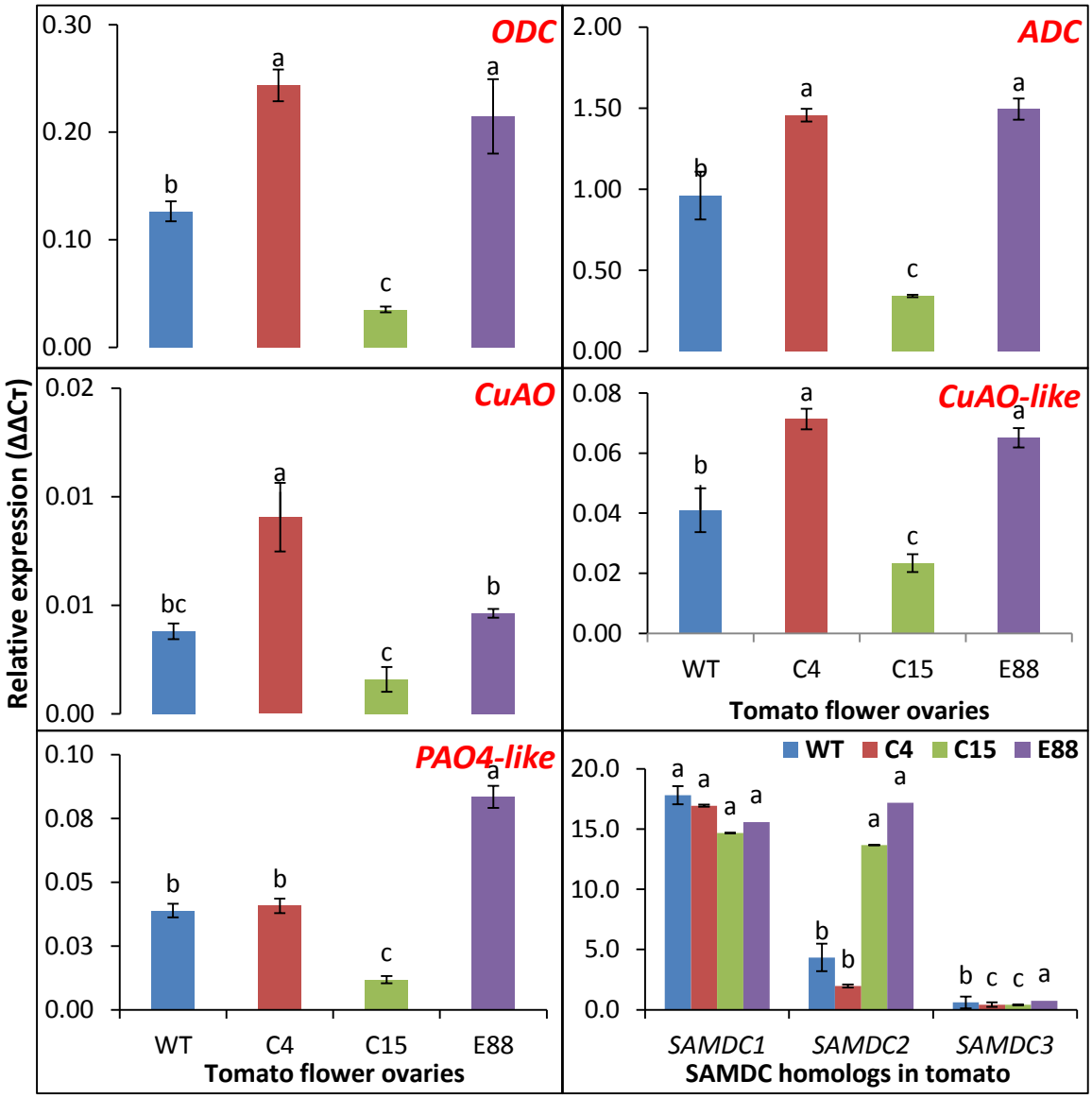


Figure 2.12**:** Steady state transcript levels of PA biosynthesis and catabolizing genes in pollinated ovaries of WT and *ySpdSyn*-expressing tomato lines at 2 DAP.

Transcripts of genes involved in PA biosynthesis (*ODC*, *ADC*, *SAMdc1*, *SAMdc2*, *SAMdc3*) and PA catabolism (*CuAO*, *CuAO-like* and *PAO4-like*) were quantified using qRT-PCR as described in Figure 2.5. Other details were same as in Figure 2.4.

2.3.8 Role of PAs biosynthetic pathway in accumulation of various forms PAs

In addition to increase in conjugated PUT in E8-8 line, expression of *ySpdSyn* greatly increased bound PUT level in all transgenic lines (Figure 2.6). To determine if this increase in conjugated and bound PUT was due to PAO-mediated back conversion of SPD and/or SPM or resulted from enhanced substrate (Arg or Orn) influx into PA pathway, we quantified transcripts of diamine oxidases (*CuAO* and *CuAO-like*) and a PA oxidase (*PAO4-like*) in WT and transgenic flower ovaries at 2 DAP. Consistent patterns of expression among the transgenic lines were not observed. Expression of *ODC*, *ADC*, *CuAO*, *CuAO-like* and *PAO4-like* genes was upregulated in C4 and E8-8, but was downregulated in C15 (Figure 2.12). The expression of *SAMdc2* was upregulated in C15 and E8-8 but downregulated in C4. In general, the correlation coefficients among the transcript levels of these genes with free and bound PUT, SPD and SPM were negative but positive with conjugated PUT, SPD and SPM at 2 DAP (Figure 2.9b). These results suggest that both anabolism and catabolism of PAs play a role in accumulation of conjugated PUT, SPD and SPM.

2.3.9 Statistical analyses accentuates role of developmental stages in changes in PA levels and gene expression

The principal component analyses were used to determine correlations among the free, conjugated and bound PUT, SPD and SPM in regulating the expression of genes associated with cell division, cell expansion and fruit shape genes (Figure 2.13a). All fractions of PUT, SPD and SPM were clustered around the center of positive quadrant of the first principal component which suggest that different fractions of PAs support biosynthesis of each other. Transcript levels of *OVATE*, *SUN1*, *CYCA2*, *CYCB2*, *CYCD3*, *CDKB2*, *WEE1*, *CCS52A* and *CCS52B* were positively associated while *FW2.2*, *CDKA1* and *FSM1* were negatively associated with different fractions of PAs. PCA analysis also showed strong association of *SUN1* with *CYCB2* transcripts (Figure 2.13a). The PCA analyses of PA levels and expression levels of genes involved in their biosynthesis or catabolism showed

that transcript levels *ODC*, *ADC*, *CuAO*, *CuAO-like*, *PAO4-like* and *SAMdc3* were more closely clustered and positively associated with conjugated PAs while *ySpdsyn* transcripts were more positively associated with *SAMdc3*, free SPD and bound and total forms of all three PAs (Figure 2.13b).

The PCA of the levels of free, conjugated and bound PUT, SPD and SPM and transcript levels of genes associated with cell division, cell expansion and known fruit shape genes showed that with a few exception, correlation among them was clustered based on flower and ovary developmental stages and not on the genotype, that is the presence of different transgenes (Figure 2.13c). These result suggest that the developmental stage trumps the free, bound and conjugated levels of PUT, SPD and SPM. This is also evident from *35S:ySpdSyn* transgene that exhibited regulated expression of transgene transcript (Figure 2.5). These results suggest that although various forms of PAs generally positively regulate expression of cell division and cell expansion genes, a delicate balance among the various forms (free, conjugated and bound) of three main PAs determines the fate of cell division in tomato fruit. Model shown in Figure 2.14 summarizes above observations to propose the regulatory targets of PAs sat gene expression level affecting cell division and cell enlargement. Both genetic and pharmacological studies have underscored the vital role of SPD is cell division (Balasundaram et al., 1991; Chattopadhyay et al., 2002; Fuller et al., 1977; Malmberg and Mcindoo, 1983), herein we provide evidence for the similar role of various PAs in determining the fate of cell cycle leading to cell division. However, further studies are needed to delineate the role of various components to understand how PAs facilitate anticlinal cell division leading to altered fruit shape.

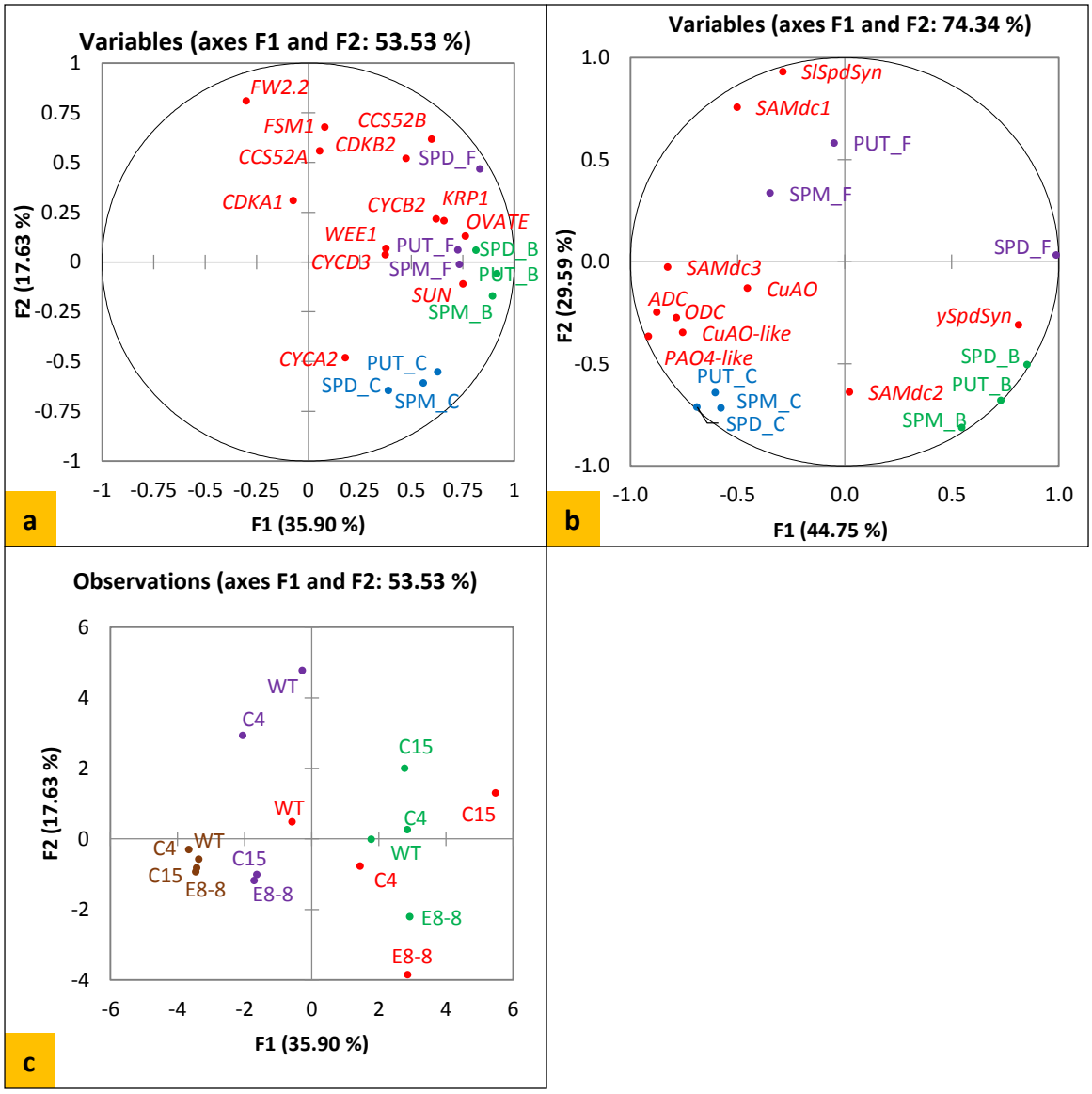


Figure 2.13: Principal component analyses of endogenous levels of PUT, SPD, SPM and total levels with transcripts of genes involved in cell cycle, cell expansion, fruit shape and PA biosynthesis and catabolism.

a) The separation of levels of various forms of PUT, SPD, SPM and total PAs and transcript levels of cell cycle, cell expansion and fruit shape genes. b) The separation of levels of various forms of PUT, SPD, SPM and total PAs and levels of transcripts of PAs biosynthetic and catabolic genes. c) The separation of samples according to developmental stage of ovaries. Color codes represent 5 days before pollination (blue), 2 days after pollination (DAP, red), 5 DAP (green), 10 DAP (purple) and 20 DAP (brown). PCA analyses and graphs were generated using XLSTAT 2014.3.05.

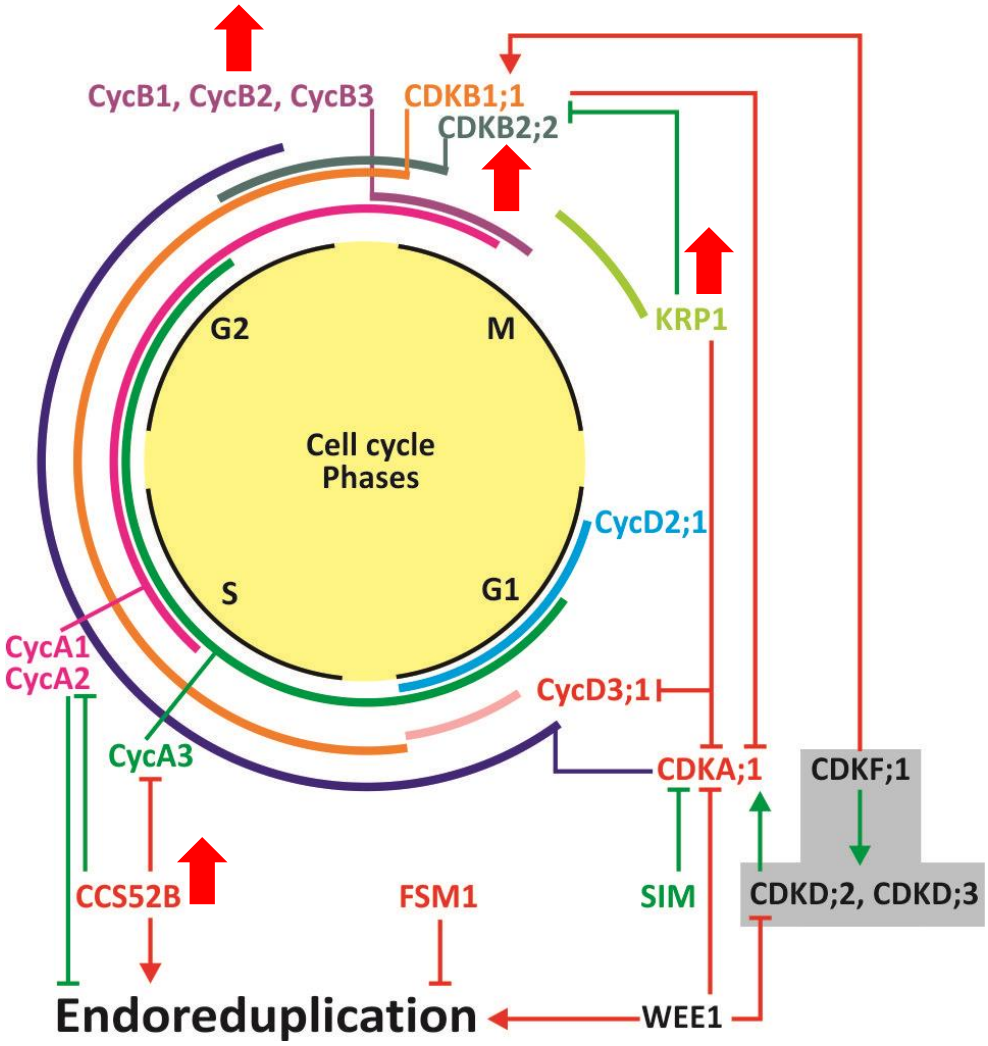


Figure 2.14**:** A model showing the PAs-mediated changes in transcript levels of genes involved in cell cycle progression and endoreduplication during tomato fruit development.

In eukaryotes, cell cycle is mainly comprised of interphase and mitosis (M phase). Interphase is further divided into three phases: i) G1 (Gap 1) during which cell increases in size and becomes ready for DNA synthesis, ii) S (Synthesis) where DNA replication occurs and iii) G2 (Gap 2) during which cell either continues to grow until ready for mitosis or enter into DNA amplification phase, called endoreduplication (Gutierrez 2009; John and Qi 2008). Expression levels of cyclins or CDKs during different phases of cell cycle progress are indicated in colors. CDK-activating kinases are highlighted in gray box. Transcript levels of genes enhanced by higher PAs (SPD/SPM) are indicated with upward-facing thick red arrows. G, gap phase; M, mitosis phase; S, synthesis phase; Rb, retinoblastoma protein; ABA, abscisic acid; Cyc, cyclin; CDK, cyclin dependent kinase; CAKs, CDK activation kinases; KRP, Kip-related proteins.

2.4 DISCUSSION

Results herein provide a strong evidence that PAs play a role in determining tomato fruit architecture by regulating expression of *SUN1*, *OVATE* and cell division and expansion genes. Transcript levels of *SUN1* were positively correlated with free SPD, conjugated PUT and bound PUT, SPD and SPM, whereas *OVATE* transcript levels were positively correlated with free SPD and bound and total SPD and SPM. Levels of the *FW2.2* transcripts exhibited low correlation with free, conjugated and bound PUT, SPD, and SPM and total PAs suggesting a limited role of this gene in PAs-altered tomato fruit shape (Figure 2.9a). SUN regulates the fruit shape by increasing cell division in the proximal-distal direction and decreases cell number in the medial-lateral direction of the fruit by modulating calcium signaling during fruit growth (Abel et al., 2005; Xiao et al., 2008). OVATE is considered to be a negative regulator of genes which enhance cell division in the proximal region of the developing ovary elongating fruit size (Hackbusch et al., 2005; Liu et al., 2002). OVATE alter fruit shape predominately before anthesis while SUN controls tomato fruit shape during post-pollination events (Tanksley, 2004; van der Knaap and Tanksley, 2001; Wu et al., 2011; Xiao et al., 2008). The highest expression of endogenous *SlSpdSyn* was at 5 DAP and developing ovaries also maintained higher levels of various PAs throughout the early fruit development (10 DBP to 5 DAP).

Figure 2.3 shows that three independent transgenic lines expressing *ySpdSyn* under CaMV 35S or E8 promoters exhibited more obovoid fruit shape compared to parental WT, and these PAs induced fruit shape changes at very early stage of fruit development (before 5 DAP) and continued to persist during fruit development and ripening as reported in *SUN1* and *OVATE* transgenic fruits (Monforte et al., 2014; Wu et al., 2011). The obovoid shape of *ySpdSyn* transgene-expressing fruit was associated with reduced pericarp thickness, decrease in cell layers and numbers per unit pericarp area and larger cells in the mesocarp during fruit expansion phase (Figure 2.4). These results indicated that expression of transgene reduced cell division in the medial-lateral direction of fruit causing more elongated shape of fruit, an effect similar to *SUN1* gene

overexpressing tomato fruit (Wu et al., 2011; Xiao et al., 2011). The parental tomato cv. Ohio 8245 used in these studies expresses a functional *OVATE* gene (Figure 2.10), which would reduce cell division at the distal end to induce more round shape of fruit. Although, *FW2.2* is also expressed, its maximum expression is delayed until 10 DAP and the gene present in all cultivated tomato has a mutation that has limited effect on fruit phenotype (Alpert et al., 1995; Nesbitt and Tanksley, 2002). We interpret these result suggesting that increase in obovoid shape of the transgenic fruit is a resultant of effect of both *SUN1* and *OVATE* genes, with *SUN1* exerting stronger effect on fruit phenotype.

Expression of *SlSpdSyn* was upregulated both during the cell division and the cell expansion phase of fruit development suggesting dual roles of PAs on both at cell division and cell expansion phase of fruit development. Interestingly, expression of *ySpdSyn* under CaMV 35S, a supposedly constitutive promoter, was also regulated by the early stages of fruit development suggesting that developmental stages trump effect on even a constitutive promoter. The SlE8 promoter, considered to be a ripening-regulated promoter, was also expressed in non-ripening tissues but at much lower rate than CaMV 35S promoter (Figure 2.5). There was up to 2- to 3-fold increase in total SPD and SPM levels with little change in total PUT levels in transgenic ovaries in response to expression of *ySpdSyn* gene (Figure 2.7). Most of these increases were due to the much higher levels of either conjugated and/or bound SPM and SPD. Although, slightly higher levels of conjugated and bound PUT, SPD and SPM were also present in C4 and C15 transgenic fruits, E8-8 developing flower and fruits accumulated much higher levels of conjugated PUT, SPD and SPM than C4 and C15 flower and fruit tissues (Figure 2.6). The molecular basis of increased conjugated PAs in E8-8 lines is not clear, but we have obtained a similar pattern in transgenic fruits expressing yeast SAM decarboxylase under SlE8 promoter indicating that an element in SlE8 promoter may be responsible of increase in the conjugated PAs (R. Anwar and AK Handa, Unpublished results). The levels of total PUT, especially bound PUT also exhibited several fold increase in transgenic flower and fruit tissues suggesting a crosstalk leading to inter-conversion among PUT, SPD and SPM to maintain PAs homeostasis. Very few enzymes are known that catalyze synthesis or

hydrolysis of PA conjugates (Tiburcio et al., 2014). Hydroxycinnamoyl transferase conjugates PUT, cadaverine, SPD and agmatine to caffeoyl-CoA, feruloyl-CoA and cinnamoyl-CoA (Negrel, 1989); and transglutaminase catalyzes covalent bonding of PAs to chloroplastic and cytoskeletal proteins (Del Duca et al., 1995; Del Duca et al., 1997). However, the molecular signals responsible for conversion of free PAs to their free and bound conjugates remained to be determined.

The chemical nature of increased conjugated and bound PAs in *ySpdSyn* transgenic lines is not known yet. However, presence of the hydroxycinnamic acids conjugate and oxidation products of PAs have been suggested to play essential roles in plant developmental processes especially abiotic and biotic stress (Tiburcio et al., 2014). Additionally, PCA-insoluble fraction of plant tissue contain dimers and trimmers of PA conjugates (Bagni and Tassoni, 2001; Bokern et al., 1995). Higher levels of conjugated than free PAs have been reported in actively dividing thin cell layers (Torrigiani et al., 1989; Torrigiani et al., 1987), apical internodes, young leaves and fertilized ovaries of tobacco (K-Sawhney and Applewhite, 1993; Slocum and Galston, 1985), developing olive fruit (Gomez-Jimenez et al., 2010a) and pea ovaries (Pérez-Amador et al., 1996), and pollinated pistils of citrus (Gentile et al., 2012) and pear (Del Duca et al., 2010). It has been suggested that conjugated forms of PAs mediate various plant growth and development processes including fruit abscission (Gomez-Jimenez et al., 2010b), petal fall (Pérez-Amador et al., 1996), plant defense against insect herbivores (Kaur et al., 2010), senescence (Gomez-Jimenez et al., 2010b; Pérez-Amador et al., 1996) and thermo-tolerance (Roy and Ghosh, 1996). However, still little is known about physiological significance and molecular roles of individual conjugated PAs (Bagni and Tassoni, 2001).

Cell cycle progression is regulated by cyclins (CYC), CDKs, CDK inhibitors and CDK-activating kinases (Harashima and Schnittger, 2010; Veylder et al., 2003). In heterodimeric protein complexes, cyclins are regulatory subunits while CDKs are catalytic subunits (Blomme et al., 2013). In mammalian and yeast cells, PAs are known to promote cell proliferation by activating these complexes (Chattopadhyay et al., 2009; Gilmour et al., 1999; Guo et al., 2003). We analyzed expression patterns of several genes shown to play

roles in plant cell division. These included *S. lycopersicum* homologs of two *CDKs* (*CDKA1*, *CDKB2*) and three cell cyclins (*CYCA2*, *CYCB2*, *CYCD3*) and interacting CDK inhibitors (*KPR1* and *WEE1*), cell expansion promoter (*CCS52A* and *CCS52B*) and a cell expansion inhibitor *FSM1* in developing floral buds and fertilized ovaries from WT and *ySpdSyn* tomato plants to determine if the *ySpdSyn* expression modified transcription rate of these genes to regulate fruit shape. In spite of variable patterns, the levels of *CDKB2*, *CYCB2, CCS52B* and *KRP1* transcripts were positively correlated with free SPD, and those of *CYCB2* and *CCS52B* with free PUT. Transcripts of *KRP1* were also positively correlated with bound SPD and SPM. No correlation was obtained for *CDKA1*, *WEE1, CYCA2* and *CYCD3*, *FSM1* and *CCS52A*. These data collectively suggest that regulation of cell division plays a role in PAs-regulated changes in fruit shape (Figure 2.9a). However, due to variability in steady state transcript levels of cell division regulating genes, it is difficult to draw a strong conclusion in favor of PAs regulated changes in fruit shape. Emerging results from transcriptome studies with various mutants and transgenic lines would help further to achieve this goal (Anwar et al., 2015).

Our results show that inter-conversion of PUT, SPD and SPM plays an important role in maintaining homeostasis of their free levels. In developing fruit, the SPD made by the expression of *ySpdSyn* transgene is sequestered into bound form of PUT, SPD and SPM. Total SPM levels (~4500 nmol in C15 at 2 DAP) were about 3-fold higher than total SPD (~ 1500 nmol in C15 at 2 DAP) and more than 2-fold higher than total PUT (~ 2000 nmol in E8-8 at 5 DBP) (Figure 2.7). The strong positive correlation of *SAMdc2* and *SAMdc3* with conjugated PUT, SPD and SPM suggests their roles in providing substrate biosynthesis for both SPD and SPM that sequestered into conjugated forms. *PAO4-like* transcripts were also strongly correlated with conjugated forms of PUT, SPD and SPM suggesting inter-conversion relationship among them. Further understanding of changes in the flux of substrate into PAs biosynthetic in transgenic lines expressing *ySpdSyn* would help understand the molecular mechanisms of inter-conversion among various PAs and their conjugates including the bound form (Lasanajak et al., 2014).

CHAPTER 3. SAM DECARBOXYLASE EXPRESSION DETERMINES THE HIGHER POLYAMINES ACCUMULATION WHICH REGULATES BIOMASS ACCUMULATION AND FRUIT FIRMNESS IN RIPENING TOMATO FRUITS

3.1 Introduction

PAs are biogenic amines and are ubiquitous biological constituents of all living organisms. PUT, SPD and SPM are three major PAs and have been implicated in cell division, longevity, stress responses and most of the vital growth and developmental processes (Handa and Mattoo, 2010; Mattoo and Handa, 2008; Nambeesan et al., 2008; Pegg, 2009). PUT is synthesized from arginine by ADC or from ornithine by ODC. Diamine PUT is converted into triamine SPD by SpdSyn and SPD is further converted into tetramine SPM by SpmSyn. The aminopropyl group required for the conversion of PUT to SPD and SPD to SPM is donated by decarboxylated SAM (dcSAM), a product of SAMdc from SAM. Ectopic expression of *SAMdc* resulted in 2- to 3-fold increase in both SPD and SPM levels indicating that SAMdc is rate-limiting step in higher PA biosynthesis. Since SAM is also a precursor for ethylene biosynthesis, a competition for SAM for the biosynthesis of ethylene and PAs was suggested (Cassol and Mattoo, 2003; Fluhr and Mattoo, 1996; Harpaz-Saad et al., 2012; Lasanajak et al., 2014; Quan et al., 2002). However, the transgenic expression of *SAMdc* or *SpdSyn* resulted in a higher production of ethylene in ripening tomato indicating that SAM is not limiting for the ethylene biosynthesis (Mehta et al., 2002; Nambeesan et al., 2010).

We have previously shown that transgenically enhanced PA levels delayed fruit ripening and increased lycopene in tomato fruits. The enhanced SPD and SPM levels in *ySAMdc*-expressing tomato fruits also increased precipitate weight ratio up to 60%, juice serum viscosity up to 47% and lycopene content up to 300% compared to WT fruits

(Mehta et al., 2002). Ectopic expression of *ySpdSyn* led to 40% increase in lycopene in red ripe transgenic tomato fruits (Nambeesan et al., 2010). In conjunction to our findings, the ectopic expression of *MdSpdSyn* in tomato fruit also resulted in over 2-fold increase in lycopene content (Neily et al., 2010). Based on the transgenic expression of *SpdSyn* or *SAMdc*, higher PAs have also been implicated in plant's responses to biotic and abiotic stresses in pear (Wen et al., 2008; Wen et al., 2011; Wen et al., 2010), tomato (Cheng et al., 2009; Hazarika and Rajam, 2011; Neily et al., 2011), sweet orange (Fu et al., 2011), potato (Kasukabe et al., 2006), *Arabidopsis* (Kasukabe et al., 2004; Marco et al., 2014), rice (Peremarti et al., 2009) and tobacco (Waie and Rajam, 2003; Wi et al., 2006). Based on changes in transcriptome of plant cells with altered PA levels, either transgenically or mutation, we have suggested that a complex network relationship exist among the three PAs and the biosynthesis and signaling pathways of plant hormones which determine the role of PAs during plant growth and development (Anwar et al., 2015). Even though, significant advances have been made in elucidating biosynthesis, catabolism and some of the biological functions of PAs, the molecular mechanisms regulating PA homeostasis are still largely unknown (Kausch et al., 2011; Paschalidis and Roubelakis-Angelakis, 2005).

In the present investigation, we have characterized transgenic tomato plants ectopically expressing either *ySpdSyn* or *ySAMdc* under CaMV 35S, a constitutive, and SlE8, a fruit-specific, promoters to evaluate changes in PAs homeostasis during fruit growth and ripening. Also, transgenic tomato fruits expressing *ySpdSyn* and *ySAMdc* under CaMV 35S and SlE8 promoters were genetically crossed to determine which of these enzymes is rate limiting for biosynthesis and homeostasis of SPD and SPM. Fruit from the transgenic lines expressing *ySpdSyn* and *ySAMdc* under CaMV 35S and SlE8 promoters were evaluated for several quality attributes, both on-planta and off-planta, including the time needed for fruit to ripen from B to R stage and subsequent postharvest shelf life. Results herein show that SAMdc is the rate limiting step in SPD and SPM biosynthesis. We also report that the free SPD and SPM levels are positively correlated with fruit firmness, accumulation of total solids and delay in fruit shriveling and inversely correlated with

fresh fruit weight, juice pH and seed number in tomato fruits. Free PUT levels exhibit trends opposite to that seen with SPD and SPM.

3.2 Material and methods

3.2.1 Plant material and growth conditions

We have previously transformed *Solanum lycopersicum* L. cv. Ohio8245 with Agrobacterium-based vectors containing the chimeric gene constructs *35S:ySpdSyn*, *E8:ySpdSyn* or *35S:ySAMdc* and have developed several independent transgenic lines, homozygous for transgenes (Mehta et al., 2002; Nambeesan et al., 2010). In present investigation, two independent transgenic lines homozygous for *35S:ySpdSyn* (designated as C4 and C15), one line homozygous for *E8:ySpdSyn* (designated E8-8), and two independent transgenic lines homozygous for *35S:ySAMdc* (designated as 566HO and 579HO) were selected for further characterization (Mehta et al., 2002; Nambeesan et al., 2010). To develop double transgenic lines harboring both *ySpdSyn* and *ySAMdc* transgenes, ovaries on C4 or E8-8 plants were fertilized with pollens from 556HO or 579HO plants. Segregating progenies from resulting heterozygote were characterized for lines homozygous for both transgenes by PCR (Mishra and Handa, 2005) and named 4x6 for C4 x 556HO cross, 4x9 for C4 x 579HO cross, 8x6 for E8-8 x 556HO cross and 8x9 for E8-8 x 579HO cross. Functional co-expression of two transgenes was determined by RT-PCR and qRT-PCR. The WT and transgenic plants were grown on high porosity potting mix (52Mix, Conard Fafard Inc., MA USA) under glasshouse environment with 23°C day/18°C night temperature and supplementary lighting was provided to maintain 16h day/8h night photoperiod. Vine-ripened red ripe fruits were randomly collected from each genotype to determine fresh fruit weight, fruit dry weight, fresh fruit density and number of seeds per fruit. For fruit firmness, pH, total titratable acidity and TSS determinations, fruits were tagged at B stage and ripened either on-planta or off-planta. USDA color classification was followed to establish B, P and R stages of tomato fruits (http://ucanr.edu/repository/a/?a=83755). Immediately after recording fruit quality

attributes, fruit pericarp tissues were slices with sterilized razor blade and immediately frozen in liquid N_2 and stored at -80°C until further use. All analyses were done in biological triplicates.

3.2.2 Fruit quality attributes

Fruit fresh weight, dry weight and pH were determined as described previously (Tieman et al., 1995). Briefly, at least three tomato fruits were diced and homogenized using kitchen blender at 20±2°C. Total acids and soluble solids contents in homogenized fruit juice were measured as described by Anwar et al. (2008) with some modifications. For determination of total titratable acid contents (citric acid as percent of fresh weight), freshly homogenized juice was diluted 10-fold in double distilled water and titrated with 0.1N NaOH up to pH 8.1. For determination of TSS, insoluble solids in freshly homogenized tomato juice were pelleted down with quick spin and amount of TSS in supernatant was determined using handheld refractometer (Atago, PAL-1, Japan). Results were obtained and analyzed in Brix, a refractive index used to measure TSS. Fruit firmness was measured with fruit pressure tester (FT 327, Mc Cormick, Italy) equipped with 8mm diameter plunger as described previously (Cutillas-Iturralde et al., 1993). To determine fresh fruit density, fruit volume was measured with water displacement method and then fruit mass was divided by fruit volume.

3.2.3 Postharvest fruit shelf life

Fruits at B stage were randomly harvested from WT and transgenic plants, immediately weighed and kept under ambient conditions (25°C±2). Each fruit was evaluated daily for changes in fruit weight and scored for signs of shriveling and decay as described earlier (Nambeesan et al., 2010).

3.2.4 Quantification of PAs by high pressure liquid chromatography

PAs in floral buds and fruit tissues of tomato plants were extracted and dansylated as described by Torrigiani et al. (2012) with some modifications. Briefly, 200 mg of finely ground tissue sample was homogenized in 800 µl of 5% cold perchloric acid (PCA) using tissue dispenser (IKT, T25 digital Ultra-Turrax, Germany) at 5000 rpm. The homogenate was left at 4°C for 60 min and centrifuged at 20,000 *g* for 30 min in an Eppendorf centrifuge. One hundred µl of the supernatant was used to determine free PAs after dansylation and another 100µl supernatant was hydrolyzed with equal volume of 6N HCl for 18 h at 110°C before dansylation to quantify free conjugated PAs. To quantify bound PAs (PCA-insoluble PAs), the pellet obtained after centrifugation was washed twice with PCA, re-suspended in 800 µl of 5% cold PCA, and hydrolyzed in equal volume of 6N HCl for 18 h at 110°C and dansylated to determine levels of PUT, SPD and SPM. For dansylation, 200µl of saturated Na_2CO_3 and 20µl of 250µM 1,7-heptanediamine (as an internal standard) were added to the 100µl of supernatant, hydrolyzed supernatant or hydrolysates from pellet and dansylated with 400µl dansyl chloride at 60°C for 60 min under dark conditions. Dansylation was terminated by adding 100µl proline (100mg/ml water) and incubating the reaction mixture at 60°C for 30 min. Dansylated PAs were extracted in 500µl toluene, air dried and dissolved in 250µl acetonitrile. Samples were diluted four times with acetonitrile, filtered through 0.45µm syringe filter (National Scientific, USA) and separated on a reversed-phase Nova-Pak C18 column (3.9 x 150mm, 4.0µm pore size) on Waters 2695 Separation Module equipped with Waters 2475 Multi λ fluorescence detector (excitation 340 nm, emission 510 nm) using a binary gradient composed of solvent A (100% water) and solvent B (100% acetonitrile) at 1 ml/min. Initial conditions were set at 60:40 (A:B) and then linear gradient was proceeded with conditions set at 30:70 (A:B) at 3 min; 0:100 (A:B) at 10 min and 60:40 (A:B) at 12 min. Column was flushed with 60:40 (A:B) for at least 3 min before next sample injection. To determine PAs recovery and generate calibration curves, standard PAs (Sigma-Aldrich, USA) were used as control. PAs were integrated and quantified using Millennium[32] 4.0 from Waters

Corporation. PCA-soluble, PCA-soluble but detectable after hydrolysis with HCl, and PCA-insoluble but quantified after hydrolysis were designated as free, conjugated and bound forms of various PAs, respectively, throughout the manuscript.

3.2.5 Statistical Analysis

Data were first tested for normality, mean comparisons were performed using Student's t-Test (two tailed, unequal variance) using algorithms in Microsoft Excel 2014 software package. Threshold level of statistical significance of the data was set at $p < 0.05$. Fisher's least significant difference (LSD) method was used for pairwise comparison of mean values. PCA and correlation coefficient values were determined with Pearson (n) method using XLSTAT Version 2014.4.06.

3.3 Results

3.3.1 Expression of *ySpdSyn* altered PA homeostasis in ripening tomato fruits

Figure 3.1 shows the changes in soluble, conjugated and bound PUT, SPD and SPM in WT and three independent transgenic lines expressing *ySpdSyn* under either CaMV 35S or SlE8 promoters at B, R, 7 DAR and 17 DAR. During the WT fruit ripening, levels of free and conjugated PUT increased while bound PUT declined. The levels of free SPD declined whereas that of conjugated and bound SPD remained similar during the WT fruit ripening. The free and bound SPM levels did not change significantly, but conjugated SPM levels exhibited decline during the WT fruit ripening. Total amounts of PUT, SPD and SPM decreased compared to B stage during WT fruit ripening (Figure 3.1).

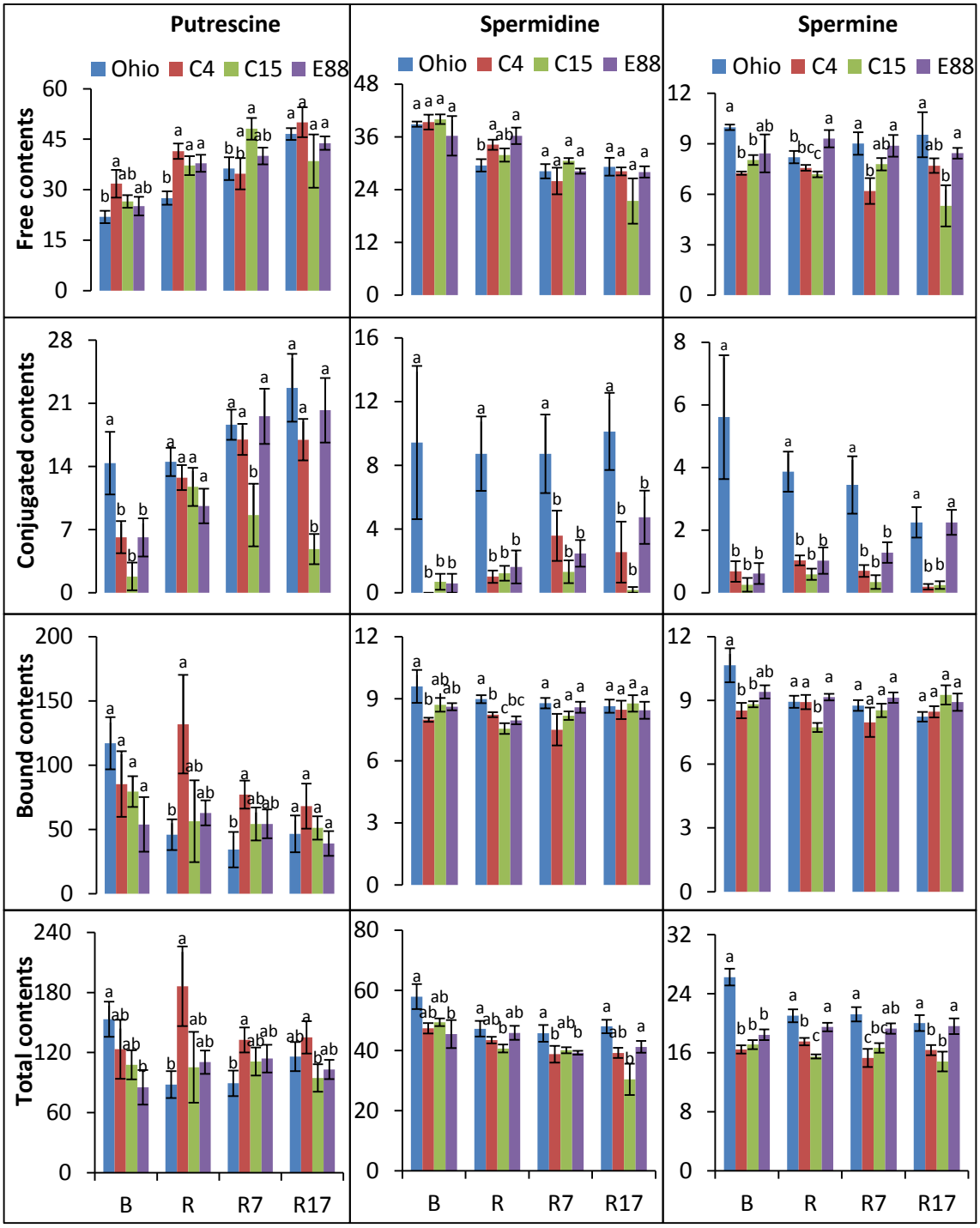


Figure 3.1**:** PA levels in WT and transgenic tomato fruits expressing *ySpdSyn*.

Fruits were tagged and ripened on plants. Shown are average ± standard error (n≥3 biological replicates). Similar letters above standard error bars indicate non-significant difference (at 95% confidence interval) among genotypes at each stage. B, breaker stage; R, red stage; R7 and 17R, 7 and 17 DAR.

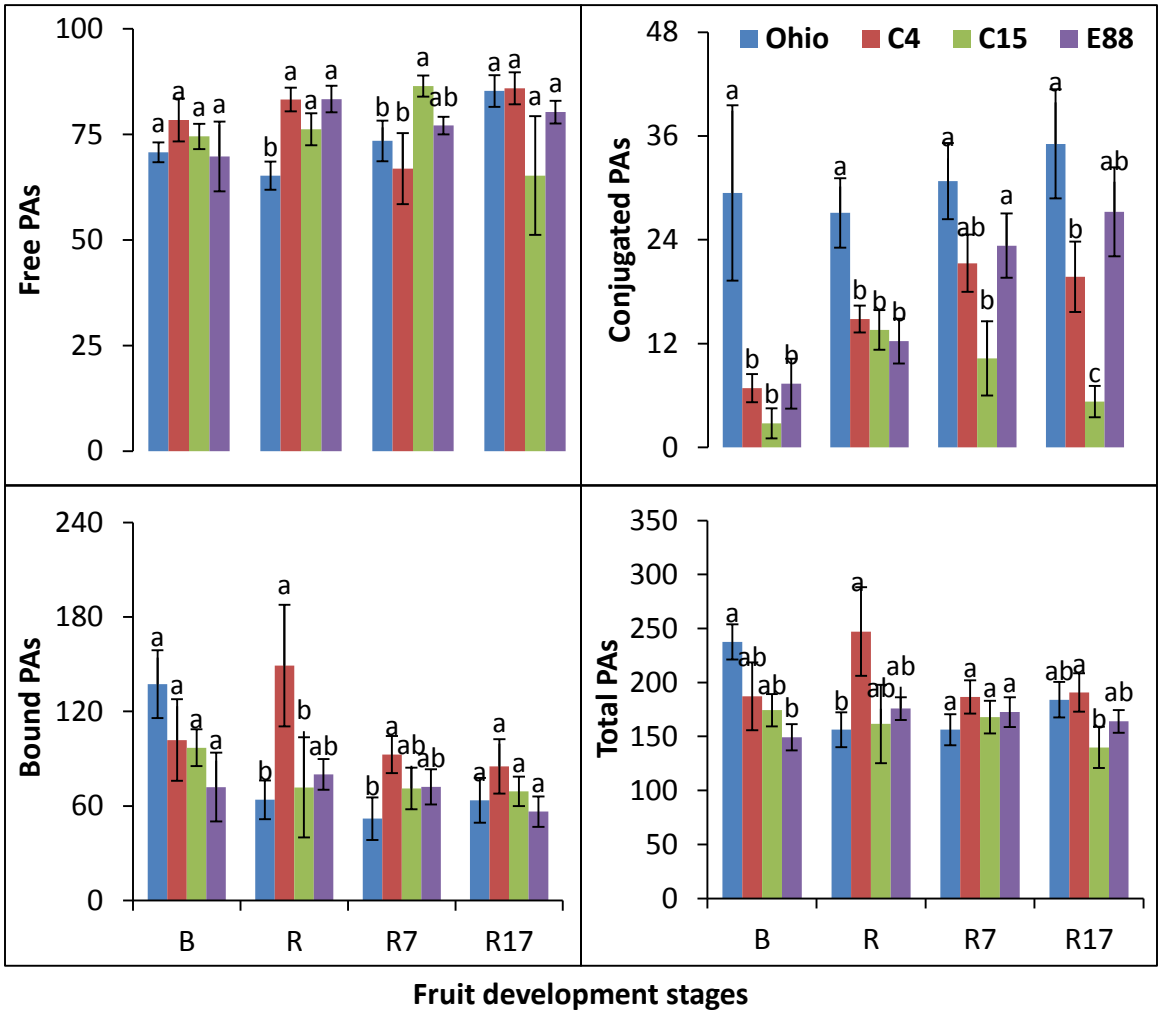


Figure 3.2**:** Total amounts of free, conjugated or bound PAs in WT and transgenic tomato fruits expressing *ySpdSyn*.

Total amounts of PUT, SPD and SPM in free, conjugated or bound fractions in Figure 3.1 were added. Total PAs in bottom right panel were calculated by adding free, conjugated and bound PAs. Other detail are given in Figure 3.1.

Expression of *ySpdSyn* transgene under both constitutive CaMV 35S and fruit/ethylene-regulated E8 promoters had a limited effect on free and bound PUT, SPD and SPM in ripening fruit but dramatically reduced levels of conjugated SPD and SPM starting from B until 17 DAR (Figure 3.1). There was an intermittent increase in free PUT as its levels in C4 fruits increased at B and R stages, in C15 fruits at R and 7 DAR and in E8-8 fruits at R stage compared to WT fruits. However, conjugated PUT was lower in C4 and C15 fruits, especially C15 fruits at B, 7 DAR and 17 DAR. The E8-8 fruits exhibited decrease in conjugated PUT at B and increased to WT levels thereafter during ripening. Bound PUT was higher at R and 7 DAR in C4 fruits while it was similar to WT fruits in C15 and E8-8 fruits at all stages of ripening. The transgenes expression increased the total PUT level in C4 fruits at R and 7 DAR but decreased in C15 and E8-8 fruits at B stage. Free SPD levels in transgenic fruits were similar to WT except an increase at R stage in C4 and E8-8. Levels of bound SPD were also similar to WT except reduction in C4 fruits at B and in all three transgenic fruits at R stage. The levels of conjugated SPD and SPM were also dramatically lower in the fruits from three transgenic lines throughout the ripening process. Thus, the total amount of SPD was reduced in transgenic fruits at almost all stages examined. The levels of total SPM were lower in all three transgenic fruits at B stage and in C4 and C15 lines at all other stages. The transgene expression had a limited effects on the total amounts of total PAs (sum of free, conjugated and bound PUT, SPD and SPM) production (Figure 3.2). The temporal and developmental trends of PAs accumulation in transgenic fruits followed patterns similar to WT, indicating that *ySpdSyn* transgene expression did not alter the biological programmed accumulation patterns of PAs in ripening tomato fruits.

3.3.2 Availability of dcSAM is the rate limiting step in SPD/SPM accumulation in ripening tomato fruits

Limited changes in levels of free, conjugated and bound PUT, SPD and SPM in transgenic fruit expressing *ySpdSyn* under both CaMV 35S and E8 promoters (Figure 3.1)

indicated that SpdSyn was not likely a rate limiting enzyme in PAs biosynthesis in ripening fruits. We have previously shown that expression of *ySAMdc* under SlE8 promoter greatly increased levels of both SPD and SPM in ripening tomato fruit (Mehta et al., 2002). To determine if the co-expression of *ySAMdc* and *ySpdSyn* transgenes would boost the levels of PAs in ripening fruits, we developed genetic crosses among *ySAMdc* and *ySpdSyn* transgenic lines and evaluated changes in accumulation of free, conjugated and bound PUT, SPD and SPM in fruits from the resulting homozygous lines. Since both transgenes were introduced into the same genetic background, the resulting homozygous plants will be isogenic to both parents, limiting any effect of genetic background on quantitative determinations.

Figure 3.3 shows that *ySAMdc* transgenic red ripe fruits contained significantly higher free SPD with corresponding decrease in free PUT compared to WT fruit, confirming the previous study (Mehta et al., 2002). Independent transgenic lines expressing either *35S:ySpdSyn* (C4), *E8:ySpdSyn* (E8-8) or *E8:ySAMdc* transgenes (556HO and 579HO) showed pattern similar to as reported above (Figure 3.1). The red ripe fruits from *35S:ySpdSyn* x *E8:ySAMdc* showed accumulation of free PUT, SPD and SPM similar to WT ripe fruits. However, red ripe fruits from *E8:ySpdSyn* x *E8:ySAMdc* showed significant increase in the levels of free SPD, a pattern similar to *E8:ySAMdc* fruits. The levels of conjugated and bound PUT were significantly higher in fruits from all three independent *ySpdSyn* transgenic lines, but this attribute was not transferred to fruits from their crosses with *E8:ySAMdc* (Figure 3.3). Furthermore, there was no increase in levels of total PUT, SPD and SPM in fruits co-expressing both *ySpdSyn* and *ySAMdc* transgenes. Principal component (PC) analysis of PA levels in WT and transgenic fruits showed a positive correlation between *ySAMdc* fruits and fruits co-expressing *ySpdSyn* and *ySAMdc* and grouped on positive quadrant of first PC*,* the WT and *ySpdSyn* fruits were clustered on negative quadrant of first PC (Figure 3.4). These results show that SAMdc is the rate limiting enzyme, for the production of higher PAs in ripening tomato fruits.

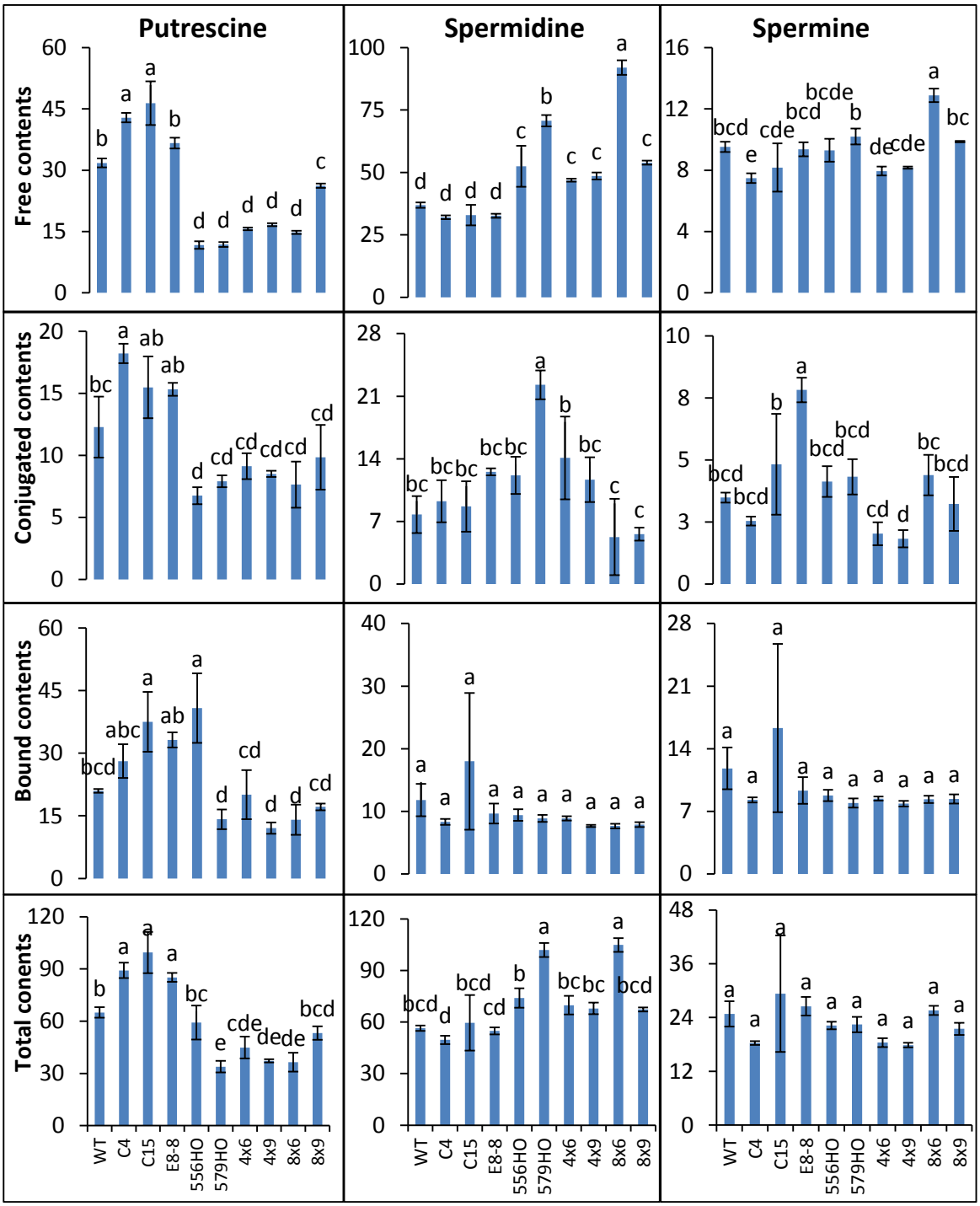


Figure 3.3**:** PA levels in red ripe tomato fruits from WT and transgenic plants expressing *ySpdSyn* or *ySAMdc* or co-expressing both transgenes.

Description of genotypes is given in 'Plant material and growth conditions' (section 3.2.1). Other detail are given in Figure 3.1.

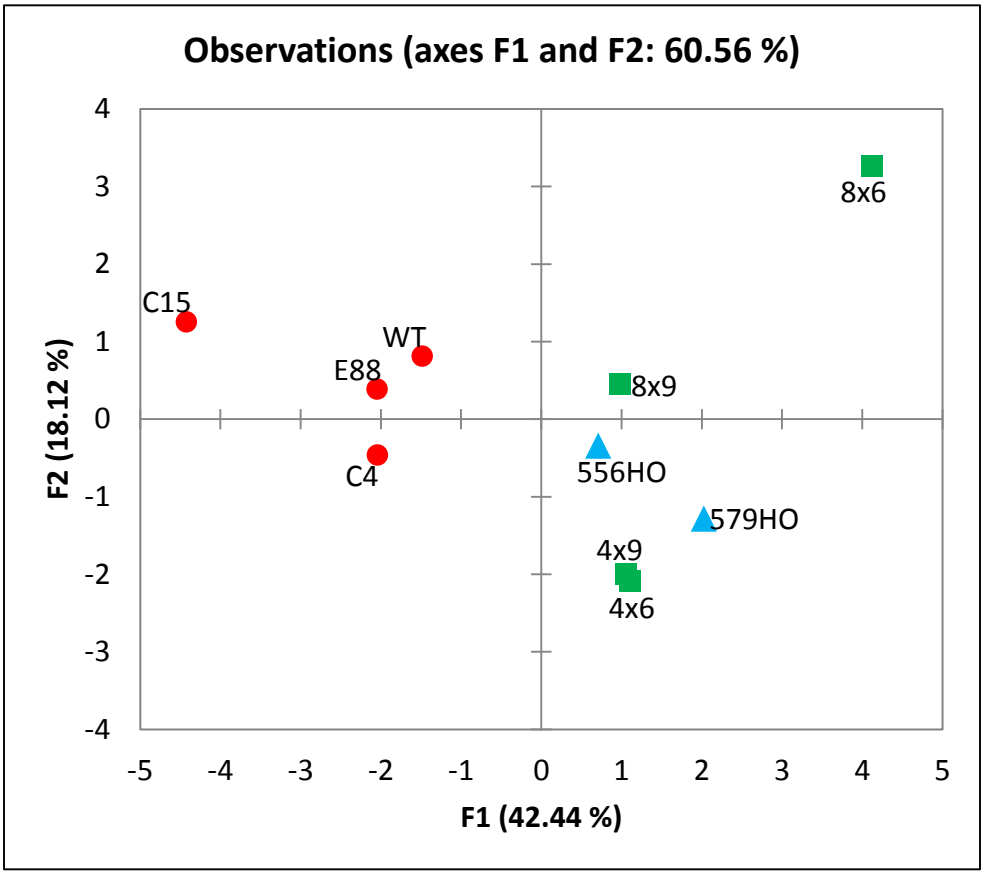


Figure 3.4**:** Principal component analysis of PA levels in WT and transgenic fruits expressing *ySpdSyn* or *ySAMdc* or co-expressing both transgenes.

PA levels in red ripe fruits from ten genotypes were analyzed with PCA using XLSTAT Version 2014.4.06. Description of genotypes is given in 'Plant material and growth conditions' (section 3.2.1).

3.3.3 Effect of transgenic expression of *ySpdSyn* and *ySAMdc* on fruit quality attributes

3.3.3.1 Firmness

Tomato fruits were either ripened on the plants or harvested at B stage and kept under ambient conditions to determined loss in fruit firmness using a fruit penetrometer. At B stage, firmness of 579HO fruits was significantly higher than WT fruits (Figure 3.5). During fruit ripening, firmness of the WT fruits declined over 2-fold in the fully ripe fruits irrespective whether fruits were ripened on-planta or off-planta (Figure 3.5). On-planta, 579HO fruits remained significantly firmer than the WT fruits until 7 DAR, whereas off-planta, they retained this advantage until the 17 DAR. The on-planta fruits from 556HO plants were slightly less firm than 579HO fruits until 7DAR, whereas off-planta, 556HO fruits exhibited firmness similar to 579HO fruits that was significantly higher than WT fruits (Figure 3.5). Firmness changes in fruits from *ySpdSyn* lines showed a mixed pattern (Figure 3.5). The absence of consistent pattern for fruit firmness in different transgenic lines suggests that the transformation event affects this phenotype (Figure 3.5), and selection of desirable events would provide genotypes/cultivars with significantly enhanced retention of fruit texture during the postharvest storage of fruits.

3.3.3.2 Total soluble solids and acid contents

Changes in TSS, acid contents and pH values during on-planta or off-planta tomato fruit ripening are given in Table 3.1. Irrespective whether the fruits were ripened on-planta or off-planta, TSS contents in WT fruits dropped at R stage compared to B stage but then remained unchanged until 17 DAR. Constitutive expression of *ySpdSyn* stimulated sporadic reduction in TSS contents in fruits at B stage that increased during on-planta fruit ripening (Table 3.1).

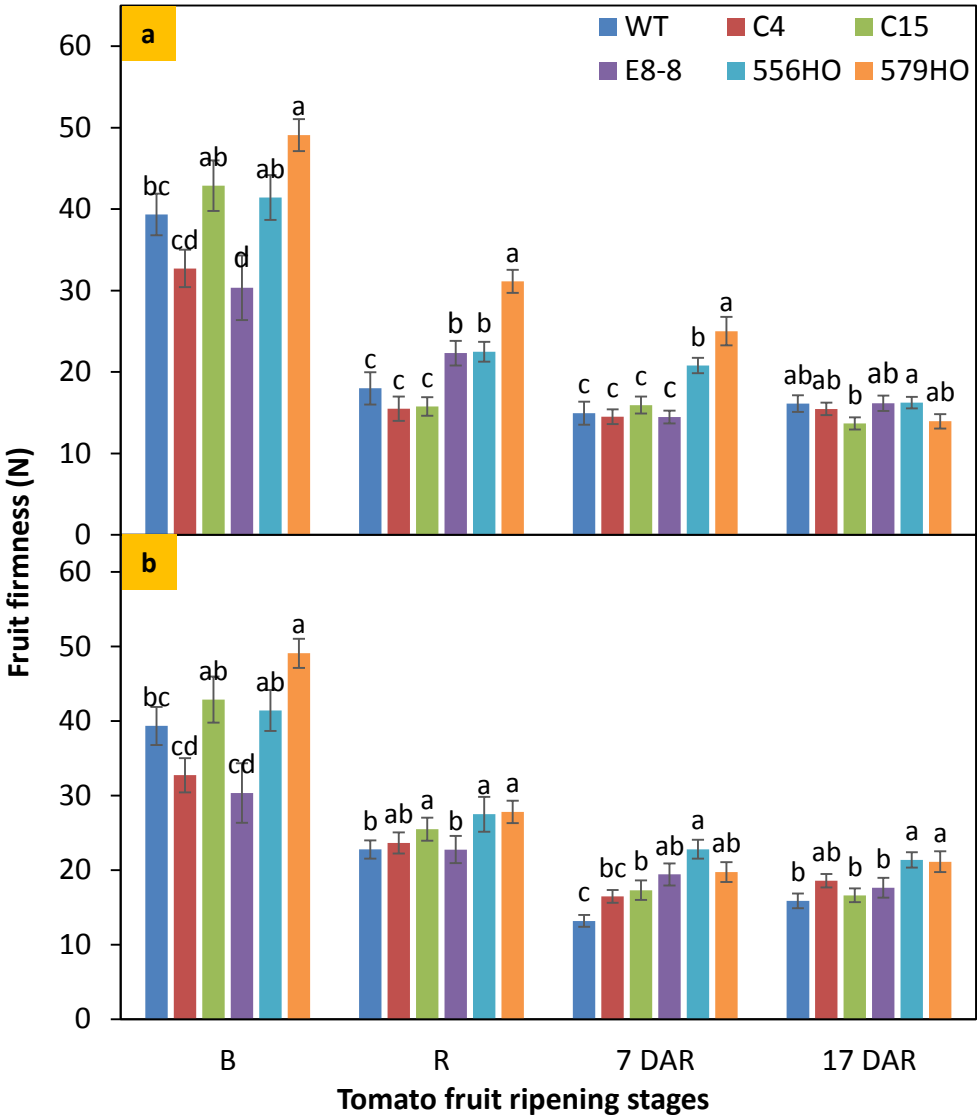


Figure 3.5**:** Changes in fruit firmness of WT and transgenic tomato fruits expressing *ySpdSyn* or *ySAMdc*.

Fruits were either tagged and ripened on the plants (a) or harvested at B stage and ripened off-vine (b) to determine changes in fruit firmness. Shown are average ± standard error (n≥9 biological replicates). Similar letters above standard error bars indicate non-significant difference (at 95% confidence interval) among genotypes at each stage. B, breaker stage; R, red stage; DAR, days after red stage.

Table 3.1**:** Fruit quality attributes in WT and transgenic tomato fruit expressing *ySpdSyn* or *ySAMdc*.

a: On-planta parameters

	pH			TSS (°Brix)			Total acids (%)			TSS:acids ratio		
Genotypes	R	7 DAR	17 DAR	R	7 DAR	17 DAR	R	7 DAR	17 DAR	R	7 DAR	17 DAR
WT	4.23^{a}	4.25^{a}	4.34^{a}	3.94^{bc}	4.23^{c}	4.01^{b}	3.62^{c}	3.59^{ab}	3.53^{a}	1.09^{b}	1.19^{c}	1.15^{ab}
C4	4.28^{a}	4.26^{a}	4.34^{a}	5.01^{a}	3.71^{c}	3.33^{b}	3.93^{bc}	2.86^{c}	2.46^{b}	1.27^{a}	1.38^{bc}	1.44^{ab}
C15	4.23^{a}	4.25^{a}	4.38^{a}	3.83^{bc}	4.16^{c}	3.06^{b}	4.19^{b}	3.08^{abc}	3.11^{ab}	0.92^{cd}	1.36^{bc}	0.98^{b}
E8-8	4.23^{ab}	4.30^{a}	4.33^{a}	4.10^{b}	6.59^{a}	3.93^{b}	4.21^{b}	2.92^{c}	2.93^{ab}	0.98^{bc}	2.37^{a}	1.52^{a}
556HO	4.14^{bc}	4.29^{a}	4.36^{a}	3.33^{c}	5.46^{b}	3.91^{b}	3.82^{bc}	3.03^{bc}	2.51^{b}	0.88^{cd}	1.79^{b}	1.57^{a}
579HO	4.13^{c}	4.17^{b}	4.40^{a}	3.84^{bc}	4.33^{c}	5.70^{a}	4.80^{a}	3.69^{a}	3.16^{ab}	0.80^{d}	1.20^{c}	1.70^{a}

b: Off-planta parameters

	pH			TSS (°Brix)			Total acids (%)			TSS:acids ratio		
Genotypes	R	7 DAR	17 DAR	R	7 DAR	17 DAR	R	7 DAR	17 DAR	R	7 DAR	17 DAR
WT	4.08^{a}	4.10^{bc}	4.11^{c}	4.51^{ab}	3.86^{a}	4.11^{ab}	4.20^{b}	3.48^{a}	2.63^{cd}	1.15^{a}	1.30^{a}	1.61^{bc}
C4	4.08^{ab}	4.13^{abc}	4.17^{bc}	4.11^{b}	3.91^{a}	4.08^{ab}	5.06^{ab}	4.43^{a}	4.36^{a}	0.81^{b}	0.92^{a}	1.11^{c}
C15	4.05^{ab}	4.19^{a}	4.25^{a}	4.25^{ab}	4.18^{a}	4.57^{ab}	4.87^{ab}	4.00^{a}	1.68^{d}	0.88^{ab}	1.35^{a}	2.80^{a}
E8-8	4.00^{b}	4.16^{ab}	4.18^{b}	4.50^{ab}	3.83^{ab}	5.15^{a}	4.70^{ab}	3.83^{a}	2.93^{bc}	0.99^{ab}	1.02^{a}	2.02^{ab}
556HO	4.03^{ab}	4.06^{c}	4.13^{bc}	3.59^{b}	2.88^{b}	3.31^{b}	5.28^{a}	4.19^{a}	3.89^{ab}	0.68^{b}	0.71^{a}	0.90^{c}
579HO	4.08^{ab}	4.07^{bc}	4.15^{bc}	5.45^{a}	3.80^{ab}	4.62^{ab}	4.63^{ab}	4.00^{a}	3.54^{abc}	1.19^{a}	1.14^{a}	1.22^{bc}

DAR, days after ripening

Total acid contents of WT fruits did not change during on-planta fruit ripening but declined during off-planta fruit ripening. Expression of *ySpdSyn* or *ySAMdc* transgenes stimulated accumulation and then reduction of total acids during on-planta fruit ripening. TSS and total acid contents during off-planta fruit ripening did not change significantly in transgenic fruits. The TSS:Acid ratio and pH of juice from WT fruits remained unchanged during on-planta and off-planta fruit ripening and transgenic fruits also did not exhibit any definite pattern in TSS:Acid ratio and pH changes in fruit juice (Table 3.1).

3.3.3.3 Fruit ripening period, weight loss and shriveling during shelf life

Number of days required by the fruits at B stage to reach R stage were also recorded under ambient conditions. All transgenic fruits showed 12 to 24 hours delay in fruit ripening while stored under ambient conditions (Figure 3.6). Color development, fruit weight and development of shriveling signs on each fruit were recorded daily until 40 days after R stage (Figure 3.7). Even though, weight loss trends of transgenic fruits during shelf life were similar to WT fruits (Figure 3.7a) but, generally, transgenic fruits shriveled slower than WT fruits (Figure 3b) except E8-8 which shriveled similar to WT fruits, comparatively.

3.3.3.4 Fresh and dry weight, density and seed production

The fresh weight of *ySpdSyn* fruits was similar to WT whereas *ySAMdc* fruits exhibited lower fresh fruit weight than WT fruits (Figure 3.8a). Co-expression of *ySpdSyn* and *ySAMdc* also resulted in decrease in fresh fruit weight compared to WT and *ySpdSyn* fruits. The *35S:ySpdSyn-4* x *E8:ySAMdc-556* (4x6) fruits had the least fresh fruit weight compared to fruits from WT and its transgenic parent lines.

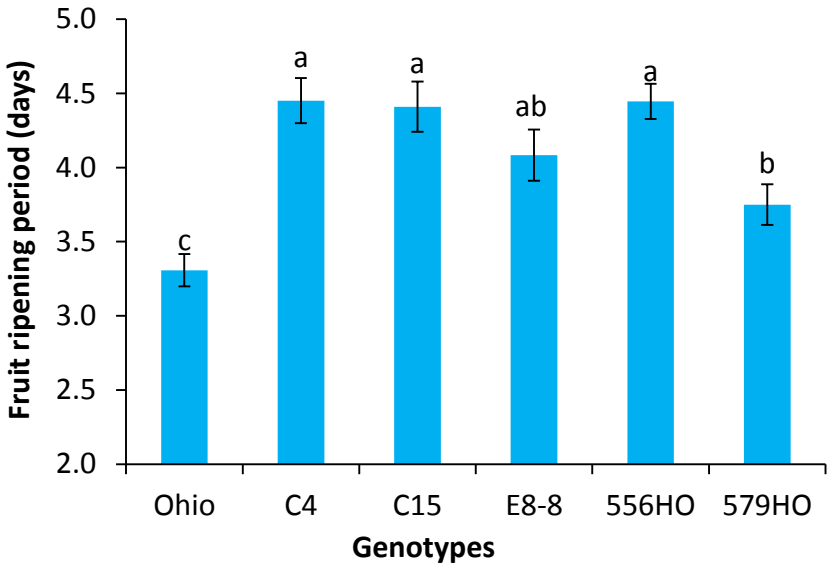


Figure 3.6**:** Fruit ripening period of WT and transgenic tomato fruits expressing *ySpdSyn* or *ySAMdc*.

Fruits were harvested at B stage and ripened off-vine to determine days required by tomato fruits to develop full red color from B stage. Other detail are given in Figure 3.5.

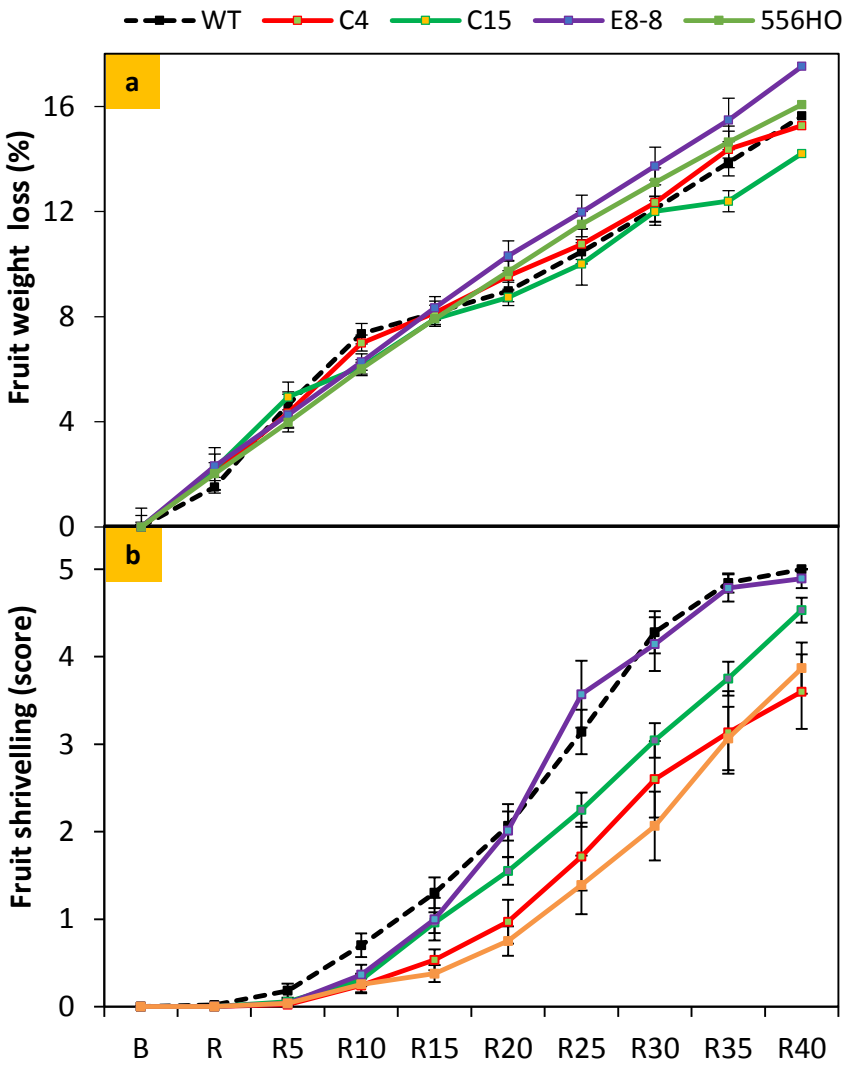


Figure 3.7**:** Fruit weight loss and development of fruit shriveling symptoms during storage of WT and transgenic tomato fruits expressing *ySpdSyn* or *ySAMdc*.

Fruits were harvested at B stage and kept at ambient temperature (25±2°C). See Material and methods section 3.2.3 for further detail. Shown are average ± standard error (n≥3 biological replicates and ≥10 fruits per replication). B, breaker stage; R, red stage; R5 to R40, 5 to 40 days after R stage.

Analysis of dry weight (percent of fresh weight) showed that C4, C15 and 579HO fruits were lower in total solids percentage whereas E8-8 and 556HO fruits were similar to WT fruits (Figure 3.8b). Among double transgenic lines, *35S:ySpdSyn-4* x *E8:ySAMdc-556* (4x6) fruits showed decrease in dry weight percentage but *E8:ySpdSyn-8* x *E8:ySAMdc-556* (8x9) and *E8:ySpdSyn-8* x *E8:ySAMdc-579* (8x9) fruits exhibited significantly enhanced dry weight percentage, indicating that co-expression under similar promoter may have much stronger impact on enhancing carbon sequestration into tomato fruits.

Fresh fruit density of all the transgenic fruits was similar to WT except E8-8 which had lower fruit density but its co-expression with 579HO enhanced fruit density comparable to WT and E8-8 fruits (Figure 3.8c). Seed production in *35S:ySpdSyn* and 579HO fruits was similar to WT fruits while E8-8 and 579HO had lower number of seeds per fruit than WT fruits (Figure 3.8d). Interestingly, co-expression of *ySpdSyn* and *ySAMdc* in *35S:ySpdSyn-4* x *E8:ySAMdc-579* (4x9) fruits resulted in 20% increase in number of seeds per fruit.

3.3.4 Statistical analyses of correlations among different forms of PAs and fruit quality attributes

Relationship between fruit quality attributes and PA levels was investigated using Pearson (n) correlation matrix and PCA (Figure 3.9). PA levels in red fruits from WT, *ySpdSyn*, *ySAMdc* and homozygous plants co-expressing both transgenes were correlated with fresh fruit weight, fruit dry weight, fresh fruit density and number of seeds per fruit. PA levels in WT and *ySpdSyn* fruits at B stage and on-planta ripened fruits at R and 7 and 17 DAR were correlated with fresh fruit firmness, TSS, pH and total acid contents.

Amounts of free PAs were more strongly correlated with fruit quality attributes than conjugated forms of PAs. Levels of free SPD and SPM were positively correlated with fruit dry weight, fruit density, firmness, TSS and total acid contents while negatively correlated with fresh fruit weight, number of seeds per fruit and pH. However, free PUT

had an opposite effect on these attributes. Even though conjugated PAs had mostly weak correlation (<0.5) with fruit quality attributes, the conjugated SPD and SPM were negatively correlated with those fruit quality parameters (except juice TSS). On the other hand, fruit quality attributes negatively correlated with free SPD/SPM were also negatively correlated with conjugated SPD/SPM (Figure 3.9).

In contrast to differential effect of free PUT and higher PAs (SPD and SPM), levels of bound PAs had similar effect on fruit quality attributes. All three titers of bound PAs were positively correlated with fresh fruit weight, fruit firmness, TSS and total acids and negatively correlated with dry weight, fruit density and juice pH. It should be noted that this correlation was not as strong as recorded in case of free PAs which suggest that free PAs have much more significant and dominant role in regulating fruit quality attributes (Figure 3.9).

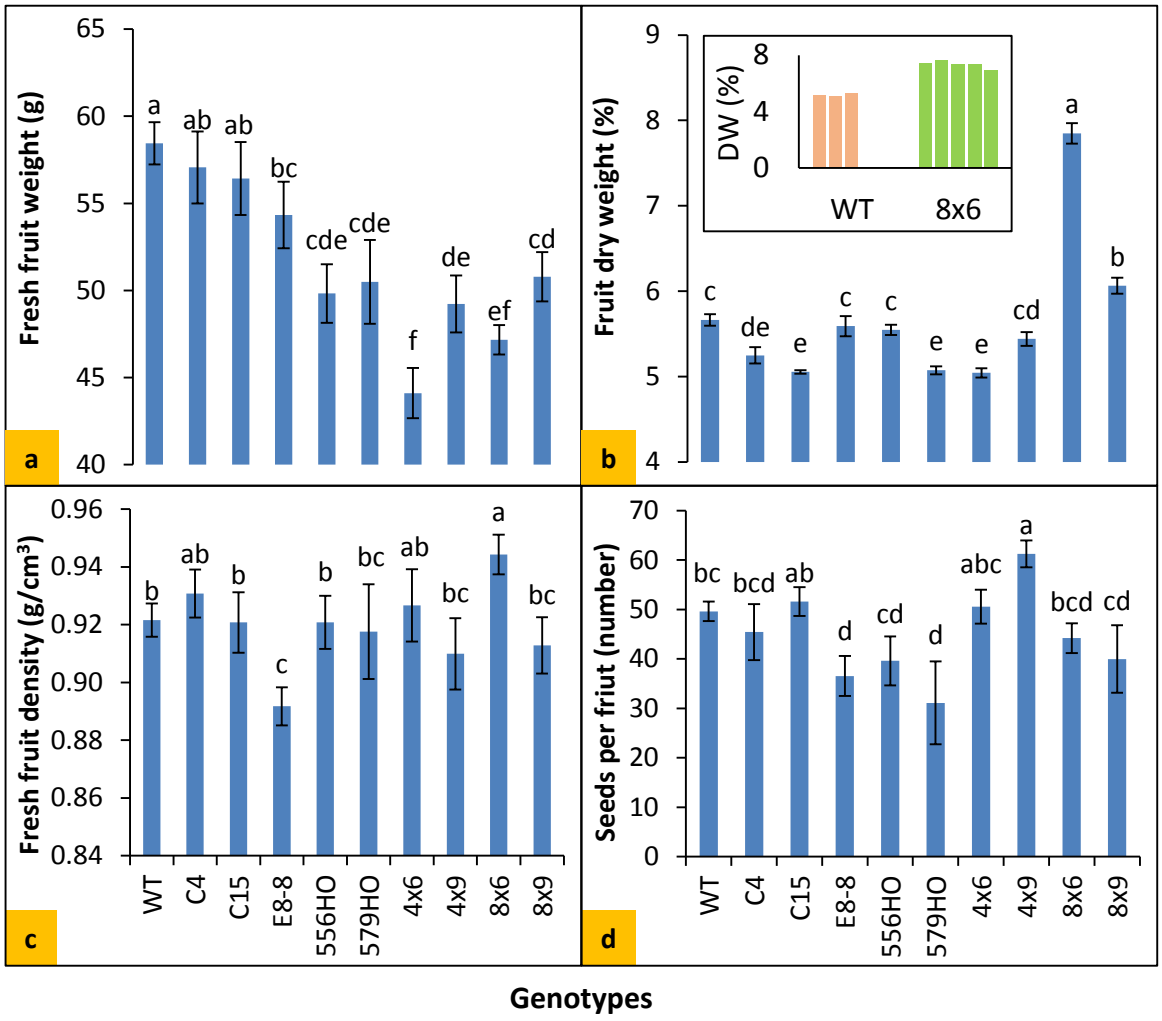


Figure 3.8**:** Fruit mass, density and seeds in red ripe tomato fruits from WT, transgenic parent lines expressing *ySpdSyn* or *ySAMdc* or co-expressing both transgenes.

Shown are average ± standard error (40-200 fruits for 'a' and 3-5 fruits for 'b, c and d' were used as biological replicates at red ripe stage. Similar letters above standard error bars indicate non-significant difference (at 95% confidence interval) among genotypes. Figure b inset, replications data used to calculate average dry weight (%) of WT (n=3) and 8x6 (n=5) fruits. Description of genotypes is given in 'Plant material and growth conditions' (section 3.2.1).

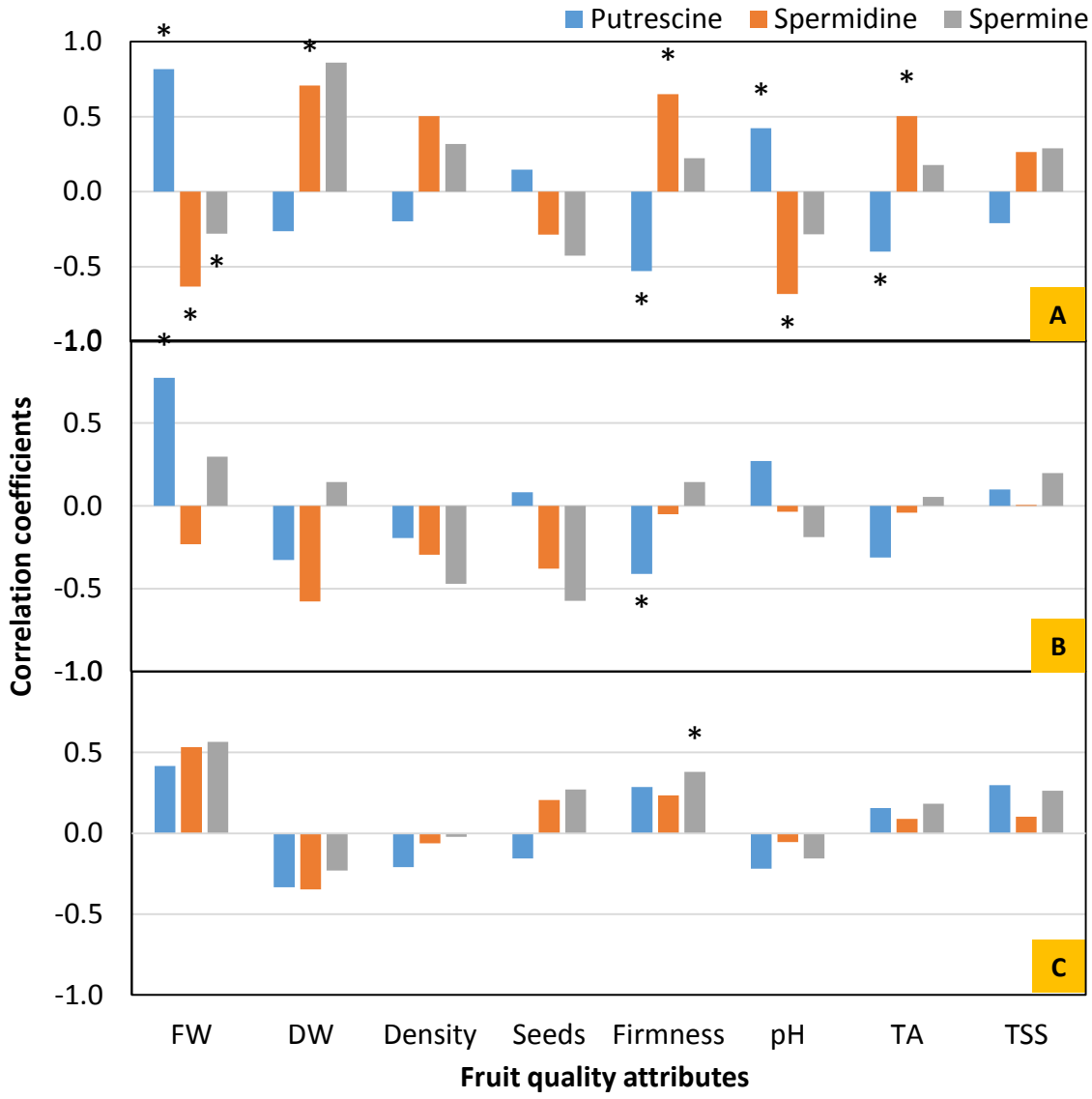


Figure 3.9: Correlation of free, conjugated and bound fractions PUT, SPD and SPM with fruit quality attributes of vine-ripened tomato fruits.

Correlation coefficient values were determined with Pearson (n) method using XLSTAT Version 2014.4.06. FW, fresh fruit weight; DW, fruit dry weight. *, different from 0 with a significance level α=0.05

3.4 Discussion

3.4.1 Limited availability of dcSAM regulates PA pools

Significant progress has been made in elucidating the biosynthetic pathway of PAs in many organisms including plants (Lasanajak et al., 2014). Although, the homeostasis of PAs during cell growth and organism's development is considered tightly regulated, the factors controlling accumulation of different forms of PAs have yet not been fully understood. Based on 2 to 3-fold increase on SPD and SPM in ripening tomato in response to expression of *ySAMdc*, we have previously proposed that this enzyme is the rate limiting step in the biosynthesis and accumulation of SPD/SPM (Mehta et al., 2002). The *ySpdSyn* expression increased SPD by 51-77% along with significant decrease in PUT and SPM levels in transgenic leaves (Nambeesan et al., 2010). However, the co-expression of *ySpdSyn* with *ySAMdc* did not appreciably changed the levels of SPD/SPM, further strengthening our earlier conclusion (Mehta et al., 2002). A similar conclusion was reported by Franceschetti et al. (2004) based on the expression of *Datura stramonium SpdSyn* in tobacco plants that increased SPD-to-PUT ratio but total PA contents remained unchanged. Other studies have led to similar conclusions (Hu et al., 2006; Lasanajak et al., 2014).

3.4.2 Higher SPD delay fruit ripening, extends shelf life, maintains high fruit quality including texture and reduces shriveling

We have previously reported delay in on-planta ripening of *ySAMdc* fruits (Mehta et al., 2002). Similarly, C4 fruits have also been shown to delay onset of fruit ripening, percentage of ripening fruits on the vine (Nambeesan et al., 2010). Here, in this study, WT and transgenic fruits at B stage were harvested from plants and ripened off-planta to determine role of PAs on tomato fruit ripening. As observed in on-planta studies, ectopic expression of *ySpdSyn* or *ySAMdc* delayed tomato fruit ripening compared to WT fruits (Figure 3.6) and delayed development of fruit wrinkling during storage ambient

conditions (Figure 3.7). Results of this study also provide genetic evidence to previously reported effects of exogenous applications of SPD and SPM that helped maintain fruit firmness, shelf life and other fruit quality parameters (Mirdehghan et al., 2007; Mirdehghan et al., 2013; Saba et al., 2012). Two major factors that regulate fruit shelf life are loss of fruit texture and loss of water leading to fruit shriveling. Fruit texture is primarily determined by the fruit firmness. Generally, ingression of a mutant gene from ripening-impaired tomato mutants *nor* (nonripening), *alc* (alcobaca), *rin* (ripening-inhibitor), *Nr* (never-ripe), Gr (green-ripe), *Cnr* (colorless, nonripening) and firme, has been used to extend the shelf life of tomato fruits (Brummell and Harpster, 2001; Giovannoni, 2004; Mutschler, 1984; Negi and Handa, 2008; Schuelter et al., 2002; Tigchelaar et al., 1973). The molecular function of most of these genes are not fully understood except that some encode transcription factors and *Nr* is an ethylene receptor. Other methods to slow down or impair fruit softening/ripening include downregulation of *SlRab11a*, a GTPase (Lu et al., 2001) or *N*-glycans processing enzymes (Meli et al., 2010), or alterations in cuticle architecture (DFD; Saladié et al., 2007). Generally, lower quality of fruits from cultivars having one of these mutations is likely due to temporal separation of ripening genes, such as ethylene-dependent and -independent genes related with ripening processes. Considering that fruits with higher SPD/SPM level exhibit improved quality attributes, the *ySAMdc* and *ySpdSyn* transgenic fruit offer a promising development.

The molecular basis of enhanced fruit texture is not yet clear. A specific relationship among fruit firmness and transcripts of cell wall degrading enzymes was not obtained in transgenic fruit expressing *ySpdSyn* (Nambeesan et al., 2010) suggesting that PAs-inhibited loss in fruit firmness is independent of cell wall degradation-machinery. PUT exist in cytoplasmic soluble fractions while SPD is bound to cell walls (Pistocchi et al., 1987). Based on the association constants between polygalacturonic acid and PUT/SPD (10^5) and SPM (10^6), D'Orazi and Bagni (1987) have suggested that strong binding of PA to pectic substances and other cell wall components may play a role in enhanced fruit firmness. PAs have been reported to strengthen links between cell wall components and

maintain overall cell integrity of tobacco thin layers (Berta et al., 1997). Taken together, these studies suggest that PAs maintain fruit firmness likely by maintaining cell wall rigidity through ionic cross-linking.

PAs have also been suggested to stabilize cell membrane by surface binding to negatively charged components in oat leaf protoplast (Altman et al., 1977). In vitro studies showed that SPD and SPM are more effective than PUT at reducing fluidity of microsomal membranes from primary leaves of bean (Roberts et al., 1986). Thus, PAs with higher cations per molecule (SPM>SPD>PUT) have more efficiency to stabilize cell membrane and cell walls (Kakkar et al., 1998; Schuber, 1989). This interaction has been suggested to play role in maintaining fruit firmness and extending fruit shelf life (Gupta et al., 2013; Madhulatha et al., 2014; Ponappa et al., 1993; Serrano et al., 2003; Valero et al., 2002). PAs-mediated delay in development of fruit shriveling symptoms (Figure 3.7) (Nambeesan et al., 2010) and maintenance of fruit firmness without compromising fruit quality (Figure 3.5, Table 3.1) is highly desired in food industry and enhancement of PAs to achieve these esteemed fruit quality attributes is very tempting.

Even though, fruit weight loss pattern in *ySpdSyn* and *ySAMdc* fruits was almost similar to WT fruits, but transgenic fruits were still shriveling slower than WT fruits stored under ambient conditions (Figure 3.7). We have previously reported that *ySpdSyn* fruits have longer shelf life than WT fruits, C4 fruits being most resistant to shriveling (Nambeesan et al., 2010). Interestingly, water loss pattern from *ySpdSyn* fruits was similar to WT fruits and expression profile of cell wall and membrane degradation-related gene was also not correlated with extended shelf life which indicates that SPD-mediated process that delayed fruit shriveling, was independent of fruit water loss and cell wall and membrane degradation-machinery (Nambeesan et al., 2010).

Our results show that expression of *SAMdc* and *SpdSyn* under the SlE8 promoter reduced the fresh weight of ripened fruit, but there was only a slight decrease in the dry weight accumulation (Figure 3.8). However, we also observed that *ySpdSyn* transgenic fruit continue to gain post-ripening fresh weights (up to 50%) with proportional increase in dry weight (Figure 4.2). There were significant changes in fruit metabolome in

transgenic fruits expressing *ySpdSyn* (CHAPTER 4) or *ySAMdc* (Mattoo et al., 2006). A number of factors, including source-sink relationship, regulate fruit fresh and dry weight accumulation (Albacete et al., 2014a; Albacete et al., 2014b). Fruit size is dependent on factors such as cell number and cell size and physiological factors regulating transport of sugar between source and sink. During rapid growth phase, fruits are the strongest sinks for assimilates, mainly hexoses and starch, which effect TSS and fruit yield (Albacete et al., 2014b; Ho, 1984). The dry weight accumulation was dramatically effected in *E8:ySAMdc* fruits (Figure 3.8), likely due to higher respiration as demonstrated earlier (Mattoo et al., 2006). Increased respiration rate would consumes more fruit carbon to generate energy needed to restore and enhance metabolic activity in ripening tomato fruits and would likely result in reduction in solid contents of transgenic tomato fruits (Figure 3.8). It has been proposed that endoreduplication plays an important role in fruit cell expansion by regulating cell cycle genes (Chevalier et al., 2011). However, endoreduplication can be uncoupled from cell expansion and may not be the prerequisite for increased cell expansion (Nafati et al., 2011). We have shown that transgenically enhanced PA levels upregulate some of the genes involved in cell division and endoreduplication (CHAPTER 2).

Several plant growth regulators are considered to regulate tomato fruit set and development (Ariizumi et al., 2013; Srivastava and Handa, 2005) and sink-related processes (Ehneß and Roitsch, 1997; Roitsch et al., 2003; Roitsch and Ehneß, 2000). Overexpression of cytokinin biosynthesis gene *IPT* or cell wall invertase gene *CIN1* enhanced sucrolytic activities and reduced ACC levels (ethylene precursor) which resulted in increased fruit weight and number of fruits per plants (Albacete et al., 2014a). ABA is likely correlated with the fruit fresh weight gain as ABA-deficient mutant fruits are small due to reduced cell expansion in the pericarp, and not due to reduction in cell number (Nitsch et al., 2012). PAs are known to slowdown plant senescence and delay fruit wrinkling during postharvest storage (Nambeesan et al., 2010). We have shown that PAs interact with a multitude of plant hormones (CHAPTER 5) (Anwar et al., 2015). It is likely that altered hormone balance coupled with inhibition in senescence-related processes

play a significant role in modifying source-sink relationships that would lead to enhanced quality attributes in *SAMdc*- and *SpdSyn*-expressing fruits. Further characterization of transcriptome and energy metabolism would help understand the physiological basis of improved fruit quality by expressing PAs biosynthetic genes.

CHAPTER 4. POLYAMINES ENHANCE FRUIT SET AND REGULATE RIPENING-ASSOCIATED FRUIT METABOLOME TO ATTENUATE RIPENING AND ENHANCE FRUIT QUALITY ATTRIBUTES IN TOMATO

4.1 Introduction

PAs are biogenic amines and ubiquitously found in almost all living organisms in µM to mM concentrations (Torrigiani et al., 2008). Recent advances have elucidated biosynthesis, catabolism and action of PAs (Anwar et al., 2015; Lasanajak et al., 2014; Paschalidis and Roubelakis-Angelakis, 2005; Tiburcio et al., 2014). PUT, SPD and SPM are major forms of PAs that have been implicated in various biological processes including cell division, cell elongation, embryogenesis, root formation, flower and fruit development, fruit ripening, senescence and biotic and abiotic stress responses (Alcazar et al., 2010; Del Duca et al., 2014; Jiménez Bremont et al., 2014; Moschou and Roubelakis-Angelakis, 2014; Nambeesan et al., 2008; Torrigiani et al., 2008). Substantial changes in metabolic contents have been associated with fruit development and ripening (Biais et al., 2009; Biais et al., 2014; Boggio et al., 2000; Carrari et al., 2006; Deluc et al., 2007; Klie et al., 2014; Osorio et al., 2011; Osorio et al., 2013). Breeding and biotechnological interventions have shown association of sugars, organic acids and certain other metabolites with fruit quality attributes (Carli et al., 2009; Carrari et al., 2006). Emerging metabolomic techniques have helped understanding the dynamics of metabolic processes during tomato fruit ripening that are associated with PAs (Mattoo et al., 2006; Neelam et al., 2008; Neily et al., 2010), ethylene (Sobolev et al., 2014), methyl jasmonate (Kausch et al., 2011), carboxylic acids (Centeno et al., 2011; Morgan et al., 2013; Osorio et al., 2013), specific QTLs (Perez-Fons et al., 2014) and plant growth conditions (Biais et al., 2014; Hohmann et al., 2014). PAs not only delay fruit ripening and extend vine/shelf

life but have been reported to enhance fruit nutrition qualities (Kolotilin et al., 2011; Mehta et al., 2002; Nambeesan et al., 2010; Neily et al., 2010). However, the full impact of PAs, especially different forms of PAs (free, conjugated and bound) on fruit metabolic processes leading to fruit quality has not yet been fully understood.

I have evaluated fruit set and on-planta fruit development, ripening ad metabolic changes in transgenic tomato fruits with higher PA due to ectopic expression of *ySpdSyn* and *ySAMdc*. The transgenic tomato plants expressing *ySpdSyn* exhibited increase in fruits set and ratio of mature green-to-ripening fruits. Whereas the parental WT fruits did not show increase in fruit fresh weight after the onset of ripening, the transgenic fruits exhibited continuous growth at least until 20 days after fully ripe stage. Significant changes in the dry weight of transgenic fruits were not obtained but there were significant changes in the metabolic profiles as determined by ^{1}H NMR spectroscopic analysis. Changes in several of these metabolites, including amino acids (Val, Ile, Glu, Gln, Asp, Trp), carboxylic acids (citrate, malate), GABA and choline were positively while sugars (sucrose, β-glucose, and fructose) and energy molecules (ATP/ADP, AMP) were negatively correlated with changes in free SPD levels. Results indicate that PAs enhance fruit quality and delay senescence-related processes by regulating multiple biochemical pathways to restore anabolic activities even in later stages of fruit ripening. Taken together these results showed that increased SPD improved postharvest fruit quality by altering many metabolic pathways and delaying senescence-related processes

4.2 Material and methods

4.2.1 Plant material and growth conditions

Tomato plants cv. Ohio8245 were transformed with yeast *SpdSyn* gene fused to a CaMV 35S promoter (C4 and C15 plants) or fruit/ethylene-specific promoter (E8-8 plants) as previously described (Nambeesan et al., 2010). Transgenic and WT plants were grown in glasshouse on high porosity potting mix (52Mix, Conard Fafard Inc., MA USA) and provided with 16h day/8h night photoperiod and 23°C day/18°C night temperature

conditions. For fresh fruit weight and metabolite analyses, fruits were tagged at B stage and harvested at P, R and 5, 10, 15 and 20 DAR. Tomato fruits were classified into B, P and R stages according to USDA color chart (http://ucanr.edu/repository/a/?a=83755). After registering fresh fruit weight, fruit tissues were either subjected to dry fruit weight or immediately frozen in liquid N_2 and stored at -80°C until further use. In each replication, at least three fruits were randomly selected from at least four plants and all analyses were run in triplicates. Standardized weeding, irrigation, plant protection and fertilization operations were carried out during the study (Tieman et al., 1995).

4.2.2 Evaluation of fruit set and vine life of tomato fruits under field conditions

The WT and transgenic plants were grown in field under randomized complete block design (RCDB). Plants were randomly selected as soon as first fruit showed sign of color change. Fruits were collected at 10, 20, 30, 45 and 52 DAB and categorized in MG, B, P and R stages. Fruit distribution percentage was calculated by dividing fruits in each category to total number of fruits.

4.2.3 Fresh and dry fruit weight

At least three fruits per replication were individually weighed and then averaged for fresh fruit weight. For dry fruit weight, fruit tissues were dried at 65°C in a dehydrator until a constant weight was obtained. Percent dry weight was calculated by dividing dry weight with fresh weight and multiplying with 100.

4.2.4 Quantification of PAs by high pressure liquid chromatography

PA levels in tomato fruit tissues were determined as described in CHAPTER 2 Material and methods section 2.2.4.

4.2.5 Quantification of fruit metabolites by nuclear magnetic resonance spectroscopy

Two-dimensional nuclear magnetic resonance techniques (HMBC, 1H-13C HSQC, COSY and TOCSY) were employed to establish spectral assignments and identification of specific metabolites as previously described (Sobolev et al., 2014). The assignment of ^{1}H NMR spectra was performed as previously described (Mattoo et al., 2006; Sobolev et al., 2003) and shown in Table 4.1.

4.2.6 Statistical analysis

A Microsoft Excel add-in statistical package XLStat (2014.3.05) was used for ANOVA, pair-wise comparison, AHC and PCA as described in CHAPTER 2 Material and methods section (2.2.5). Fisher's least significant difference with confidence interval of 95% was used for pair-wise comparison analysis within genotypes at each sample stage.

Table 4.1**:** List of metabolites and chemical shifts (in ppm) of its characteristic signals.

Variable No.	Chemical Shift (ppm)	Compound
1	1.02	Ile
2	1.05	Val
3	1.34	Thr
4	1.49	Ala
5	2.08	Glu
6	2.30	GABA
7	2.48	Gln
8	2.53	Citrate
9	2.80	Asp
10	2.91	Asn
11	3.21	Choline
12	3.24	β-glucose
13	3.30	Myo-inositol
14	4.02	Fructose
15	4.29	Malate
16	5.42	Sucrose
17	6.52	Fumarate
18	6.92	Tyr
19	7.44	Phe
20	7.75	Trp
21	7.84	Nucl1
22	7.87	UDP-NAcGLU
23	8.15	His
24	8.36	Adenosine
25	8.46	Formic acid
26	8.53	ATP/ADP
27	8.59	AMP
28	9.13	Trigonelline

4.3 Results

4.3.1 Ectopic expression of *ySpdSyn* increased fruit set and extended vine life of tomato fruits

The WT and transgenic plants were grown under field conditions to evaluate their performance for production of fruits and impact on fruit ripening (Figure 4.1). Total number of fruits on WT and transgenic plants were similar at 10 DAB. There was no further increase in fruit set in WT and E8-8 plants after the first sign of onset of fruit ripening on a plant, but the *35S:ySpdSyn* transgenic plants (C4 and C15) continued to set fruits until 52 DAB and exhibited up to 50 % increase in fruit number per plant (Figure 4.1a). Percentages of fruits at G, B, P and R stages on WT and transgenic plants were also determined and the percentage of green fruits is presented in Figure 4.1b. Both WT and transgenic plants exhibited similar percentage of green fruits (>90%) at 10 DAB. Thereafter, both WT and E8-8 plants exhibited steady decline in percentage of green fruits which reached <40% at 52 DAB. This decline in percentage of green fruits was much slower in *35S:ySpdSyn* plants and >50% fruits were still at green stage on C4 and C15 plants at 52 DAB (Figure 4.1b). Percent share of mature green, B, P and R fruits in WT and transgenic plants at 52 DAB in given in Figure 4.1c. The WT and E8-8 fruits had almost similar percentage of fruits at different ripening stages while C15 plants had highest percentage of mature green fruits (71%) and lowest percentage of red fruits (26%) followed by C4 plants which exhibited 58% mature green fruits and 39% red fruits (Figure 4.1c). Taken together data indicate that constitutive expression of *ySpdSyn* continue to stimulate new fruit set resulting in higher percentage of green fruits in C4 and C15 plants. However, at this stage, we cannot rule out contribution of delayed fruit ripening due to changes in PAs profile in C4 and C15 transgenic lines.

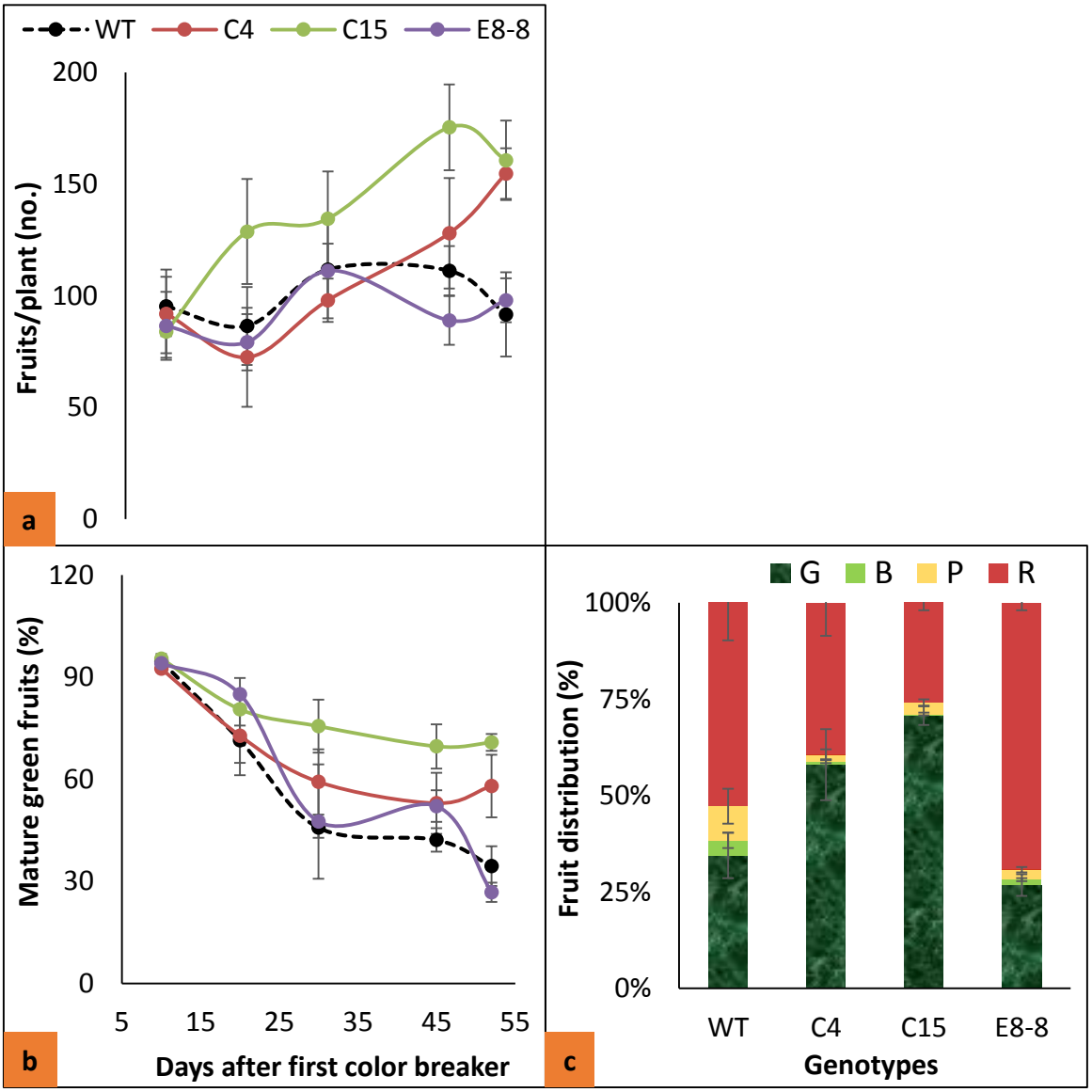


Figure 4.1: Fruit production trend and percent share of fruits at different stages of fruit development and ripening on WT and transgenic plants grown under field conditions.

Number of fruits at G, B, P and R stages were harvested and counted at 10, 20, 30, 45 and 52 days after first sign of breaker stage on a plant (DAB). Total number of fruits per plant (a) and percent share of green fruits in total number of fruits (b) on WT and transgenic plants at 10, 20, 30, 45 and 52 DAB. Percent share of G, B, P and R fruits in total number of fruits on WT and transgenic plants at 52 DAB (c). Vertical bars represent ±SE (n≥3 biological replicates), $p<0.05$.

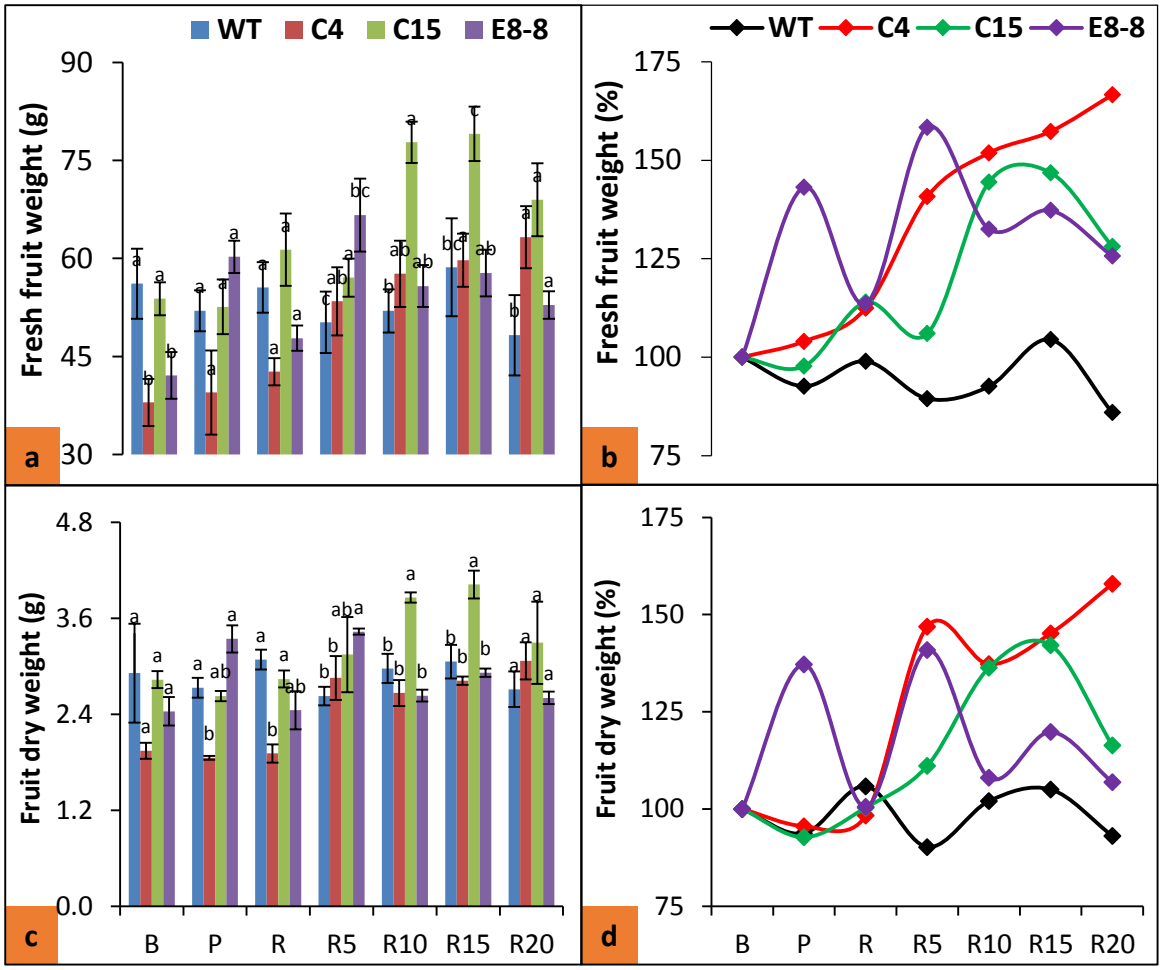


Figure 4.2: On-planta expression of *ySpdSyn*, both under CaMV 35S and SlE8 promoters, continued to increase fruit fresh and dry weight.

Upper panels represent fresh fruit weight (a) and % change in fresh fruit weight after the fruit reach B stage (b). The lower panels represent whole fruit dry weight (c) and % change in dry fruit weight after the fruit reach B stage (d). Mean values in panels 'a' and 'c' were used to calculate % change in fresh weight (b) and dry weight (d) from B stage. Vertical bars represent ±SE=3 (3-4 fruits in each replication), $p<0.05$.

4.3.2 Transgenic expression of *ySpdSyn* altered biomass accumulation in tomato fruits

The fresh fruit weights of WT tomatoes remained similar from B to 20 DAR stages. The C4 and E8-8 fruits fresh weight were significantly lower than WT fruits at B stage, but they continued to gain fresh weight after B stage and their 20 DAR fruit exhibited significantly higher fresh weight than 20 DAR WT fruits (Figure 4.2a). The C15 fruits exhibited fresh fruit weight similar to WT until P stage, but thereafter, fresh weight of its fruits was significantly higher until the termination of experiment (20 DAR). Changes in dry weight (whole fruit, g) of fruits from all genotypes were proportional to changes in their fresh fruit weight (Figure 4.2c). Figure 4.2b shows post-B stage % change fresh fruit weight in on-planta fruits until the 20 DAR. Whereas, the fresh weight of WT fruit remained unchanged during post-B stages until 20 DAR, fruits from all transgenic genotypes exhibited increase in as much as 40% to 60% gain in fresh weight during post-B stages (Figure 4.2b). The post-B stage % change in dry weight of WT fruits remained similar from B to 20 DAR stages, whereas fruits from all transgenic genotypes exhibited as much as 50% increase in dry weight during on-planta post-B stages until 20 DAR compared to B stage (Figure 4.2d).

4.3.3 Effect of *ySpdSyn* on fruit metabolome

Figure 4.3 shows the on-planta changes in amino acids in WT and transgenic fruits during ripening and post ripening period as determined by ^{1}H NMR. Changes in 12 amino acid levels were similar in WT and transgenic fruits as both showed gradual decline in Ile, Val, Gln, and incline in Ala, Glu, Asp and Trp. The levels of Thr, Asn and His did not change significantly during fruit ripening. Tyr and Phe showed gradual decline in WT and transgenic fruits until 10 DAR but with an increase at 15 DAR that declined in 20 DAR. In transgenic fruits, Tyr and Phe levels also increased at 15 DAR but maintained higher levels at 20 DAR. Among the 12 amino acids quantified, the expression of *ySpdSyn* enhanced accumulation of Ile, Val, Thr, Asn, Glu, Gln, His, Tyr, Phe and Trp whereas Ala and Asp levels remained unaltered.

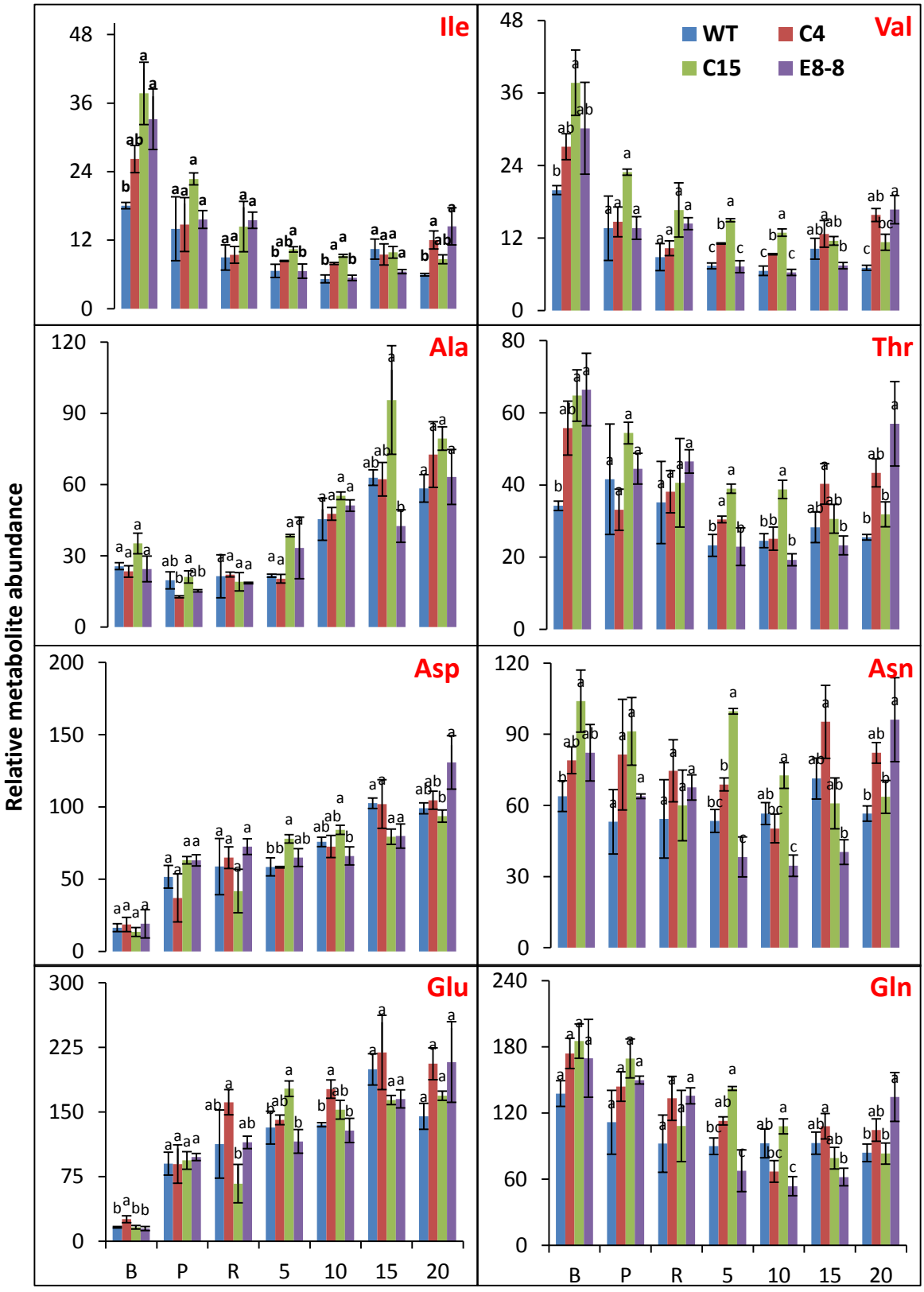


Figure 4.3: continued…….

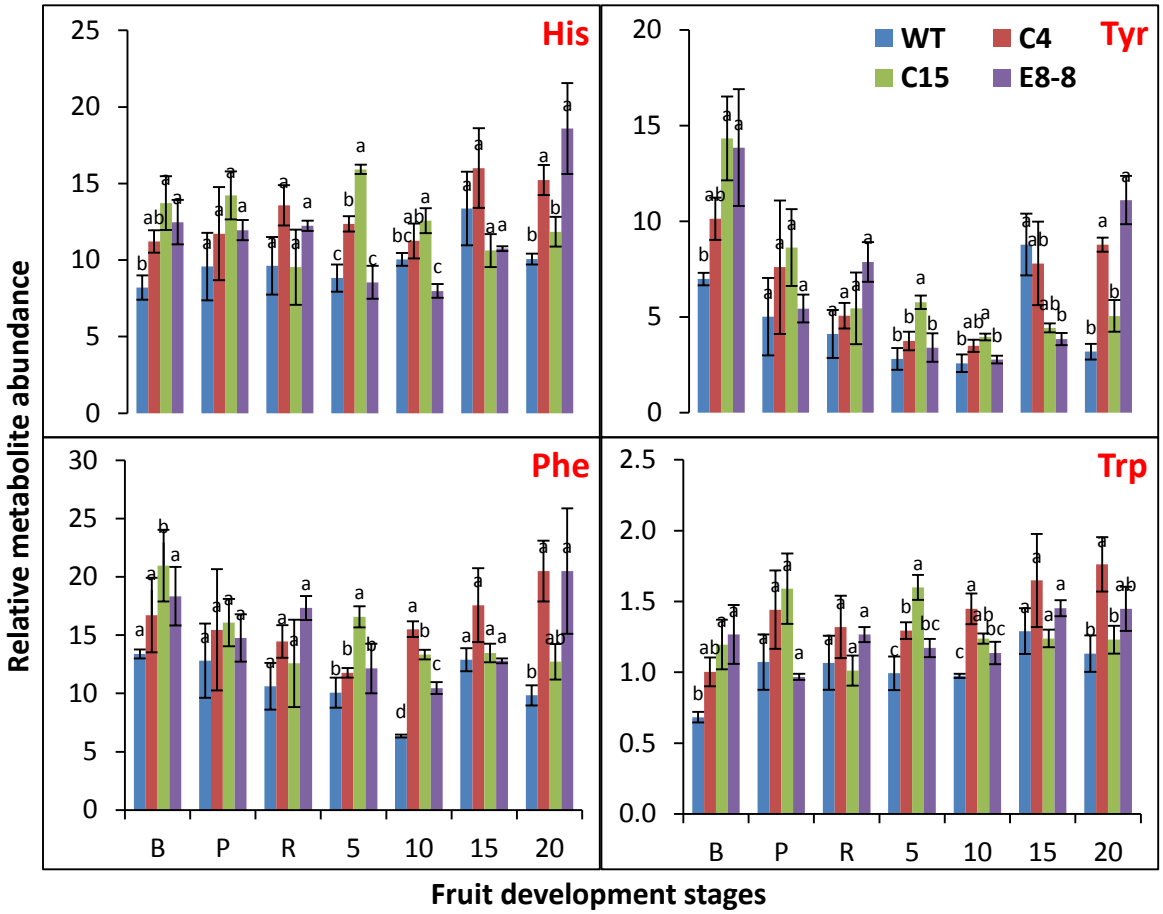


Figure 4.3: Changes in amino acids profiles of WT and *ySpdSyn*-transgenic tomato fruits during on-planta ripening and post-ripening storage.

Relative molecular abundance is based on signal intensity of identified metabolite in ^{1}H NMR spectrum as described in Material and methods section 4.2.5. Vertical bars represent ±SE=3 biological replicates (3-4 fruits in each replication). Similar letters above standard error bars indicate non-significant difference (at 95% confidence interval) among genotypes within a fruit ripening stage. B, breaker; P, pink stage; R, red; R5 to R20, 5 to 20 days after red stage of tomato fruits.

4.3.4 Profile of organic acids and sugars

Figure 4.4 shows the on-planta changes in citrate, malate, fumarate and formic acid in WT and transgenic fruits during ripening and post-ripening period as determined by ^{1}H NMR. Like amino acids, molecular abundance profile of organic acids were similar in WT and transgenic fruits. Citrate and malate levels declined while formic acid accumulated during tomato fruit ripening. Fumarate level remained unaltered during this time period. The ectopic expression of *ySpdSyn* resulted in sporadic but significant changes in organic acids. The *ySpdSyn*-expression slowed down the decline of citrate in C4 fruits at R and 10 DAR while in E8-8 fruits at R stage only. C4 fruits also had higher level of malate at B, P and 20 DAR while C15 and E8-8 fruits exhibited increase in malate at P and B stages, respectively. The *ySpdSyn*-expression also inhibited the increase of formic acid in all three transgenic fruits at 20 DAR. Fumarate levels in WT and transgenic fruits were almost similar during observed fruit ripening stages (Figure 4.4).

Both in WT and transgenic fruits, sucrose content decreased while β-glucose and fructose levels remained unaltered during on-vine tomato fruit ripening (Figure 4.5). Expression of *ySpdSyn* lead to decline in sucrose contents at R, 10 and 20 DAR while fructose contents in transgenics remained almost similar to WT except a single decrease at 10 DAR. β-Glucose level decreased at P, 10 and 20 DAR in C4 fruits (Figure 4.5). Both WT and transgenic fruits exhibited gradual decline in ratio of organic acids (citrate and malate) to sugars (β-glucose, fructose and sucrose). Among transgenic lines, only C4 fruits showed slight increase in acid:sugar ratio at R and 10 DAR. Gradual increase in UDP-NAcGLU contents during tomato fruit ripening was also consistent among WT and transgenic fruits. Slight increase in UDP-NAcGLU contents was observed in C4 fruits at R and 10 DAR and in C15 fruits at 10 DAR compared to WT fruits (Figure 4.5).

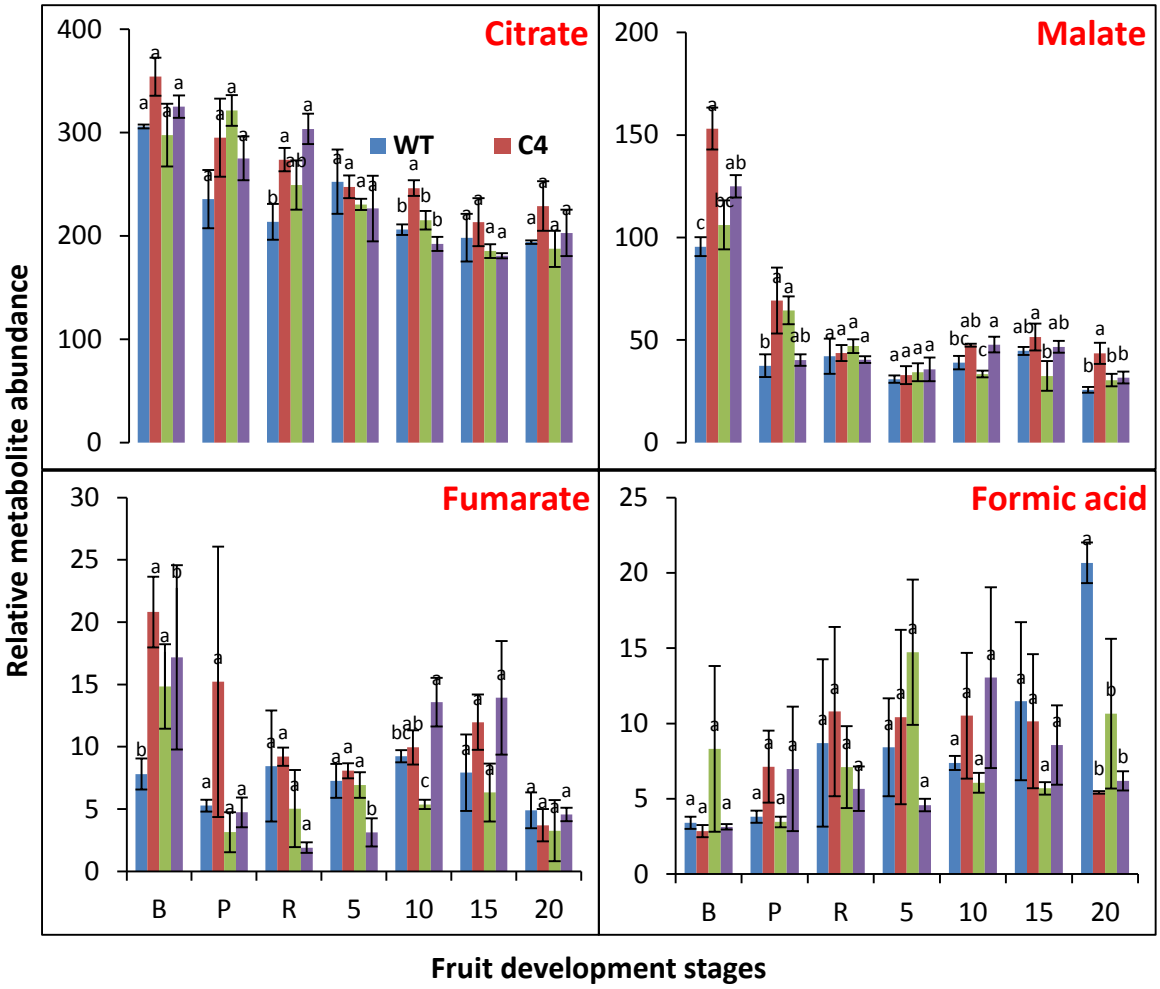


Figure 4.4**:** Changes in organic acids profiles of WT and *ySpdSyn*-transgenic tomato fruits during on-planta ripening and post-ripening storage.

Other details are given in Figure 4.3.

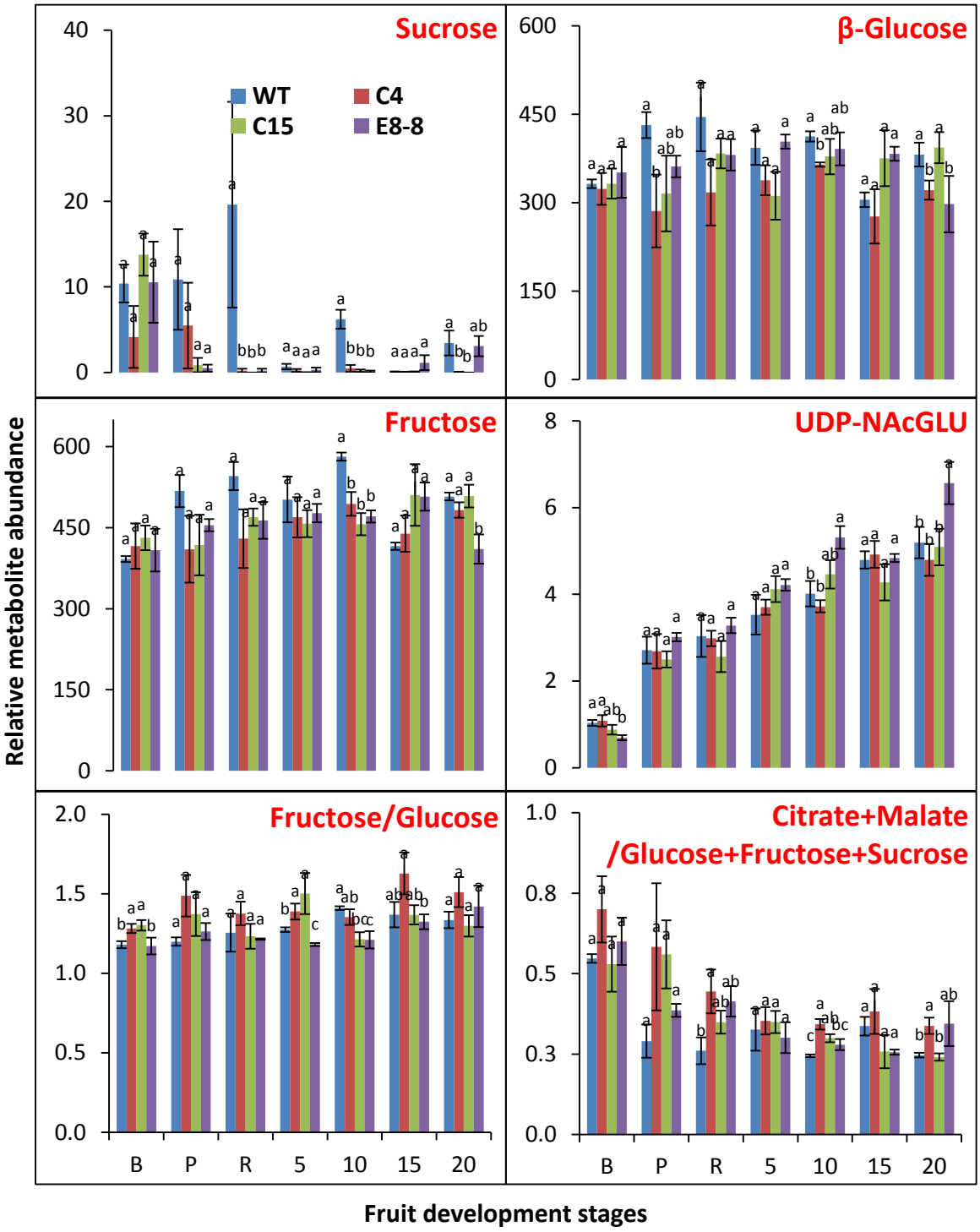


Figure 4.5: Metabolomic profile of sugars and acid:sugar ratio in WT and *ySpdSyn*-transgenic tomato fruits during on-planta ripening and post-ripening storage. Other details are given in Figure 4.3.

4.3.5 Profile of GABA, choline, myo-inositol, trigonelline, adenosine, ATP/ADP, AMP and Ncl1

Profiles of GABA, choline, myo-inositol, trigonelline, nucleoside adenosine, nucleotides (ATP/ADP, AMP) and Nucl1 during on-planta fruit ripening and post-ripening are shown in Figure 4.6. Both WT and transgenic fruits showed decline in GABA and escalation in AMP and Nucl1 levels while myo-inositol, choline and trigonelline levels remained unaltered during tomato fruit ripening. Adenosine and ATP/ADP levels increased from B to 10 DAR and then decreased at 15 and 20 DAR. Interestingly, adenosine levels showed 2-fold surge at P and onward ripening fruits compared to B stage. Expression of *ySpdSyn* resulted in further increase in GABA, myo-inositol, choline and Nucl1 but reduction in adenosine, ATP/ADP and AMP while trigonelline levels remained unaltered compared to WT fruits.

4.3.6 Statistical analyses discriminated metabolic profiles in three distinct clusters

AHC analysis of ^{1}H NMR data in fruit tissues from all four genotypes clustered metabolites in three distinct clusters, designated I to III (Figure 4.7a). Cluster I comprised of fructose, β-Glucose, Ala, Glu, Nucl1, Asp, UDP-NAcGLU, formic acid, adenosine, ATP/ADP and AMP. Cluster II included choline, Trp, Insositol, trigonelline, Phe, Asn and His while cluster III included Ile, Val, Thr, Tyr, GABA, citrate, Gln, sucrose, malate and fumarate. Correlation coefficient analysis showed significant positive correlation (≥0.5) between metabolites within a cluster (Figure 4.7a). The metabolites in cluster I had strong negative correlation with most of metabolites in cluster III while fructose, β-Glucose and adenosine in cluster I were also negatively correlated (≥0.5) with metabolites in cluster II. Metabolites in clusters II and III had a positive correlation (≥0.5) among them.

4.3.7 Metabolites are differentially regulated by different fractions of PUT, SPD and SPM

Free, conjugated and bound levels of PUT, SPD and SPM were also determined in vine-ripened WT and transgenic fruits (Figure 3.1). A pair-wise correlation matrix between different fractions of PAs and 28 determined metabolites was generated (Figure 4.7b). Free PUT was positively correlated (≥0.5) with Glu, Nucl1, UDP-NAcGLU, Trp but negatively correlated (≤-0.5) with sucrose. Conjugated PUT was positively correlated with UDP-NAcGLU, ATP/ADP but negatively correlated with Ile, Val and GABA. Bound PUT was positively correlated with choline, trigonelline and citrate but negatively correlated with fructose and β-Glucose. Free SPD was positively correlated with trigonelline, Ile, Val, citrate, Gln and malate but negatively correlated with fructose, Ala, Glu, Nucl1, Asp, UDP-NAcGLU and AMP. No significant correlation was observed between metabolites and conjugated or bound levels of SPD in tomato fruit tissues. Among different fractions of SPM, only conjugated SPM was negatively correlated with Trp. AHC-based clustering of metabolites was also embedded in correlation matrix (Figure 4.7b). Generally, metabolites in cluster I were positively correlated with PUT but negatively with SPD and metabolites in cluster II were negatively correlated with PUT but positively with SPD which suggests that PUT and SPD have antagonistic role in regulating metabolites in cluster I and II while SPM has least impact on regulating these metabolites.

PCA of PAs and metabolites also showed close clustering of free SPD and metabolites in cluster III on positive quadrant of first principal component while free and conjugated PUT were clustered closely with metabolites in cluster I on negative quadrant of first principal component (Figure 4.7c).

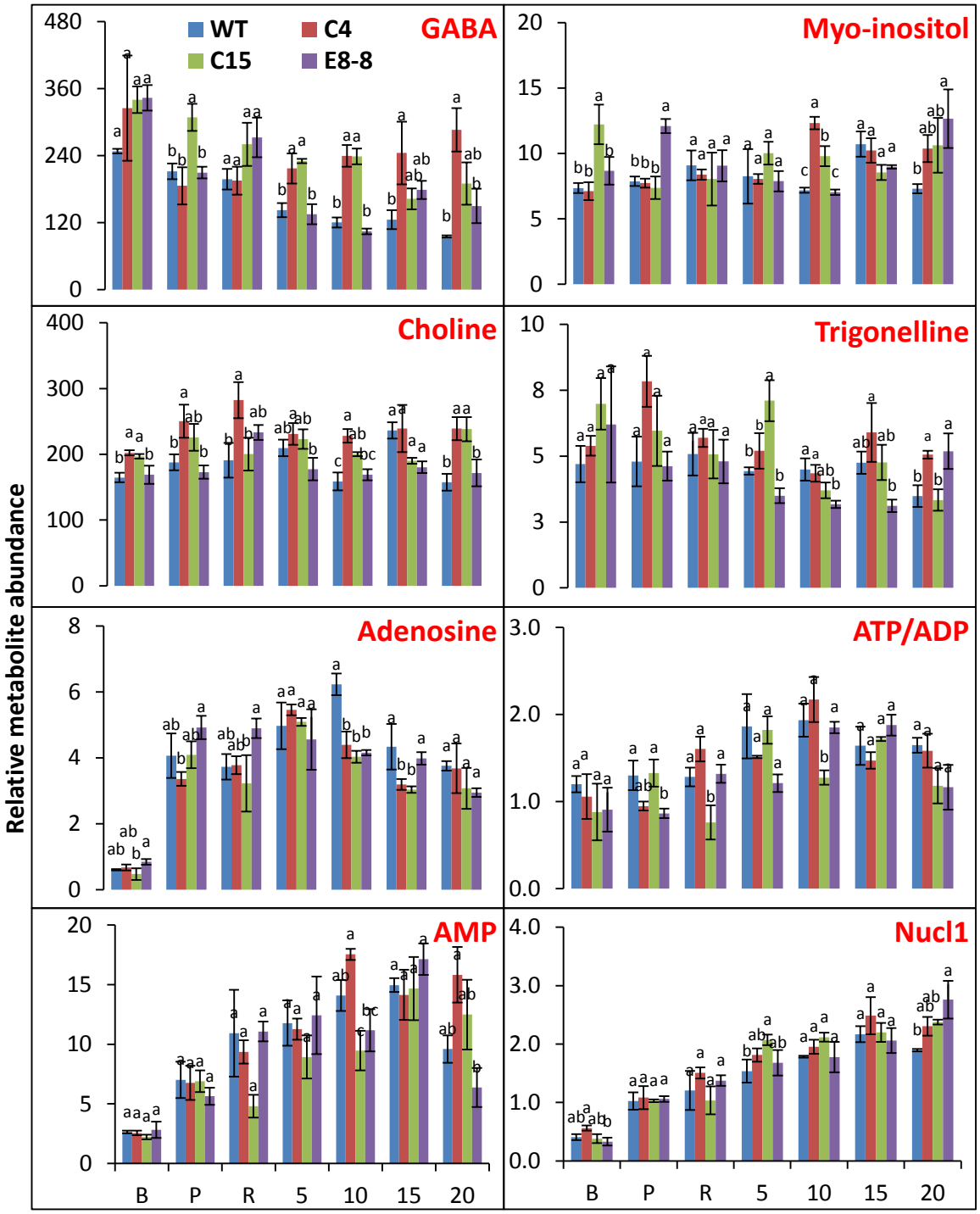


Figure 4.6: Changes in γ-aminobutyric acid (GABA), inositol, choline, trigonelline, andosine, ATP/ADP, AMP and Nucl1 profiles of WT and *ySpdSyn*-expressing transgenic tomato fruits during on-planta ripening and post-ripening storage.

Other details are given in Figure 4.3.

Figure 4.7: Discrimination analyses of metabolic profiles and their differential regulation by different fractions of PUT, SPD and SPM.

The ^{1}H NMR data from on-vine ripened tomato fruits of all four genotypes at seven different time points of fruit ripening were analyzed for AHC and correlation coefficient between metabolites (a) using XLStat version 2014.04.06. Three distinct clusters of metabolites identified with AHC were named as I, II and III on bottom and left of correlation coefficient heat map (a). Free (F), conjugated (C) and bound (B) levels of PUT, SPD and SPM and amounts of 28 determined metabolites in tomato fruits from four genotypes at seven different fruit ripening stages were used to generate correlation matrix (b) and PCA two-dimensional score plot (c). Significant correlation coefficient values in blue (positive) or red (negative) are different from 0 with a significance level α=0.05 (a).

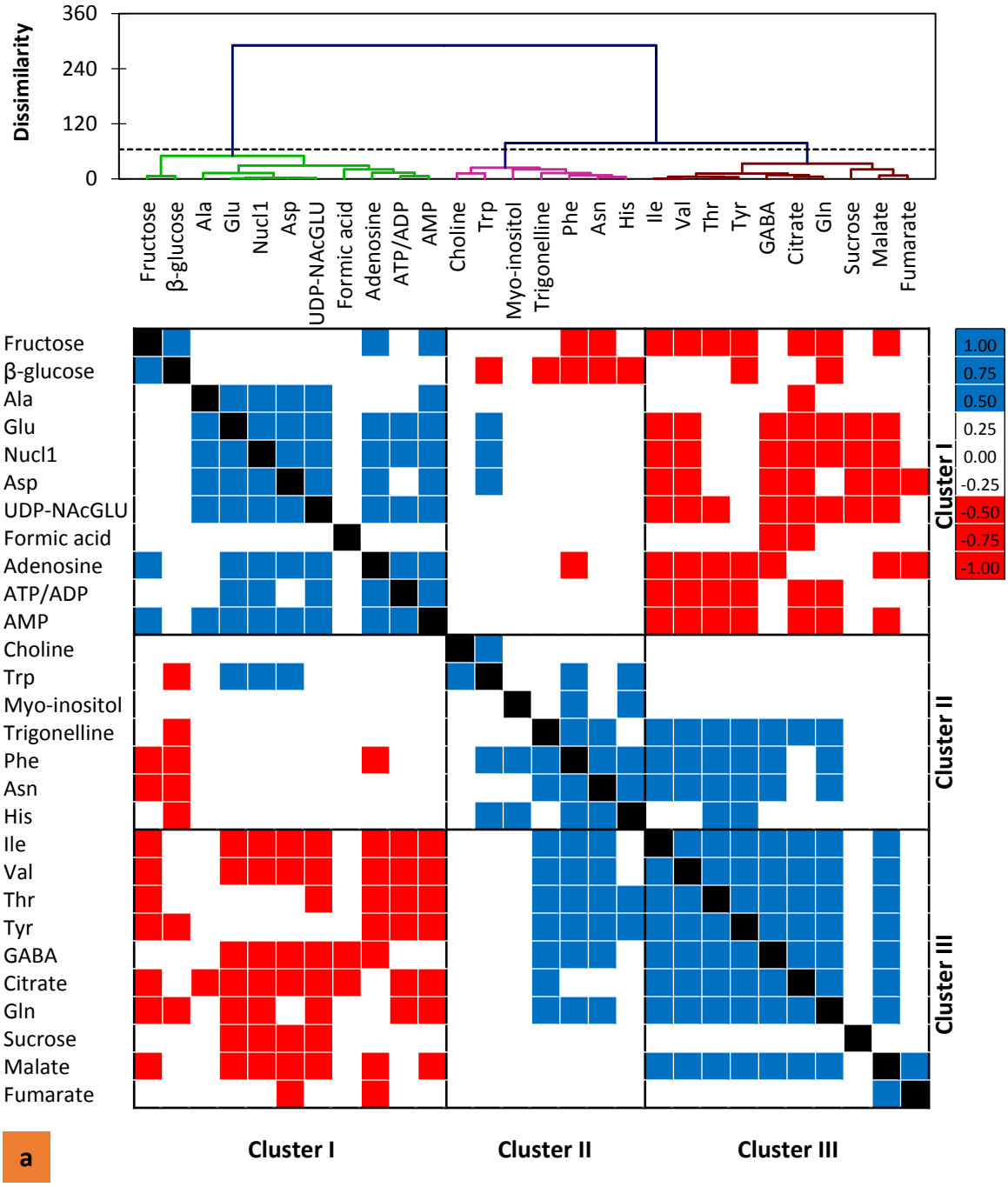
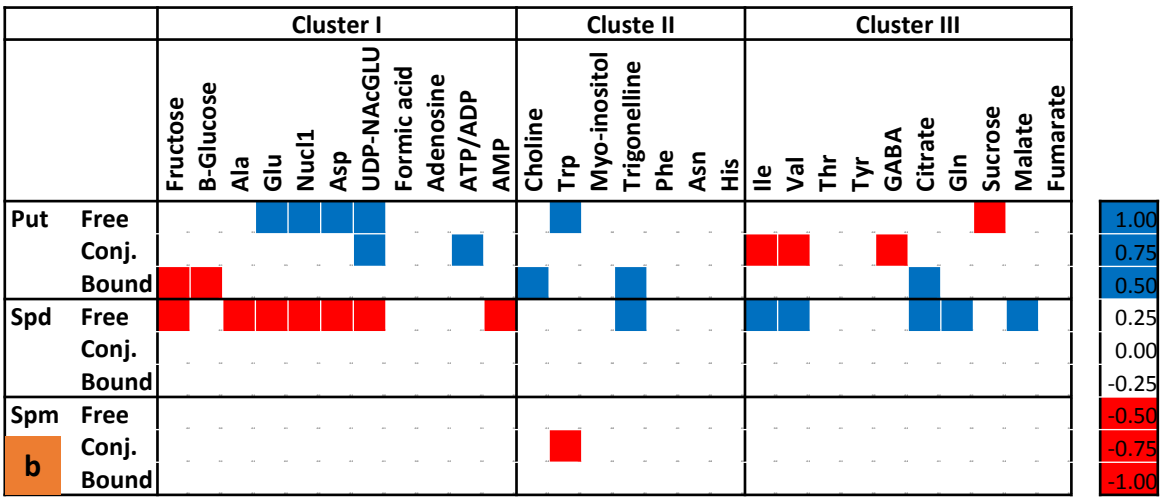

Dissimilarity
360
240
120
0
Fructose
β-glucose
Ala
Glu
Nucl1
Asp
UDP-NAcGLU
Formic acid
Adenosine
ATP/ADP
AMP
Choline
Trp
Myo-inositol
Trigonelline
Phe
Asn
His
Ile
Val
Thr
Tyr
GABA
Citrate
Gln
Sucrose
Malate
Fumarate
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
Cluster I
Cluster II
Cluster III
a

Figure 4.7


Cluster I
Cluste II
Cluster III
Fructose
B-Glucose
Ala
Glu
Nucl1
Asp
UDP-NAcGLU
Formic acid
Adenosine
ATP/ADP
AMP
Choline
Trp
Myo-inositol
Trigonelline
Phe
Asn
His
Ile
Val
Thr
Tyr
GABA
Citrate
Gln
Sucrose
Malate
Fumarate
Put
Spd
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Free
Conj.
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0.75
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0.00
-0.25
-0.50
-0.75
-1.00
b

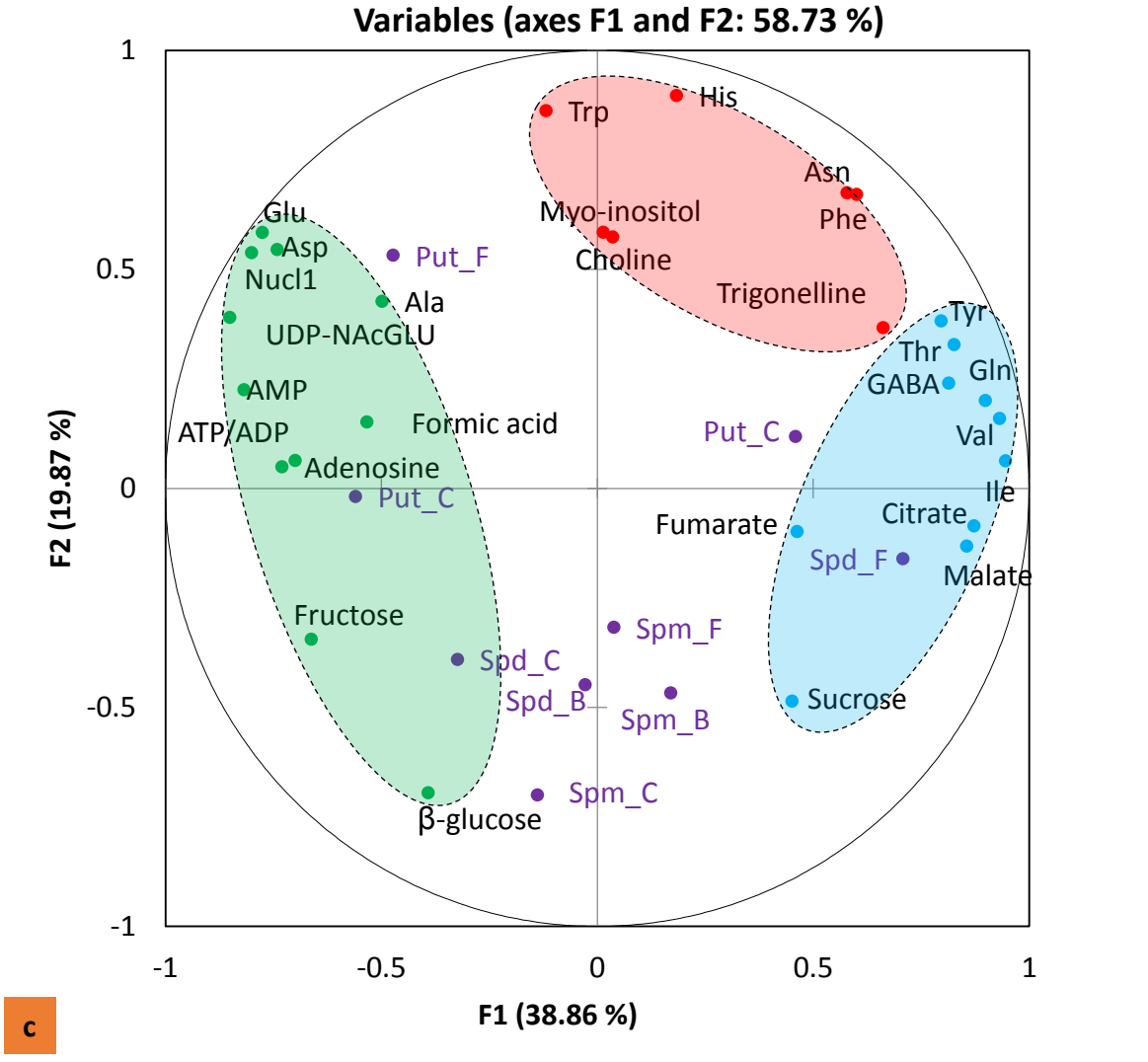
Variables (axes F1 and F2: 58.73 %)
F2 (19.87 %)
F1 (38.86 %)
1
0.5
0
-0.5
-1
-1
-0.5
0
0.5
1
Trp
His
Asn
Phe
Myo-inositol
Choline
Trigonelline
Glu
Asp
Nucl1
Put_F
Ala
UDP-NAcGLU
AMP
ATP/ADP
Formic acid
Adenosine
Put_C
Fructose
β-glucose
Spd_C
Spd_B
Spm_F
Spm_B
Spm_C
Tyr
Thr
GABA
Gln
Val
Ile
Put_C
Fumarate
Citrate
Spd_F
Malate
Sucrose
c

Figure 4.7

4.3.8 Metabolite levels in tomato fruits are developmentally regulated

Metabolite levels in four genotypes at seven fruit ripening stages were analyzed with PCA to determine if changes in their levels in transgenic fruits were still developmentally regulated (Figure 4.8). Two-dimensional score plot from PCA showed distinction in metabolite variability between fruit ripening stages along first principal component and accounted for 34.54% variability in the data. Tomato fruit samples at B had highest score while samples at 20 DAR had lowest scores along first principal component. Samples from other ripening stages were partially mixed between these two extremes but more towards negative quadrants for 20 DAR cluster. The discrimination of samples along second principal component, which accounted for 21.27% variability in the data, corresponded to genotypic variability in samples. The WT samples were clustered towards positive quadrants while C4 and C15 samples were clustered towards negative quadrants while E8-8 samples were clustered between WT and *35S:ySpdSyn* samples. Considering the percentage of variability associated with the first principal component (34.54%) and second principal components (21.27%), results indicate that changes in metabolites are principally regulated by fruit ripening stage while ectopic expression of *ySpdSyn* is a secondary but strong cause of altering metabolic profiles (Figure 4.8).

4.3.9 PAs regulate primary metabolic pathways during tomato fruit ripening

To discern PA-dependent changes in metabolites during tomato fruit ripening, metabolites exhibiting strong correlation with different fractions of PAs were selected (Figure 4.7b). Metabolic pathways associated with these PA-regulated metabolites were obtained from KEGG pathway database (http://www.genome.jp/kegg/pathway.html) and a model was generated to explain PA-regulated changes in metabolites and their associated pathways during tomato fruit ripening (Figure 4.9). Node-edge network clearly showed dominant role of PUT and SPD in regulating tomato fruit metabolome during ripening. Results indicate that PAs regulate primary metabolic pathways during tomato fruit ripening which include metabolisms of amino acids, pyruvate, 2-oxocarboxylic acid,

glyoxylate, pantothenate and CoA, glucosinolate, alkaloids, nicotinamides, glutathione, purine and pyrimidine, and glycerophospholipids. This model also indicated that PAs enhance N metabolism, C metabolism and fixation and TCA cycle. Free and bound forms of PAs have been shown to enhance functionality of photosynthetic complexes suggesting role of PAs in C fixation and ATP synthesis (Ioannidis and Kotzabasis, 2014; Ioannidis et al., 2012). Thus, enhanced photosynthetic activity in PA-accumulating tomato plants might have role in delaying chloroplast degradation in tomato fruit peel (Figure 4.1c).

4.4 Discussion

Higher accumulation of PAs, especially SPD and SPM, had shown potential to delay fruit ripening and improve fruit quality. We have previously reported that ectopic expression of *ySpdSyn* stimulated vegetative fresh weight in transgenic lines as much as 42% in C4 and delayed the onset of fruit ripening on plants without any change in fruit set compared to WT plants (Nambeesan et al., 2010). The *ySpdSyn*-expression also delayed fruit ripening, extended shelf life by delaying decay symptoms without impairing ethylene production (Nambeesan et al., 2010) and enhanced lycopene contents in tomato fruits (Nambeesan et al., 2010). In another independent study, 2-fold increase in free PUT, SPD, SPM contents in *35S:MdSpdSyn* tomato fruits resulted in upregulation of lycopene biosynthesis genes, 2.2-fold increase in lycopene contents and 1.6-fold increase in ethylene production (Neily et al., 2010). Similarly, transgenic expression of *ySAMdc* under SlE8 promoter delayed fruit ripening (Mehta et al., 2002) in spite of higher ethylene production, increased lycopene by 2 to 3-fold and enhanced precipitate weight ratio and serum viscosity in tomato fruit juice (Mehta et al., 2002). These fruit also exhibited increase in carotenoids (70% at 27 DAB) , *cis*-10-heptadecanoic acid (50%), linolenic acid (20%) and nervonic acids (28%) (Kolotilin et al., 2011).

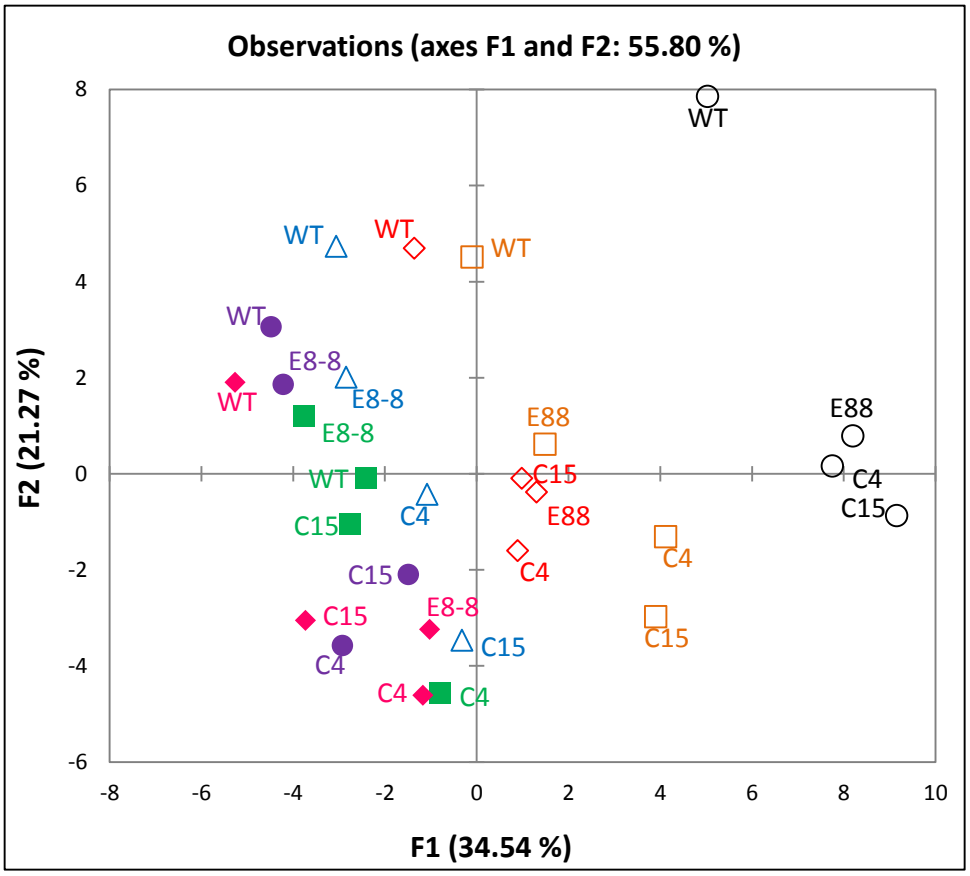

Figure 4.8**:** Principal component analysis (PCA) of genotypes and fruit ripening stages. Free, conjugated and bound levels of PUT, SPD and SPM and metabolite levels were used as variables for analysis for PCA of tomato fruit samples from four genotypes at seven different ripening stages. **Black**, breaker stage; **brown**, pink stage; **red**, red stage; **blue**, 5 days after red stage (DAR); **purple**, 10 DAR; **green**, 15 DAR; **pink**, 20 DAR. Other detail are same as in Figure 4.7.

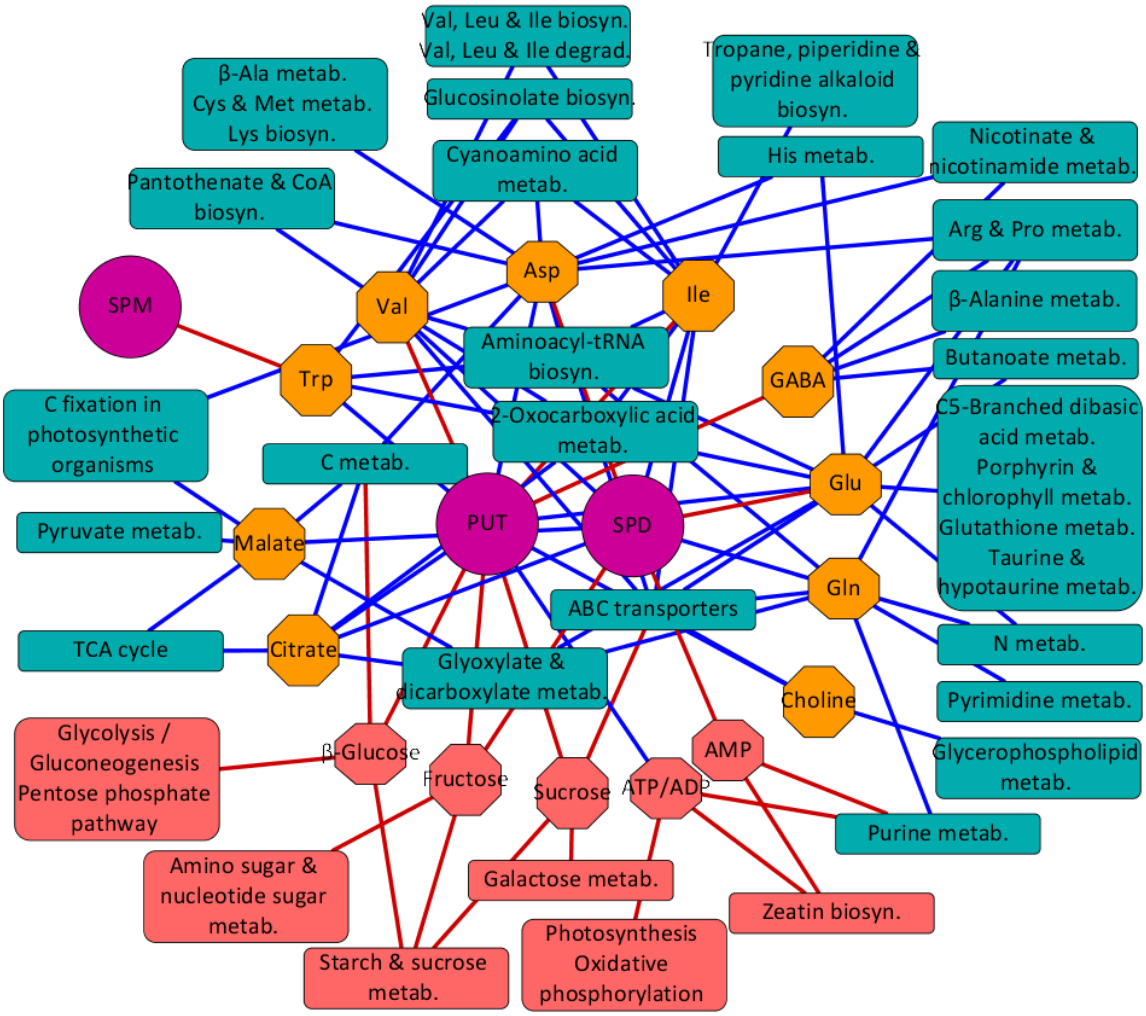

Figure 4.9**:** Ripening-associated metabolic pathways altered by PAs in transgenic tomato fruits expressing *ySpdSyn*.

Metabolites (octagons) with significant negative (≤-0.5, **red**) or positive (≥-0.5, **brown**) correlations with PUT, SPD and SPM (**purple** circles) were considered. Pathways (**dark teal** rectangles) associated with different metabolite were obtained from KEGG pathway database (http://www.genome.jp/kegg/pathway.html). Node-edge network among PAs, metabolites and their associated pathways was generated with Cytoscape 3. Edge colors represent PA-mediated increase (**blue**) or decrease (**red**) in metabolites or their associated pathways.

Table 4.2**:** Metabolite profiles quantified in different tomato species.

Changes in metabolite profile is indicated with ↑ (increase, blue), ↓ (decrease, red) or ↔ (no change, green) arrows during tomato fruit ripening.

Reference	Present study	Sobolev et al. (2014)	Carrari et al. (2006)	Mattoo et al. (2006)	Neelam et al. (2008)	Akihiro et al. (2008)	Neily et al. (2010)	Osorio et al. (2011)
Metabolite	Ohio 8245	Ohio 8245	Moneymaker	Ohio 8245	Ohio 8245 (556AZ)	Micro-Tom	Micro-Tom	Ailsa Craig
Glu	↑, ↓	↑	↑	↑	↑	↑	↑	↑, ↓
Asp	↑	↑	↑	↑	↑	↑	↑	↑
Trp	↑	↑	↑					
Nucl1	↑	↑						
ATP/ADP	↑	↔						
Formic acid	↑	↔						
Adenosine	↑	↑						
AMP	↑	↑						
UPD-NAcGLU	↑							
Ala	↑	↓	↑	↓	↓	↑	↑	↑
Tyr	↓	↓	↑					
Myo-Inositol	↔	↔	↓				↑	
Sucrose	↓	↓	↓	↓	↓		↓	↑
Val	↓	↓	↓	↓	↓			↔
Ile	↓	↓	↓	↓	↓		↓	↑
Phe	↓	↓	↓	↓	↓		↑	↓
Malate	↓	↓	↓	↓	↓	↓	↓	↓
GABA	↓	↓	↓	↓	↓	↓	↓	↓
Citrate	↓	↔	↓	↓	↔	↔	↑	↓
Gln	↓	↔		↓	↓		↑	
Fumarate	↔	↓	↔	↓		↔		
Thr	↔	↔	↔	↓	↓			↓
Asn	↔	↔	↓	↔	↓			
Choline	↔	↔		↔	↔			
His	↔	↔						
β-Glucose	↔	↔	↔	↔	↔		↑	↑
Fructose	↔	↔	↔	↔	↔		↑	↑
Trigonelline	↔	↔						

Here in this study, we further evaluated growth kinetics of *ySpdSyn* tomato plants under field conditions. Although, total number of fruits produced on WT and transgenic plants were similar at 10 DAB but, in contrast to stagnancy in fruit set by WT plants, *35S:ySpdSyn* lines (C4 and C15) exhibited as much as 50% increase in total number of fruits at 52 DAB (Figure 4.1a). This increase was not obtained in *E8:ySpdSyn* line E8-8. Thus the constitutive expression of *ySpdSyn* had a stimulatory impact on fruit set during later stages of plant growth compared to WT and E8-8 genotypes. This might be one of the reasons, for the significant increase in proportion of green fruits in C4 (24%) and C15 plants (37%) compared to WT plants at 52 DAB (Figure 4.1b,c). The other explanations include delayed onset of ripening thus extending vine life of fruits (Figure 4.1b,c) (Mehta et al., 2002). SPD has been reported to promote longevity and extend life span of yeast, flies, worms and mammalian cells (Eisenberg et al., 2009; Madeo et al., 2010) and the whole-plant senescence is suppressed in *ySpdSyn*-expressing transgenic plants (Nambeesan et al., 2010). We attribute the extended fruit set in C4 and C15 plants to delay senescence-related developmental processes of tomato plants due to higher SPD and SPM levels in the transgenic lines (Mattoo and Handa, 2008).

Tomato ovaries have a period of active cell division until 7-14 after fertilization (Gillaspy et al., 1993; Joubes et al., 1999). Beyond this stage, polyploidy-associated cell expansion initiates and ovary cells just keep expanding (up to 20-fold) until one week before ripening after which fruit growth ceases with a little or no further increase in fresh or dry weight of WT fruits (Cheniclet et al., 2005; Tanksley, 2004). This pattern was observed for the WT fruits, but there was consistent increase in fresh fruit weight with corresponding increase in dry weight in transgenic fruits from all three genotypes (Figure 4.2). This novel physiological role of PAs in the transgenic fruit development suggests that enhanced levels of SPD and SPM (Figure 2.6), continue to stimulate metabolic processes in transgenic fruit much after they cease in the WT fruits. The cell expansion in plant cells is generally attributed to changes in osmotic potential due to increased cellular solutes levels. Sugar unloading metabolism and carboxylic acids establish total osmotic water potential that drive the water uptake into expanding cells (Liu et al., 2007; Mitchell et al.,

1991). The sugar required for this process either be coming from the mother plant or transgenic fruits are utilizing sugar reserves and accumulating carboxylic acids as observed in this investigation (Figure 4.4 and Figure 4.5)

During tomato fruit ripening, WT fruit tissues (cv. Ohio 8245) exhibited increase in Ala, Asp, Glu, Trp, formic acid, adenosine, AMP, ATP/ADP, Nucl1 and UDP-NAcGLU and decrease in Ile, Val, Tyr, Phe, Gln, GABA, malate, citrate and sucrose, but no alternations in Thr, Asn, His, β-glucose, fructose, fumarate, myo-inositol, choline and trigonelline. We matched these metabolic profiles with previously published patterns in same cv. Ohio 8245 (Table 4.2). Out of 17 metabolites commonly quantified, profiles of Ile, Val, Phe, Asn, Asp, Glu, GABA, sucrose, β-glucose, fructose, malate and choline were similar in all three studies (present study; Mattoo et al., 2006; Sobolev et al., 2014). Profiles of Tyr, Trp, His, Thr, adenosine, AMP, Nucl1, myo-Inositol and trigonelline quantified in present study were also similar to those previously reported (Sobolev et al., 2014) and patterns of Gln and citrate in our study were also similar to those reported by Mattoo et al. (2006) but not to those reported by Sobolev et al. (2014) who observed no change in these levels during tomato fruit ripening. Profiles of fumarate and Ala in present study also contrast with previous reports where decrease in their levels was observed during tomato fruit ripening (Mattoo et al., 2006; Sobolev et al., 2014). Metabolic profiles of Ile, Val, Asp, Phe, Glu, Gln, Sucrose, β-glucose, fructose, malate, GABA and choline in WT fruits (this study) were also similar to non-transgenic azygous fruits with cv. Ohio 8245 background (Neelam et al., 2008). Comparison of the metabolite profiles of cv. Ohio 8245 fruits (present study) with those of cv. Ailsa Craig (Osorio et al., 2011) and cv. Moneymaker (Carrari et al., 2006) showed that out of 13 metabolites (Table 4.2) that were quantified in these varieties, the levels of Ala, Asp, Glu, GABA and malate were similar in all three studies (present study; Carrari et al., 2006; Osorio et al., 2011) while Ile, Val, Phe, Thr, sucrose, β-glucose and fructose levels matched only with cv. Moneymaker (Carrari et al., 2006) and not with cv. Ailsa Craig (Osorio et al., 2011). Citrate level decline during fruit ripening of cv. Ohio 8245 (present study; Mattoo et al., 2006) but display highly variable behavior in cv. Moneymaker (Carrari et al., 2006) and cv. Ailsa Craig (Osorio et al., 2011). Metabolite

profiles of Ala, Ile, Glu, Asp, sucrose, GABA and malate determined in cv. Ohio8245 fruits were also similar to those quantified in cv. Micro-Tom (Akihiro et al., 2008; Neily et al., 2010). With a few exceptions, most metabolic profiles were consistent among various cultivars, indicating that ripening-associated metabolism is well preserved among tomato species.

To discriminate among accumulation pattern of different metabolites during tomato fruit ripening, the ^{1}H NMR data from WT and transgenic fruits were analyzed with agglomerative hierarchical clustering, Pearson's correlation matrix and PCA (Figure 4.7). Metabolites involved in energy/salvage pathway (adenosine, AMP, ATP/ADP) increased (Cluster I) at the expense of sucrose degradation (sucrose), Krebs cycle (malate, citrate, GABA) and pyruvate metabolism (Val, Ile) (Cluster III) during fruit ripening. PCA analyses showed that these changes in metabolite profiles are associated with temporal stages of fruit ripening (Figure 4.8). Such tight regulation of metabolic pathways by fruit development stages is independent of fruit ripening conditions, for example, on-planta or off-vine ripening (present work; Mattoo et al., 2006) or even diverse plant growth conditions, for example, standard practices, water deficit and shade production (Biais et al., 2014). Samples at B stage clustered on positive quadrant while samples at 20 DAR clustered on negative quadrant of first principal component that indicates a significant shift in metabolic processes during onset of fruit ripening (Figure 4.8).

Even though the ripening process is a major factor that influence metabolic profile of tomato fruit, a good separation of genotypes along second principal components suggest a considerable influence of transgene on fruit ripening-associated changes in quantified metabolites (Figure 4.8). Student's t-Test and Fisher's least significant difference in metabolite level showed that amino acids including Ile, Val, His, Tyr, Thr, Trp and Phe were most influenced by transgenic expression of *ySpdSyn* and exhibited increase in their levels at B, 5, 10 and 20 DAR compared to WT fruits. Common fruit ripening stages where transgenic expression of *ySpdSyn* enhanced GABA, myo-inositol and choline levels included, but not limited to, P and 10 and 20 DAR. Effect of *ySpdSyn*-expression on other metabolites was noticeable at one to three fruit ripening stages out of seven stages

examined. In total, out of 28 metabolites quantified in tomato fruit tissues, levels of 18 metabolites were altered whereas 10 metabolites remained unaltered by constitutive or fruit/ethylene-specific expression of *ySpdSyn*. This indicates that fruit metabolome is under control of fruit developmental stage and ripening-associated changes in metabolites are both PA-dependent and independent. Recently, metabolic profiles of tomato fruits impaired in ethylene production (2AS-AS) and 2AS-AS fruits with *E8:ySAMdc* ingression also showed that ripening-related shifts in metabolites are not only ethylene- or PA-dependent but are also regulated by other processes independently (Sobolev et al., 2014).

Many amino acids, including aromatic amino acids (Tyr, Phe, Ile, Val), aspartate family of amino acids (Asp, Asn, Thr, Gln, GABA), in addition to TCA cycle intermediates (citrate and fumarate) increased significantly during tomato fruit ripening, whereas decrease in energy/salvage pathway metabolites (adenosine, ATP/ADP) was observed during on-planta ripening of transgenics than WT fruits. Changes in metabolite profiles due to expression of *ySpdSyn* were evaluated with those previously reported. Accumulation patterns of metabolites in transgenic tomato fruit expressing *MdSpdSyn* (Neily et al., 2010) or *ySAMdc* (Mattoo et al., 2006; Neelam et al., 2008) are given in Table 4.3. Increase in Glu, Gln and GABA and decrease in sucrose, β-glucose and fructose were found to be a common feature in PA-accumulating transgenic tomato fruits and thus, can be considered as unique metabolite signatures of PAs. Other differences in metabolite profiles of these transgenic fruits might be due to differences in PA levels, fruit ripening conditions and sampling techniques. The metabolic pathways associated with PA-regulated metabolites suggest that free and bound forms of PAs enhance functionality of photosynthetic complexes suggesting role of PAs in C fixation and ATP synthesis as reported by other investigators (Ioannidis and Kotzabasis, 2014; Ioannidis et al., 2012). Thus, enhanced photosynthetic activity in PA-accumulating tomato plants might have role in delaying chloroplast degradation in tomato fruit peel as observed in the present investigation (Figure 4.1c).

Table 4.3**:** Metabolite profiles in transgenic tomato fruits ectopically expressing *SpdSyn* or *SAMdc*.

Changes in metabolite profile is indicated with ↑ (increase, **blue**), ↓ (decrease, **red**) or ↔ (no change, **green**) arrows during tomato fruit ripening.

Reference	Present study	Neily et al. (2010)	Neelam et al. (2008)	Mattoo et al. (2006)
Metabolite	*E8/35S:ySpdSyn*	*35S:MdSpdSyn*	*E8:ySAMdc*	*E8:ySAMdc*
Myo-Inositol	↑	↑		
Gln	↑	↑	↑	↑
GABA	↑	↑	↑	↔
Glu	↑	↑	↑	↔
Thr	↑		↑	↔
Asn	↑		↑	↑
Choline	↑		↑	↑
Fumarate	↑			↑
Trp	↑			
Tyr	↑			
His	↑			
Nucl1	↑			
Ile	↑	↑	↓	↔
Phe	↑	↑	↓	↔
Malate	↑	↓	↑	↑
Asp	↑	↓	↓	↓
Citrate	↑	↔	↔	↑
Val	↑		↓	↓
ATP/ADP	↓			
Formic acid	↓			
Adenosine	↓			
Sucrose	↓	↓	↓	↔
β-Glucose	↓	↓	↓	↓
Fructose	↓	↓	↔	↔
AMP	↓			
UPD-NAcGLU	↔			
Trigonelline	↔			
Ala	↔	↑	↓	↓

PA levels in transgenic lines were positively correlated with levels of Glu, Gln and GABA (Figure 4.7, Figure 4.9), three predominant N-forms in tomato fruits (Boggio et al., 2000; Scarpeci et al., 2007; Valle et al., 1998). PA levels are strictly maintained within stringent limits by cooperative action of many cellular mechanisms including PA catabolism (Moschou et al., 2008). Oxidation of PUT and SPD/SPM produce pyrroline and diaminopropane, respectively. Pyrroline and diaminopropane are converted into GABA and Ala, respectively. Thus, higher amounts of GABA and Ala in transgenic fruits may also be an indication of subsequent degradation of PAs in *ySpdSyn*-expressing and higher PA-accumulating transgenic fruits. In accordance to these findings, PA degradation has been associated with increase in GABA (Yang et al., 2013). Thus, in either case, PAs, as biogenic amines in nature, contribute significantly in nitrogen metabolism (Moschou et al., 2012).

GABA shunt converts GABA into succinate, a Krebs cycle intermediate (Cavalcanti et al., 2014; Shelp et al., 2012). Therefore, higher accumulation of GABA in PA-accumulating transgenics compared to WT fruits lead to increase in production of fumarate, malate and citrate in Krebs cycle. Citrate and malate are major acid metabolites in tomato fruit and are critically important in determining quality of fresh tomato fruits (Etienne et al., 2013; Morgan et al., 2013). For example, citric acid, precursor of citrate, is also positively correlated with smell in tomato fruits (Carli et al., 2009). And, increase in malate contents decrease transitory starch and soluble sugars in transgenic tomato fruits (Centeno et al., 2011).

Phosphoenolpyruvate carboxylase (PEPC) and cytosolic isocitrate dehydrogenase (ICDH) stimulate flux of soluble sugars and starch into production of Glu, Gln and malate in response to N assimilation (Scheible et al., 1997; Scheible et al., 2000). Transcript levels of these C:N metabolism modulating enzymes were also found upregulated in *ySAMdc*-expressing tomato fruits (Mattoo et al., 2006). Accumulation of PAs in transgenic tomato fruit triggers its nitrogen sensing/signaling mechanism which lead to stimulation of carbon metabolism (Figure 4.9) and increased carbon sequestration into fruits (Figure 4.2d) (Mattoo et al., 2006). This result in lower β-glucose and higher citrate, malate and fumarate levels during on-planta (present work) and off-vine ripening of transgenics

compared to control fruits (Mattoo et al., 2006). PAs stimulate O_2 consumption (Andronis et al., 2014) and this is likely by stimulating production of citrate, malate and fumarate (Mattoo et al., 2006). Since, we have also observed similar profiles of these TCA cycle intermediates in *ySpdSyn*-expressing tomato fruits (Figure 4.4), it would be tempting to determine respiration rate during ripening of *ySpdSyn* fruits.

Interestingly, six out of 10 metabolites (Val, Asp, Ile, Glu, Gln and choline) positively correlated with PAs were associated with a membrane transport system of ATP-binding cassette (ABC) transporters. Energized from ATP hydrolysis, ABC transporters transport sugars, lipids, peptides/proteins, ions and sterols across membranes which implicate their role in regulating cellular levels of metabolites and hormones (Bailly, 2014; ter Beek et al., 2014). In addition, *ySpdsyn*-stimulated increase in Ile, a major precursor of aroma volatiles, suggests potential in PAs to improve tomato fruit flavor.

Accumulation of PAs in transgenic tomato fruits resulted in enhancements in lycopene, carotenoids and fatty acids (Kolotilin et al., 2011; Mehta et al., 2002; Nambeesan et al., 2010). Proteins related to aroma volatiles, carotenoids, fatty acids, Calvin cycle and other amino acids have been found in chromoplast proteome of red tomato fruit (Barsan et al., 2010). Together with observed increase of phytonutrients in PA-accumulating tomato fruits, this data suggest that PAs maintain structural integrity and biological functions of chromoplasts which result in prolonged biosynthesis of these phytonutrients. Chromoplasts also contribute in ATP synthesis when mitochondria-derived ATP synthesis is diminishing during fruit ripening (Pateraki et al., 2013; Renato et al., 2014). Higher anabolic activities instead of lower β-glucose and ATP/ATP levels in transgenic fruits indicate that PAs fuel metabolic activities in transgenic fruits through chromorespiration and chemiosmosis (Ioannidis and Kotzabasis, 2014; Renato et al., 2014). Altogether, PA-regulated changes of distinct-in-nature metabolites also suggests that PAs influence multiple cellular pathways in diverse subcellular compartments including cytoplasm, chloroplasts, chromoplasts and mitochondria during fruit ripening (Mattoo et al., 2007).

The transcriptome studies have revealed massive changes in global gene expression in PA-accumulating tomato fruits (Cheng et al., 2012; Kolotilin et al., 2011; Srivastava et al., 2007) and *Arabidopsis* plants (Alcazar et al., 2005; Gonzalez et al., 2011; Kasukabe et al., 2004; Marco et al., 2011a; Marco et al., 2011b) which lead to alteration in plant architecture, delay in fruit ripening and senescence and induction of plant's responses against biotic and abiotic stresses (Alcazar et al., 2005; Cheng et al., 2012; Gonzalez et al., 2011; Kasukabe et al., 2004; Mitsuya et al., 2009; Nambeesan et al., 2012). PAs regulate gene expression by promoting action of histone acetyltransferases and hyperacetylating chromatin in proliferating epidermal and fibroblast cell types (Hobbs and Gilmour, 2000). SPD post-transcriptionally downregulates expression of p53 in rat cells (Li et al., 2001) and SPM enhances the interaction between nuclear receptor HNF4α and vitamin D receptor-interacting protein 205 (DRIP205) and decrease interaction of HNF4α with p160 co-activator glucocorticoid receptor interacting protein 1 (GRIP1) (Maeda et al., 2002). SPD is required in synthesis of hypusine and, thus, essential for post-translational modification of eukaryotic translation initiation factor 5A (eIF5A) which is an mRNA-binding protein and is involved in translational elongation (Park, 2006). Also, PAs can stimulate various stages of protein synthesis by promoting assembly of 30 S ribosomal subunits and Ile-tRNA formation and elevating +1 ribosomal frame shift efficiency at the retro transposon ty1 frame shift site and at antizyme frame shift site (Igarashi and Kashiwagi, 2000 and references therein). At cellular level, SPM regulate intrinsic gating of strong inward rectifier K^+ channel by directly plugging it pore (Kurata et al., 2006). SPM also potentiate function of *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor by binding to its amino-terminal domain and stabilizing its open-channel state (Kumar and Mayer, 2013). A plurality model of PA action has been proposed which describe impact of PAs on chromatin integrity, transcription, translation initiation and translation, protein structure and function and their ultimate effect on cellular metabolism leading to modification in phenotype (Handa and Mattoo, 2010).

CHAPTER 5. POLYAMINE INTERACTIONS WITH PLANT HORMONES: CROSSTALK AT SEVERAL LEVELS

5.1 Introduction

PAs are biogenic amines with aliphatic polycationic properties and ubiquitous in all living organisms. Although PAs were discovered more than 300 years ago (Vanleeuwenhoek, 1978), it is only within the past few decades that significant progress has been made in understanding their role in plant growth and development (Bachrach, 2010; Evans and Malmberg, 1989; Galston and Sawhney, 1990; Martin-Tanguy, 2001). PAs are essential for cell division and proliferation in all organisms and implicated in diverse growth and development processes including chromatin function, protein synthesis, structural integrity of nucleic acids, and cellular membrane dynamics (Handa and Mattoo, 2010; Kusano et al., 2008; Matthews, 1993; Theiss et al., 2002; Thomas and Thomas, 2001; Wallace, 2009). Pharmacological evidence through exogenous application of PAs and recent molecular inroads through perturbation of endogenous PA levels by transgenic approach have demonstrated the important role of PAs in seed germination, tissue lignification, organogenesis, flowering, pollination, embryogenesis, fruit development, ripening, abscission, senescence and stress responses (Alcazar et al, 2005; Gomez-Jimenez et al., 2010a; Imai et al., 2004; Kusano et al., 2008; Mattoo et al., 2007; Minocha

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et al., 2014; Nambeesan et al., 2008; Takahashi and Kakehi, 2010; Tisi et al., 2011; Urano et al., 2005). However, in spite of the myriad of effects of PAs confirmed in many organisms including plants, the molecular mechanisms involved are not yet understood completely (Torrigiani et al., 2008).

The electrostatic or covalent binding of PAs to various macromolecules that causes conformational changes in chromatin, DNA, RNA, and protein structures and culminates in altered gene expression and physiological cellular responses led to the suggestion that they are important components of cellular proliferation (Thomas and Thomas, 2001), and impact plant growth and developmental processes (Garufi et al., 2007; Kasukabe et al., 2004; Srivastava et al., 2007). Covalent binding of PAs to proteins, such as cationization and crosslinking, hypusine synthesis (a cofactor of eIF-5A), and accumulation of cytotoxic lipophilic PA-derivativesmay, in fact, lead to metabolic shifts in organisms (Seiler and Raul, 2005; Takahashi and Kakehi, 2010). Another property that is being researched is the ability of PAs, particularly SPM, to scavenge free radicals and thus impact reactive oxygen species (ROS) and redox signaling (Das and Misra, 2004; Ha et al., 1998; Løvaas, 1996). In plants, engineering the levels of SPD and SPM at the cost of PUT was shown to affect glucose metabolism and carbon:nitrogen (C:N) signaling and alter cellular energy balance in fruits (Mattoo et al., 2007; Mattoo et al., 2006; Pirinen et al., 2007).

An interplay of plant hormone during ripening of fleshy fruits has been suggested (Gillaspy et al., 1993; Kumar et al., 2014; McAtee et al., 2013). Suggestions for a crosstalk among plant hormones and PAs emanated from studies in which many physiological and developmental processes were found synergistically or antagonistically modulated by PAs vis a vis plant hormones (Bitrián et al., 2012; Milhinhos and Miguel, 2013). Biosynthesis of PAs, SPD and SPM, starts with the substrate, SAM, which is also a substrate for the plant hormone ethylene (Mattoo and White, 1991). In situations when this substrate might become limiting, it could determine the outcome for which pathway, whether ethylene or SPD/SPM biosynthesis, would dominate (Harpaz-Saad et al., 2012; Lasanajak et al., 2014).

Another example of a hormone that appears to crosstalk with PAs is ABA in regulating abiotic stress responses, integrating ROS and nitric oxide (NO), and altering ion homeostasis, especially Ca^{2+} (Alcazar et al., 2010). Neither a specific receptor nor a signal transduction mechanism is as yet discerned for PAs. Little is known or understood about the signal transduction pathways regulating a myriad of PA effects. We, therefore, analyzed and collated the information on changes in transcriptome obtained on transgenics where metabolic engineering of PA pathway was carried out as well as data on mutants of PA biosynthesis, and studies in which various PAs were exogenously applied to plant tissues. These analyses, summarized here, indicate that a complex network regulates interactions of both PAs and plant hormones.

5.2 Altered endogenous PA levels affect transcriptome

Transgenic expression of yeast *SAMdc* under a fruit/ethylene-specific promoter E8 (*E8:ySAMdc*) resulted in 2 to 3-fold increase in SPD and SPM content of ripening tomato fruit while lowering the concentration of PUT to a minimum (Mehta et al., 2002). Comparison of the transcriptome of the WT and the *E8:ySAMdc* transgenic ripening tomato fruits revealed a massive change in the gene expression between the two genotypes, attributed to the increased levels of SPD and SPM in *E8:ySAMdc* lines (Srivastava et al., 2007). The metabolome of these genotypes showed corresponding changes as well (Mattoo et al., 2006). These data were compared for correlation analysis with changes in amino acids of poplar cells transformed with a constitutively expressed mouse *ODC* (*mODC*) gene that accumulated PUT (Mohapatra et al., 2010). It was revealed that PUT and SPD/SPM had mostly opposite effects, and therefore it was concluded that each PA may have a unique role in plant metabolome, as well as at the level of the transcriptome (Handa and Mattoo, 2010; Mattoo and Handa, 2008; Mattoo et al., 2010a). Similarly, although SPD/SPM levels were positively correlated with changed transcript levels of early ripening genes, PUT exhibited a negative correlation with transcript accumulation of many of the same genes (Handa and Mattoo, 2010; Mattoo and Handa,

2008; Mattoo et al., 2010a); this was also true for several fruit quality attributes (Handa and Mattoo, 2010). Evaluation of transcriptome changes associated with high SPD/SPM level, the *E8:ySAMdc* transgenic tomato, in comparison with the parental WT fruit (Mehta et al., 2002) have also been carried out using the TOM1 transcriptional microarray (Kolotilin et al., 2011). These investigators also found significant changes in gene expression patterns of *E8:ySAMdc* transgenic fruits compared to WT fruits as originally published (Srivastava et al., 2007). Effect of exogenous application of SPD to mature green tomato fruit with or without exposure to increasing temperature stress on gene expression using the Affymetrix microarray also revealed upregulation of several genes related to defense responses, oxidation reduction, signal transduction, and hormone biosynthesis (Cheng et al., 2012).

Engineered constitutive expression of *Curubita ficifolia SpdSyn* in *Arabidopsis* plants also increased SPD and SPM contents by about 2-fold. The transgenic plants had reduced chilling stress that correlated with enhanced expression of stress-responsive transcription factors and proteins (Kasukabe et al., 2004). Constitutive overexpression of *ADC* gene (*35S:AtADC2*) in *Arabidopsis* led to levels of PUT in the transgenics more than 16-fold higher without significantly changing SPD and SPM contents (Alcazar et al., 2005). Accumulation of PUT inhibited gibberellic acid (GA) biosynthesis, which resulted in dwarf stature and delayed flowering in transgenic *Arabidopsis* plants (Alcazar et al., 2005). Transcriptome analysis of *35S:AtADC2* and WT plants showed that overexpression of *ADC2* downregulated expression of dioxygenases genes (*GA20ox1*, *GA3ox1* and *GA3ox3*), which are involved in the final step of GA metabolism, whereas transcription of genes involved in early steps of GA biosynthesis remained unaltered (Alcazar et al., 2005). Similarly, *Arabidopsis* plants constitutively overexpressing *SpmSyn* (*35S:AtSPMS-9*) had about 3-fold increase in SPM contents without perturbing PUT and SPD levels in 15-day old leaves (Gonzalez et al., 2011), an observation in agreement with results from a T-DNA insertion mutant of *Arabidopsis* in *SpmSyn* (*spms-2*) that showed about 2-fold decrease in SPM without alteration in PUT and SPD contents (Gonzalez et al., 2011). Thus, it appears that each step in individual PA biosynthesis can dictate changes in their respective levels,

with and without affecting the level of the other PAs. SPM levels in *35S:AtSPMS-9* transgenic *Arabidopsis* plants were positively correlated with plant resistance to *Pseudomonas viridiflava* (Gonzalez et al., 2011). Microarray analysis of transcriptional changes in the gene expression in *35S:AtSPMS-9* revealed that overproduction of SPM enhanced transcription of several transcription factors, kinases, nucleotide- and DNA/RNA-binding proteins, and the genes involved in pathogen perception and defense responses (Gonzalez et al., 2011). In another study, transcriptome profile of *SAMdc1*-overexpressing (*35S:AtSAMdc1*) was compared with those of *35S:AtADC2* and *35S:AtSPMS-9 Arabidopsis* plants to investigate role of PAs in regulating abiotic stress pathways (Marco et al., 2011a; Marco et al., 2011b). The Affymetrix ATH1 microarray-generated transcriptome showed that the functional enrichment of genes related to pathogen defense and abiotic stresses were commonly upregulated in PUT- or SPM-accumulating *Arabidopsis* plants (Marco et al., 2011a; Marco et al., 2011b). We further evaluated these global transcriptome profiles along with that obtained from SPD/SPM-accumulating tomato fruits (*E8:ySAMdc*) to delineate the roles of endogenous PA levels on regulation of plant hormone biosynthesis and signaling pathway genes. Exogenous application of SPM also induced defense response in *Arabidopsis* against cucumber mosaic virus by modulating expression of genes involved in photorespiration, protein degradation, defense, protein folding, and secretion (Mitsuya et al., 2009).

5.3 PA-Ethylene crosstalk

PAs are considered as anti-senescence growth regulators that seem antagonistic to ethylene-promoted leaf senescence, fruit ripening, and biotic stresses (Abeles et al., 1992; Alba et al., 2005; Alexander and Grierson, 2002; Cheong et al., 2002; Evans and Malmberg, 1989; Galston and Sawhney, 1990; Giovannoni, 2001; Giovannoni, 2004; Klee, 1993; Nambeesan et al., 2008; Tieman et al., 2000). Ethylene is involved in leaf epinasty, flower fading, abscission, fruit ripening, and senescence. Because SAM is the common substrate for ethylene, SPD and SPM biosynthesis, that exogenous application of PAs

inhibit ethylene production in diverse plant tissues, and that ethylene inhibits activities of enzymes in PA biosynthesis pathway, a crosstalk among their biosynthesis pathways as well as during plant development was suggested (Apelbaum et al., 1981; Cassol and Mattoo, 2003; Harpaz-Saad et al., 2012; Li et al., 1992; Mattoo and White, 1991). However, the rate of ethylene production in *E8:ySAMdc* transgenic tomato fruits, that accumulated 2- to 3-fold higher SPD/SPM, was much higher than the azygous control fruit (Mehta et al., 2002), which demonstrated that availability of SAM *in vivo* is not rate limiting for the biosynthesis of either ethylene or SPD/SPM and that both pathways could run simultaneously. Interestingly, in spite of higher ethylene production in the y*SAMdc*-expressing transgenic tomato fruit, a delay in on-vine ripening of transgenic fruits was observed. These results indicated a dominant role of SPD/SPM over ethylene during the fruit ripening process. This inference was also supported by investigations on another genetic event that involved expression of *ySpdSyn*, driven also by E8 promoter (Nambeesan et al., 2010). The *E8:ySpdSyn* fruit also had increased SPD as well as ethylene, and yet had extended shelf life, lower shriveling rate, and delayed decay compared to WT tomato fruits.

The high endogenous SPD/SPM concentrations in the *E8:ySAMdc* tomato fruit were accompanied by about 2-fold increase in *ACS* transcripts compared to WT fruits (Mattoo et al., 2007). The *35S:AtSPMS-9* transgenic *Arabidopsis* had also increased *ACS* transcript levels in the leaves (Gonzalez et al., 2011), but *ACS6* transcripts were about 3-fold downregulated in the PUT accumulating leaves of *35S:AtADC2* transgenic *Arabidopsis* leaves (Alcazar et al., 2005). Reduction in *ACS* transcripts in *35S:AtADC2 Arabidopsis* leaves and increase in *E8:ySAMdc* tomato fruits and *35S:AtSPMS-9 Arabidopsis* leaves (Alcazar et al., 2005; Gonzalez et al., 2011; Kolotilin et al., 2011; Mattoo et al., 2007) support the contrasting roles played by PUT and SPD/SPM in plant growth and development, as proposed earlier (Handa and Mattoo, 2010; Mattoo et al., 2010a). However, more data from other transgenic plants and PA mutants are needed to unequivocally prove this hypothesis.

The microarray data obtained from plant tissues with altered PA levels support the contention that the biogenic amines alter ethylene response by modulating expression of ethylene signaling pathway components (Figure 5.1). Transcriptome data indicate potential of PAs to increase ethylene production by enhancing expression of *ACS*. The expression of *S-ADENOSYLMETHIONINE SYNTHETASE* (*MAT*) and *MITOGEN-ACTIVATED PROTEIN KINASE KINASE* (*MAPKK*) were also upregulated in high *SPD*/SPM accumulating transgenic tomato fruit (Kolotilin et al., 2011). It is now known that the ethylene response is negatively regulated by its receptors. In the absence of ethylene, ethylene receptors (ETRs, ERS, EIN4) interact with ER-localized family of Raf-like serine/threonine kinases (CTRs: CONSTITUTIVE TRIPLE RESPONSE factors) and suppress the ethylene signaling cascade (Zhong et al., 2008). Five ethylene receptors and one CTR1 have been identified in *Arabidopsis*, whereas six ethylene receptors and three CTRs are present in the tomato genome (Chen et al., 2010; Klee and Tieman, 2002). As shown in Figure 5.1, RESPONSIVE-TO-ANTAGONIST1 (RAN1) delivers Cu^{+} cofactor to ethylene receptor to make the multi-protein complex functional (Hirayama et al., 1999; Woeste and Kieber, 2000). Constitutive overexpression of tomato *Green-ripe* (*GR*) or an *Arabidopsis reversion-to-ethylene sensitivity 1* (*RTE1*) homologue reduced ethylene responsiveness while its mutant exhibited weaker ethylene insensitivity in tomato fruit, indicating that, similar to receptors and CTRs, repression of GR is also required to perceive ethylene action in tomato (Barry and Giovannoni, 2006). To activate signaling pathway, ethylene binds to its receptors, causing conformation change and inactivation of CTRs; this releases downstream signal transducer ETHYLENE INSENSITIVE 2 (EIN2) from suppression (Ju et al., 2012) and induces transcription of EIN3 and EIN3-like transcription factors (EILs) (Ji and Guo, 2012). EIN3 and EILs differentially regulate transcription of ethylene response factors (ERFs) that regulate transcription of ethylene-responsive target genes (Guo and Ecker, 2004). The high endogenous levels of PUT or SPM had no effect on the transcript levels of either ethylene receptors or *CTR1* in *Arabidopsis* leaves (Alcazar et al., 2005; Gonzalez et al., 2011). However, higher endogenous SPD/SPM levels in *E8:ySAMdc* tomato fruits correlated with downregulation of ethylene receptors *ETR2* and

ETR3 (*NEVER-RIPE*), but *ETR4* and *ETR6* were upregulated (Kolotilin et al., 2011). *CTR* homologues were also either upregulated by 2- to 3-fold or remained unaltered in *E8:ySAMdc* tomato fruits (Kolotilin et al., 2011).

Based on the absence or presence of putative transmembrane domains, ethylene receptors have been broadly divided into subfamilies I and II, respectively (Hall et al., 2007). Tomato ETR2 and ETR3 belong to subfamily I and ETR4 and ETR6 belong to subfamily II (Binder, 2008). Increase in the expression of *ETR4*, *ETR6* and *CTRs* in SPD/SPM-accumulating fruits is analogous to increase in the abundance of receptor–CTR complexes that would strengthen the negative regulation of ethylene. This analogy would also explain why, in spite of higher ethylene production in *ySAMdc* and *ySpdSyn* transgenic tomato fruits, the ethylene responsiveness manifested as fruit ripening was reduced in these fruit (Mehta et al., 2002; Nambeesan et al., 2010). It is possible that the higher expression of subfamily II receptors is also a compensatory response to decreased *ETR3* expression (Tieman et al., 2000).

The transcriptome changes in response to exogenously applied PAs support the afore-discussed results that PAs alter expression of ethylene signaling pathway genes. Application of 1mM PUT or SPD to abscising mature olive fruit increased transcript levels of ethylene receptor *ERS1*, even though *CTR1* expression showed slight decrease and abrogated the ethylene signaling pathway (Parra-Lobato and Gomez-Jimenez, 2011). Exogenous application of 0.1mM SPD to peach fruit mesocarp lowered ethylene production and led to higher transcript levels of *ETR1* and *ERS1* (Ziosi et al., 2006). Peach fruits treated with 1mM SPD had reduced ethylene perception with enhanced expression of subfamily II receptors and CTRs transcripts, which is indicative of PAs nexus with ethylene signaling (Torrigiani et al., 2012). In the absence of ethylene, EIN3-regulating F-box proteins (EBF1 and EBF2) degrade EIN3 by the ubiquitin/proteasome pathway (Guo and Ecker, 2003). Silencing of *EBF1* and *EBF2* has been reported to cause constitutive ethylene response phenotype in tomato (Yang et al., 2010). However, the *E8:ySAMdc* tomato fruit pericarp was neutral for the expression of *EBF1* (Kolotilin et al., 2011). None

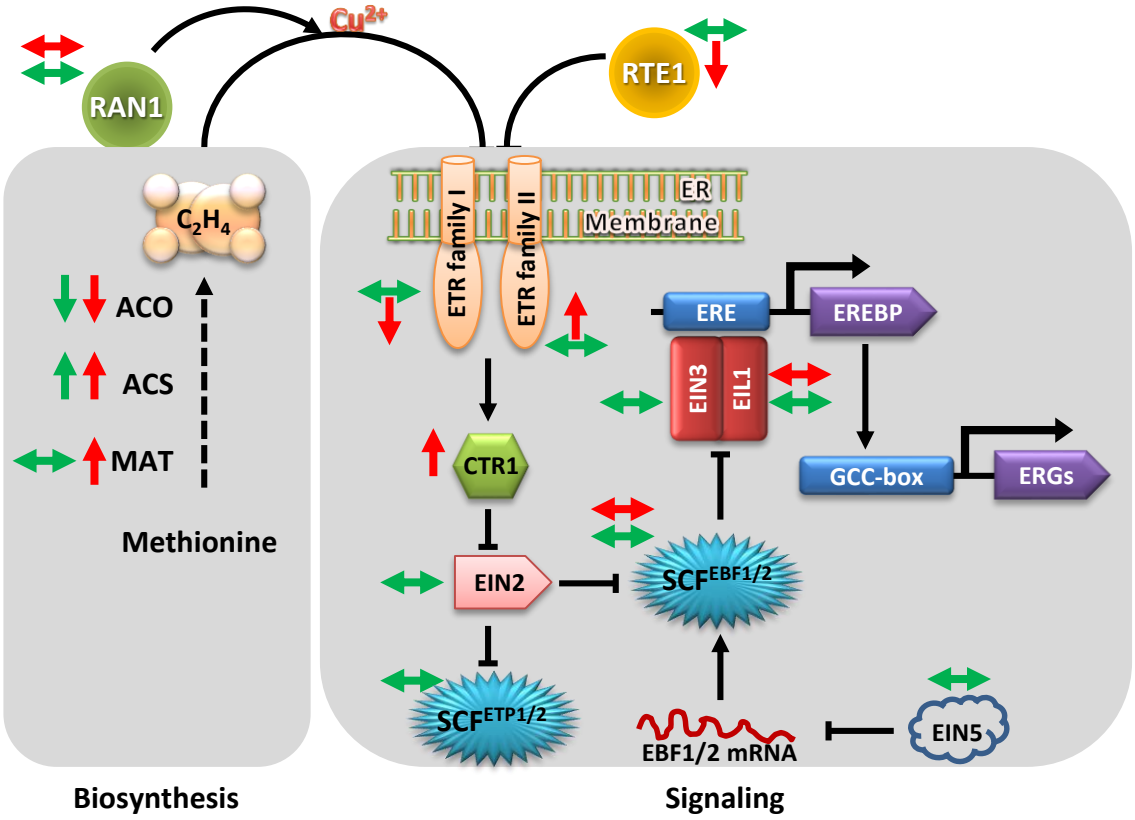

Figure 5.1: Consensus effects of SPD and SPM on ethylene metabolism and signaling cascade.

Green (*Arabidopsis* leaves) or red (tomato fruit) vertical arrows indicate up- and downregulated gene transcripts by SPD/SPM. Black arrows and blunt heads indicate stimulatory or inhibitory effects, respectively. *ACO*, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase; *ACS*, ACC synthase; *CTR1*, constitutive triple response 1; *EBF1*, EIN3-binding F-box protein 1; *EBF2*, EIN3-binding F-box protein 2; *EIL1*, ethylene-insensitive3-like 1; *EIN2*, ethylene insensitive 2; *EIN3*, ethylene insensitive 3; *EIN5*, ethylene insensitive 5; *ERE*, ethylene response element; *EREBP*, ethylene response element-binding protein (including ERFs); *ERGs*, ethylene responsive genes; *ETP1*, EIN2 targeting protein 1; *ETP2*, EIN2 targeting protein 2; *ETR*, ethylene receptor; *MAT*, *S*-adenosylmethionine synthetase; *RAN1*, responsive-to-antagonist 1; *RTE1,* reversion-to-ethylene sensitivity 1.

the less, grapes treated with guazatine, an inhibitor of PA oxidase, accumulated PUT and were upregulated in *EIN3* and *EBF2* expression (Agudelo-Romero et al., 2014).

Taken together these results indicate a very complex crosstalk between PAs and various components of ethylene action. The complexity is partly driven by the presence of a family of genes for both the biosynthesis and action of ethylene, each under a complex developmental and environmental regulation. Homologues of various gene families not only are redundant in some cases and take over the function of their counterpart, but also their transcription can compensate the loss/inexpression of a family member (Tieman et al., 2000).

5.4 PA-Jasmonate crosstalk

Jasmonates (JAs), mainly derived from linolenic acid, are oxylipin signaling molecules that regulate a wide range of plant developmental and growth processes including male fertility, root growth, tendril coiling, fruit ripening, and inducing plant defense responses (Farmer and Ryan, 1990; Srivastava and Handa, 2005; Wasternack, 2007; Yan et al., 2007). The early enzymatic reactions catalyzed by plastid-localized lipoxygenases (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) yield *cis*-(+)-12-oxophytodienoic acid (OPDA). Conversion of OPDA to JA in peroxisomes is catalyzed in series by 12-oxophytodienoate reductase 3 (OPR3), acyl-CoA oxidase (ACX), multifunctional protein (MFP), *L*-3-ketoacyl-CoA thiolase (KAT), and acyl-thioesterase (ACH) (Creelman and Mullet, 1997; Schaller and Stintzi, 2009; Wasternack, 2007). Jasmonoyl-*L*-isoleucine synthetase (JAR) and JA carboxyl methyltransferase (MJT) catalyze the conversion of JA to jasmonate-isoleucine (JA-Ile) and methyl ester of JA (MeJA), respectively (Fonseca et al., 2009). The JAs-induced changes in PA biosynthesis seem to delay fruit ripening (Yoshikawa et al., 2007; Ziosi et al., 2007; Ziosi et al., 2009), stimulate tolerance against pathogens and insect herbivores (Kaur et al., 2010; Walters et al., 2002), induce wound response (Pérez-Amador et al., 2002), and decrease low-temperature injuries (Wang and Buta, 1994; Yoshikawa et al., 2007). JAs have also been

implicated in the production of conjugated PAs in vegetative plant tissue (Keinänen et al., 2001; Tebayashi et al., 2007; Zhang et al., 2007).

MeJA was reported to upregulate expression of *ADC*, *ODC* and *SAMdc*, increase oxidation and conjugation of PAs, and inhibit shoot formation in tobacco thin layers (Biondi et al., 2001). Either MeJA application or mutations imparting constitutive JA signaling resulted in increased production of caffeoyl-PUT in tomato leaves, whereas *jasmonate insensitive1* (*jai1-1*) and *coi1* mutants defective in JA perception were inhibited in the production of caffeoyl-PUT in tomato and tobacco leaves, respectively (Chen et al., 2006; Paschold et al., 2007). That MeJA induces conjugation of PAs and involves R2R3-MYB8, a JA responsive transcription factor, was revealed through RNAi-mediated silencing of R2R3-MYB8 (Kaur et al., 2010). In these transgenic tobacco plants, downregulation of *SpdSyn* was accompanied by lack of phenylpropanoid-PA conjugates, caffeoyl-PUT and dicaffeoyl-Spd (Kaur et al., 2010; Onkokesung et al., 2012). Transcription of PUT *N*-methyltransferase (PMT), an enzyme that converts PUT into *N*-methyl-PUT, is stimulated by MeJA (Shoji et al., 2000) and inhibited by herbivore-induced ethylene production (Winz and Baldwin, 2001). It has been discovered that a TA-rich region and a GCC motif in the promoter of tobacco *PMT* regulate MeJA-induced transcription of *PMT* (Xu and Timko, 2004). In fruit tissues, however, MeJA induced accumulation of free PAs without altering the levels of conjugated PAs. Application of *n*-propyl dihydro jasmonate (PDJ), a synthetic derivative of MeJA, enhanced accumulation of free PAs by 30-60% in epicarp and mesocarp of peach fruit, whereas MeJA application had negligible effects on PA and ethylene production. In both treatments, PCA-soluble and PCA-insoluble PAs could not be detected in peach fruits (Ziosi et al., 2009). Co-suppression of *SlLOXB* resulted in 60-90% reduction in MeJA and 50% reduction in free PAs in transgenic compared to WT tomato fruits (Kausch et al., 2011). Increase in free SPD and SPM in MeJA-treated fruits correlates with low-temperature stress tolerance in zucchini squash (Wang and Buta, 1994), mango (González-Aguilar et al., 2000), and apples (Yoshikawa et al., 2007), suggesting a role of free SPD and SPM in fruit ripening and low-temperature stress tolerance.

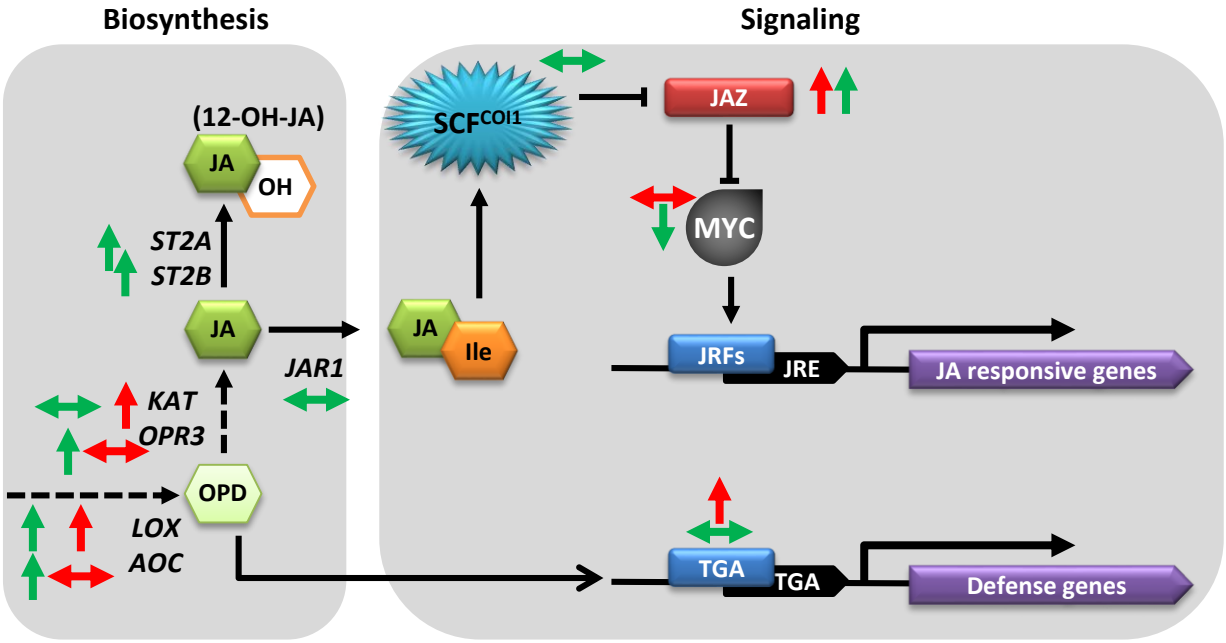

Figure 5.2: Consensus effects of SPD and SPM on jasmonic acid metabolism and signaling cascade.

Description same as in legends to Figure 5.1. *12-OH-JA*, 12-hydoxyjasmonic acid; *AOC,* allene oxide cyclase; *COI1,* coronatine insensitive 1; *JA-Ile*, jasmonate (JA)-isoleucine conjugate; *JAR1,* jasmonate resistant 1; *JAZ,* jasmonate-zim-domain protein; *JRE,* jasmonate response element; *JRFS,* jasmonate response factors; *KAT,* 3-keto-acyl-coenzyme A thiolase; *LOX,* lipoxygenase; *MYC* and *TGA,* transcription factor proteins; *OPDA, cis*-(+)-12-oxophytodienoic acid; *OPR3,* oxophytodienoate reductase 3; *ST2A* and *ST2B,* sulfotransferase.

Little is known about the effects of PAs on production, conjugation, perception, and signal transduction of JAs. Ectopic expression of y*SAMdc* intensified accumulation of ω-3 fatty acids in ripening tomato fruit, with α-linolenic acid (C18:3 n3) levels increasing to more than 50% of total fatty acids (Kolotilin et al., 2011). High SPD/SPM also increased transcript levels of *LOX* and *3-KETO-ACYL-COENZYME A THIOLASE (KAT)* in tomato fruit (Kolotilin et al., 2011; Srivastava et al., 2007). Constitutively expressed *ySAMdc* caused a 24- to 90-fold increase in *LOX* transcripts whereas expression of *SpmSyn* (*35S:AtSPMS-9*) in *Arabidopsis* increased the levels of *LOX*, *AOC3* and *OPR3* transcripts (Gonzalez et al., 2011; Marco et al., 2011a). In addition to upregulation of JA biosynthesis genes, transcript levels of JA-conjugating sulfotransferases (ST2A, ST2B) were also increased in *35S:AtSPMS-9 Arabidopsis* plants, suggesting that higher PAs also stimulate conjugation of JAs (Gonzalez et al., 2011). The over-accumulation of PUT in *ADC2* transgenic *Arabidopsis* plants showed about 3-fold downregulation of the *LOX* gene, an effect opposite to that of SPD and SPM (Alcazar et al., 2005).

Studies using an *Arabidopsis* mutant (*coi1*) resistant to coronatine, a phytotoxin structurally similar to jasmonate, have helped our understanding of the JAs signaling pathway in plants (Santner and Estelle, 2009). Binding of jasmonate-isoleucine (JA-Ile) to COI1, a F-box protein that forms E3 ubiquitin ligase complex (SCF^{COI1}), promotes binding to JAZ proteins and facilitates their degradation (Figure 5.2); this liberates JIN1/MYC2 from repression, activating the jasmonate response (Chini et al., 2009; Sheard et al., 2010; Thines et al., 2007; Yan et al., 2007). MYC proteins are transcription factors that belong to group IIIe of the bHLH family. MYC2/JIN1 binds with conserved G-box *cis*-elements in the promoter region of target transcriptional activators or repressors to regulate JA-induced gene expression (Figure 5.2) (Fonseca et al., 2009; Kazan and Manners, 2008; Pauwels and Goossens, 2011; Turner et al., 2002). Transcript levels of genes encoding JAZ proteins were upregulated by higher SPD/SPM in transgenic tomato fruits and *Arabidopsis* leaves, but downregulated in PUT-accumulating *35S:AtADC2 Arabidopsis* leaves (Alcazar et al., 2005). However, expression of neither *JAR1* nor *COI1* changed in *35S:AtSPMS-9* or *spms-2 Arabidopsis* leaves (Gonzalez et al., 2011). It is noted here that

the JAZ proteins repress EIN3 and EIL1 in the ethylene signaling pathway, suggesting that PAs can inhibit ethylene signaling by positively regulating transcription of JAZ proteins (Pauwels and Goossens, 2011). JERF3 is a two-way acting regulatory hub induced by JA, ethylene, ABA, salt, and cold, and binds with both dehydration-responsive element DRE and GCC-box to regulate expression of genes in multiple defense mechanisms (Wang et al., 2004). The report that *JERF3* transcripts were downregulated in *E8:ySAMdc* transgenic tomato supports an inhibitory role of PAs on jasmonate and ethylene signal transduction pathways (Kolotilin et al., 2011).

OPDA induces plant resistance against wounding and pathogen infection by activating expression of genes via a COI1-independent signaling pathway dependent on TGA transcription factors (Mueller et al., 2008; Stintzi et al., 2001). OPDA-specific response genes (ORGs) that were strongly upregulated by OPDA but less so by JA or MeJA have been identified (Taki et al., 2005). Among the OPDA-responsive genes differentially transcribed in *35S:AtSPMS-9 Arabidopsis* leaves, expression of 29 genes was positively correlated with *SpmSyn* expression (Gonzalez et al., 2011) whereas 17 OPDA-specific genes were downregulated in the PUT-accumulating *35S:AtADC2 Arabidopsis* leaves (Alcazar et al., 2005). OPDA-regulated TGA transcription factors bind to the TGA motif in promoter regions to enhance expression of defense response genes (Zhang et al., 1999). Increased transcript levels of *TGA* transcription factors in *E8:ySAMdc* transgenic tomato fruits that accumulate high SPD/SPM suggest that PAs might have a role in OPDA-mediated signal transduction in tomato fruit (Kolotilin et al., 2011).

5.5 PA-Auxin crosstalk

Auxins are well-known plant growth hormones involved in plant processes including embryogenesis, apical patterning, stem elongation, development of vascular tissues, fruit set, growth, maturation and ripening, and root initiations that mediate gravitropic and phototropic responses in plants (Chapman and Estelle, 2009; El-Sharkawy et al., 2014; Guillon et al., 2008; Kang et al., 2013; Kumar et al., 2014; Vanneste and Friml,

2009). Auxin and ethylene act synergistically during fruit ripening (Gillaspy et al., 1993; Jones et al., 2002; Liu et al., 2005; Tatsuki et al., 2013; Trainotti et al., 2007) and SPD impair ethylene- and auxin-related metabolism and signaling to slow down fruit ripening (Torrigiani et al., 2012). Auxin concentration and distribution in plant tissues is regulated by its biosynthesis, conjugation, modification, and transport (Korasick et al., 2013). Indole-3-acetic acid (IAA) is a predominant bioactive form of auxin derived from tryptophan, mainly through tryptophan aminotransferase and YUCCA/flavin monooxygenase pathways. The auxin signaling pathway is a complex, with Aux/IAA proteins being the major responsive proteins that negatively regulate auxin signaling by associating with carboxyl-terminal dimerization domain of auxin response factors (ARFs) and repressing their transcriptional activity (Figure 5.3) (Chapman and Estelle, 2009; Vanneste and Friml, 2009). Auxin binds and activates F-box receptors TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and TIR1-related AUXIN SIGNALING F-BOX (AFB) proteins. The activated TIR1 and AFB proteins, as a part of ubiquitin E3 ligase complex ($SCF^{TIR1/AFB}$), interact with Aux/IAA and direct them to 26S proteasome-mediated degradation (Chapman and Estelle, 2009; Vanneste and Friml, 2009). This event releases ARFs transcription factors from repression and thus depresses or activates transcription of auxin response genes (Chapman and Estelle, 2009). ARFs are *trans*-acting factors that bind with TGTCTC-containing auxin-response *cis*-elements (AuxREs) within the promoter region of auxin-regulated genes (Hagen and Guilfoyle, 2002). Among hundreds of gene regulated by auxin, major classes of auxin early response genes, in addition to *Aux/IAA*, include *SMALL AUXIN UP RNA* (*SAUR*) and *GH3* proteins (Hagen and Guilfoyle, 2002). Until now, at least 34 *Aux/IAA*, 23 *ARFs*, and 20 *GH3* genes have been identified in *Arabidopsis* (Hagen and Guilfoyle, 2002), and 36 *Aux/IAA* and 17 *ARFs* have been identified in tomato (Audran-Delalande et al., 2012; Kumar et al., 2011; Wu et al., 2012b).

The nexus of PAs with auxin came about through transgenic research and new information through transcriptomic analysis. Hyposensitivity of *bud2* mutant plants, with *AtSAMdc4* loss-of-function, to auxin and induction of endogenous *BUD2* by auxin indicate that PAs regulate plant architecture through auxin response in *Arabidopsis* (Cui et al.,

2010). The overexpression of *SpmSyn* (*35S:AtSPMS-9)* in *Arabidopsis* leaves resulted in differential expression of the family of YUCCA genes: more than 2-fold decrease in the expression of *YUC1* and *YUC10* occurred and a 2- to 3-fold increase in *YUC5* and *YUC8* (Gonzalez et al., 2011). However, higher PA levels in *E8:ySAMdc* tomato fruit did not alter steady-state transcript levels of *YUC-LIKE FLAVIN MONOOXYGENASE* and *ToFZY*, a putative orthologue of *YUC4* and *FLOOZY (FZY)* (Expósito-Rodríguez et al., 2007; Kolotilin et al., 2011). Also, application of SPD did not induce accumulation of free and bound levels of IAA in radish seedlings that were either unstressed or stressed with copper (Choudhary et al., 2012b) or chromium (Choudhary et al., 2012a). *SpmSyn* transgenic leaves had twice as many transcripts of *IAA2*, *IAA3*, *IAA6*, *IAA19*, *IAA20*, *IAA29* and *IAA33* whereas transcripts for *ARF6*, *ARF8* and *ARF13* were actually downregulated as compared to the control WT plants (Gonzalez et al., 2011). It is noted here that, in contrast to these data, levels of *IAA3, IAA6* and *IAA25* transcripts were lower in *E8:ySAMdc* transgenic tomato fruit. In conjunction, exogenous application of SPD to early developing peach fruit also downregulated *IAA* (ctg57) and *GH3* (ctg1993) transcripts at during fruit maturation and ripening (Torrigiani et al., 2012).

Distribution of auxin and establishment of its gradient within tissues is crucial for some of the auxin-regulated plant growth and development processes (Ikeda et al., 2009; Tivendale et al., 2014; van Berkel et al., 2013). Several auxin influx and efflux carriers have been characterized that regulate cell-to-cell transport of auxin. Auxin influx into the cell is either passive or regulated by AUX1/LAX (Auxin Permease/Like AUX) family of H^+ symporters (Pattison and Catala, 2012; Peret et al., 2012). Auxin efflux carriers have been classified into two major families. The PIN (PIN-FORMED) family of auxin efflux carriers is plant specific and regulates polar auxin transport. MDR/PGP (multi-drug resistance/P-glycoprotein) family of auxin exporters belongs to the ATP-Binding Cassette (ABCB) superfamily of transporters that ubiquitously handle distribution of various molecules and nutrients (Zazimalova et al., 2010). How PAs affect auxin transport has not yet been studied. Ectopic expression of *35S:AtSAMdc1* or *35S:AtSPMS*-9 in *Arabidopsis* downregulated two auxin efflux carriers, *PGP10* and *PIN5,* and one auxin influx carrier

LAX3 (Gonzalez et al., 2011; Marco et al., 2011a). Decrease in SPM in *spms-2* mutant leaves also downregulated *PIN1*, *PIN7* and *ABCB4* (Gonzalez et al., 2011). On the other hand, *ADC2* overexpression upregulated *ABCB4* by 4-fold in *Arabidopsis* leaves. However, it needs to be noted that *Arabidopsis* overexpressing *SpmSyn* had 3-fold increase in free SPM levels over the controls whereas the *spms-2* mutant, deficient in SPM, had 2-fold lower free SPM content than its WT (Gonzalez et al., 2011). Suppression of xylem vessel differentiation is also a unique characteristic of a structural isomer of SPM, thermoSPM (Kakehi et al., 2008; Muñiz et al., 2008; Takano et al., 2012). Higher SPM levels in *35S:AtSPMS-9* enhanced expression of *ACL5*, a thermoSPM-encoding gene, by more than 8-fold (Gonzalez et al., 2011). ThermoSPM and auxin act antagonistically to fine tune the temporal and spatial pattern of xylem differentiation (Yoshimoto et al., 2012). Increase in the expression of *ACL5* further explains the mechanism behind downregulation of auxin in *Arabidopsis* leaves. Increase in *ABCB4* in the PUT-accumulating line also adds to the hypothesis that functions of SPD and SPM are opposite to that of PUT (Handa and Mattoo, 2010; Mattoo et al., 2010a). In contrast to downregulation of auxin carriers in *Arabidopsis* leaves, transcript levels of *PIN1-type* proteins were upregulated by 2-fold in *E8:ySAMdc* tomato fruit (Kolotilin et al., 2011). It appears that the interaction of PAs with auxin in plant development may, in fact, be tissue specific.

Members of the *SAUR* family are plant-specific and constitute a major set of auxin-responsive genes (Wu et al., 2012a). In *35S:AtSPMS-9 Arabidopsis* leaves, most of the *SAUR* genes including *SAUR8*, *SAUR11*, *SAUR35*, *SAUR36*, *SAUR37*, *SAUR38*, *SAUR51*, and *SAUR56* were downregulated. In parallel, SPM deficiency in *spms-2* leaves downregulated *SAUR21* (Gonzalez et al., 2011). The *ySAMdc*-tomato fruit pericarp was twice richer than the controls in the transcripts for SAUR protein genes, *SAUR1*, *SAUR36* and solyc06g053290 (Kolotilin et al., 2011). *Arabidopsis* and tomato contain more than 72 and 98 *SAUR* genes, respectively. Although, some members in the SAUR family have been implicated in hypocotyl elongation during shade avoidance (Roig-Villanova et al., 2007), plant response to high temperature (Franklin et al., 2011), and cell expansion and tropic responses (Spartz et al., 2012), most *SAUR* genes have not yet been functionally

characterized, possible because *SAUR* mRNA and proteins are unstable, short-lived (Gil and Green, 1996; Knauss et al., 2003; Newman et al., 1993; Zenser et al., 2003), and do not bear similarity with any motif of known biochemical function (Spartz et al., 2012).

SPM inhibits expression of several auxin carriers, *Aux/IAA*, *ARF* and *SAUR* genes, in *Arabidopsis* (Gonzalez et al., 2011), whereas higher SPD and SPM levels enhance expression of some of the auxin-regulated genes in tomato fruit (Kolotilin et al., 2011). Higher PUT in *Arabidopsis* leaves upregulates *GH3.4*, *GH3.6* and *GH3.17* transcripts and over-accumulation of SPM upregulates *GH3.3* and *GH3.5*, and *IAMT1*, an IAA carboxyl methyltransferase that catalyzes the methylation of IAA (Li et al., 2008). In *E8:ySAMdc* tomato fruit, higher SPD/SPM also increased *GH3.8* and *IAA-AMINO ACID HYDROLASE 4* (*ILL4*) transcripts (Kolotilin et al., 2011). Further characterization of the role of PAs in regulating auxin function is needed to shed more light on the role of PAs in the auxin biology.

5.6 PA-Gibberellins crosstalk

Gibberellins (GAs) are tetracyclic, diterpenoid carboxylic acids that regulate many cellular processes in plants including seed dormancy breakdown, stem and root elongation, trichome development, leaf expansion, pollen maturation, flowering, sex expression, fruit setting, parthenocarpic fruit development, and fruit ripening (Itoh et al., 2008). As many as 136 GA compounds have been identified in plants, fungi, and bacteria. GA biosynthesis is regulated by various internal and external stimuli such as auxin, brassinosteroids, cytokinins, light, stratification, salinity, and cold (Hedden and Thomas, 2012). Terminal steps in GA biosynthesis are catalyzed by two key enzymes: GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) (Figure 5.4). These enzymes belong to the 2-oxoglutarate (2OG) and Fe(II)dependent oxygenase superfamily (Hedden and Thomas, 2012).

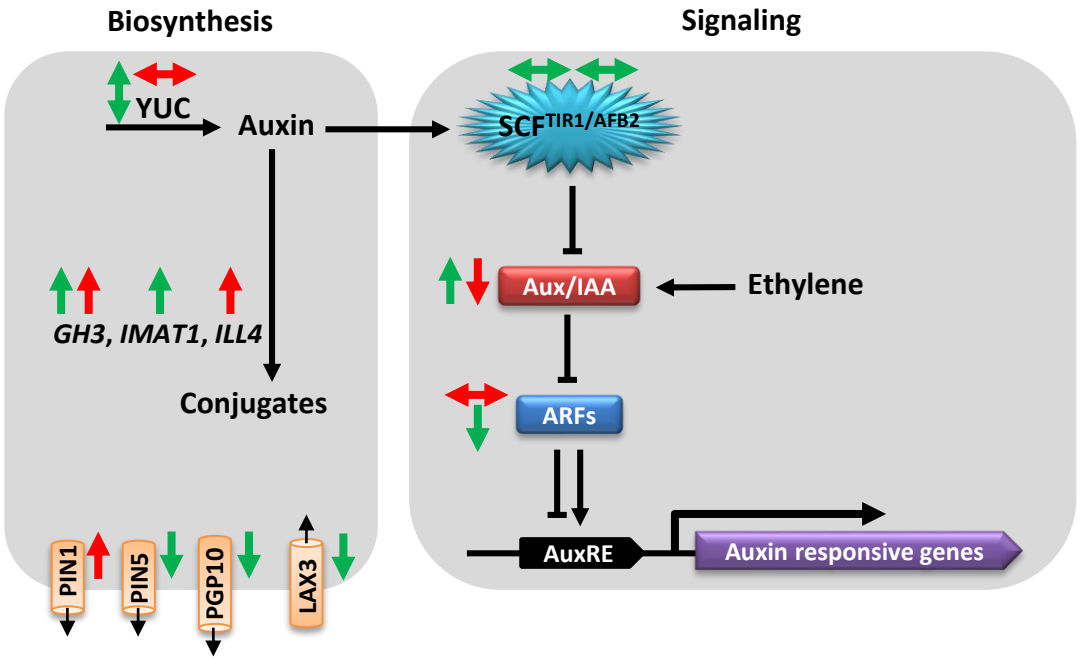

Figure 5.3: Consensus effects of SPD and SPM on auxin metabolism and signaling cascade.

Description is same as in legends to Figure 5.1. *AFB2*, auxin signaling F-box protein 2; *ARFs*, auxin response factors; *Aux/IAA*, indole-3-acetic acid inducible (members of Aux/IAA protein family); *AuxRE*, auxin response *cis*-elements; *GH3,* IAA-amido synthetase; *IAMT1,* IAA carboxyl methyl transferase 1; *ILL4,* IAA amidohydrolase; *LAX3,* like AUX1 3; *PGP10,* P-glycoprotein 10 (auxin efflux carrier); *PIN1,* PIN-formed 1; *PIN5,* PIN-formed 5; *TIR1,* transport inhibitor response 1; *YUC,* YUCCA.

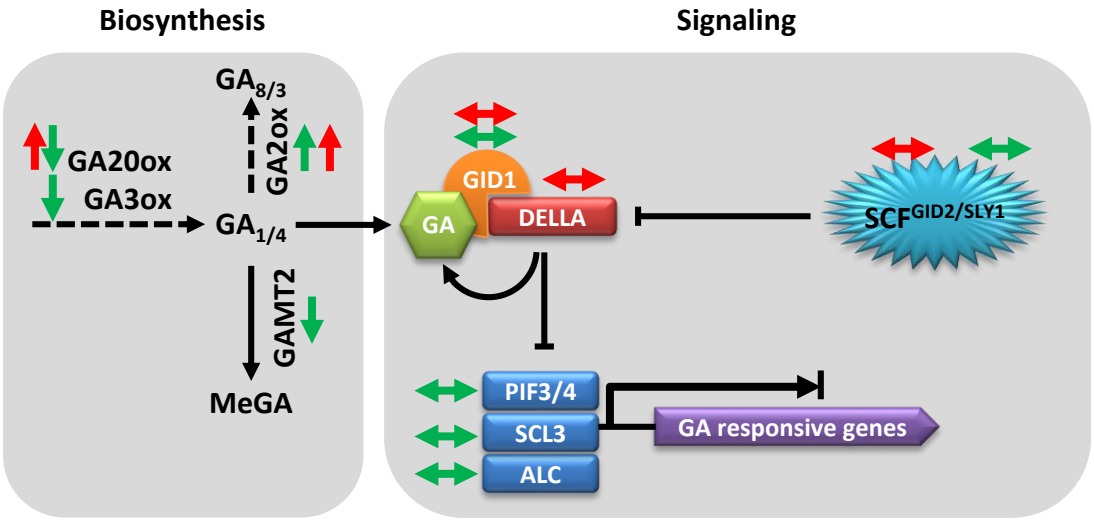

Figure 5.4: Consensus effects of SPD and SPM on gibberellin metabolism and signaling cascade.

Description is same as in legends to Figure 5.1. *ALC,* Alcatraz; *GA,* gibberellin; *GA2ox,* GA 2-oxidase; *GA20ox,* GA 20-oxidase; *GA3ox,* GA 3-oxidase; *GAMT2,* gibberellic acid methyltransferase 2; *GID1,* GA insensitive dwarf 1; *GID2,* gibberellin insensitive dwarf 2; *MeGA,* methyl ester form of GA; *PIF3,* phytochrome interacting factor 3; *PIF4,* phytochrome interacting factor 4; *SCL3,* scarecrow-like 3; *SLY1,* Sleepy 1.

The connection between PAs and GA arose serendipitously during characterization of transgenic *Arabidopsis* plants that overexpressed *35S:AtADC2* and accumulated high amounts of free and conjugated PUT (Alcazar et al., 2005). These transgenic plants were dwarf with delayed flowering and exhibited reduced expression of *GA3ox3* and *GA20ox1* as well as reduced production of GA_1 (Alcazar et al., 2005). The accumulation of SPM in *35S:AtSPMS-9 Arabidopsis* leaves also correlated with downregulation of *GA 20-oxidases* and *GA 3-oxidase* transcripts; in addition, GA catabolism was enhanced by upregulation of *GA 2-oxidases* transcripts (Gonzalez et al., 2011). Downregulation of *GIBBERELLIC ACID METHYLTRANSFERASE 2* (*GAMT2*), an enzyme that converts active GA_1 and GA_4 into its methyl ester forms, MeGAs, was observed in *35S:AtSPMS-9 Arabidopsis* leaves (Gonzalez et al., 2011). On the other hand, *E8:ySAMdc* tomato fruit was upregulated in the transcript levels for both the GA 20-oxidase and GA 2-oxidases during fruit ripening (Kolotilin et al., 2011), which suggests that SPD/SPM promote GAs conjugation and their conversion into inactive forms. Accumulation or deficiency of PUT or SPD/SPM has not thus far been found to alter expression of any GA singling gene in transgenic *Arabidopsis* leaves or tomato fruits (Figure 5.4).

5.7 PA-Cytokinin crosstalk

Another plant hormone with important function in growth and development as well as in environmental responses is a group called cytokinins (CKs). CKs are involved in processes such as seed development, tuber formation, shoot and meristem development, chloroplast biogenesis, vascular differentiation, leaf expansion, leaf senescence, nutrient balance, fruit set and growth and stress tolerance (Hwang et al., 2012; Kumar et al., 2014; Sakakibara et al., 2006). CKs and PAs mutually regulate several common physiological and developmental processes as tested by physiological and pharmacological approaches (Galston, 1983). The reports that both CKs and PAs generate nitric oxide (NO), an intra- and intercellular gaseous messenger involved in regulation of biotic and abiotic stress

responses, has attracted some attention (Wimalasekera and Scherer, 2009). Although CKs have been reported as potential inducers of NO, whether NO regulates the CKs signaling pathway is yet to be determined (Romanov et al., 2008). Similarly, SPD/SPM but not PUT induce production of NO (Moreau et al., 2010; Tun et al., 2006), but any potential links between PAs and NO are yet to be verified (Yamasaki and Cohen, 2006).

CKs enhance ADC activity and PUT accumulation in excised cucumber cotyledons in culture (Suresh et al., 1978), rice embryos (Choudhuri and Ghosh, 1982), and etiolated pea seedlings (Palavan et al., 1984). Treatment of etiolated cucumber cotyledons with kinetin increased PA oxidase (PAO) activity, decreased SAMdc activity along with a decrease in SPD levels, and increased PUT content (Sobieszczuk-Nowicka et al., 2007). CK treatment of lettuce cotyledons, dark-grown cucumber cotyledons (Walker et al., 1988), and soybean suspension cultures (Mader and Hanke, 1997) induced accumulation of free PUT, but not free SPD and SPM (Cho, 1983). However, higher PUT levels were not required for CK-induced greening of cucumber cotyledons (Walker et al., 1988). Earlier, PUT and CK were reported to act synergistically during embryogenesis, but increase in SPD and SPM levels seemed to play an important role in embryo development and plantlet formation in celery (Danin et al., 1993). CK enhanced free PUT and reduced SPD and SPM levels during expansion of excised cucumber cotyledons and gametophore bud formation in moss (Legocka and Zarnowska, 2002). Interestingly, deficiency of CKs does not affect PUT biosynthesis, but reduction in *trans*-zeatin, an active form of cytokinin, was accompanied by increases in free PUT, SPD, and SPM during and after germination of maple seedlings (Walker et al., 1989). In kinetin-treated moss, decrease in SPD was also accompanied by increase in PCA-insoluble levels of all three PAs (Legocka and Zarnowska, 2002).

The loss-of-function of *SAMdc4* in *bud2-2 Arabidopsis* mutant led to an increase in PUT (11.5%) with a corresponding decrease in SPD (9.3%) and SPM (13.3%) and exhibited altered root and shoot architecture due to hyposensitivity to auxin but hypersensitivity to cytokinin (Cui et al., 2010). In another study, exogenous SPM prevented expression of *ARR5* in *Arabidopsis* (Romanov et al., 2002). However, no change

in transcript levels of CK biosynthetic genes was found in *Arabidopsis* leaves expressing either *35S:AtADC2*, *35S:AtSPMS-9* or *35S:AtSAMdc1* or in tomato fruits expressing *E8:ySAMdc* (Alcazar et al., 2005; Gonzalez et al., 2011; Kolotilin et al., 2011; Marco et al., 2011a).

CK dehydrogenase (CKX, previously known as cytokinin oxidase) cleaves the CK side chain, producing aldehydes and adenine derivatives. The transcripts of CKX genes were found elevated for *CKX1* and *CKX6* in *35S:AtSPMS-9*, for *CKX6* in *35S:AtSAMdc1 Arabidopsis* leaves (Gonzalez et al., 2011; Marco et al., 2011a), and for *CKX2* in *E8:ySAMdc* tomatoes (Kolotilin et al., 2011), suggesting roles for PAs in CK degradation and homeostasis. Zeatin-*O*-glucosyltransferase, which glucosylates *trans*-zeatin into *trans*-zeatin-*O*-glucoside, was downregulated in *E8:ySAMdc* tomatoes (Kolotilin et al., 2011). In contrast, *CKX* transcripts remained unaffected in *35S:AtADC2 Arabidopsis* plants, suggesting that PUT may have a limited role in CK biosynthesis (Alcazar et al., 2005).

The canonical CK signaling cascade in *Arabidopsis* includes three histidine kinase receptors: AHK2, AHK3, and AHK4/CRE1/WOL (Hwang et al., 2012) (Figure 5.5). CK triggers autophosphorylation of conserved histidine residue in cytoplasmic kinase domain of AHKs. Histidine-containing phosphotransfer proteins (AHPs) accept a phosphoryl group via aspartic acid residue on the AHK receiver domain and transfer it to an aspartic acid residue on the N-terminal receiver domain of type-B response regulators (RRs). Phosphorylation of type-B RRs releases repression of their C-terminal DNA-binding domain and activates type-A RRs CK response factors (CRFs) and other response genes. As a negative feedback regulation, type-A RRs negatively regulate phosphorelay circuitry (Argueso et al., 2010; Hwang et al., 2012). In the absence of CK, AHK4 dephosphorylates AHPs to inhibit phosphorelay in signaling cascade (Mähönen et al., 2006). F-box KISS ME DEADLY (KMD) family proteins negatively interact with type-B RRs and target them for SCF/proteasome-mediated degradation (Kim et al., 2013; Kim et al., 2012).

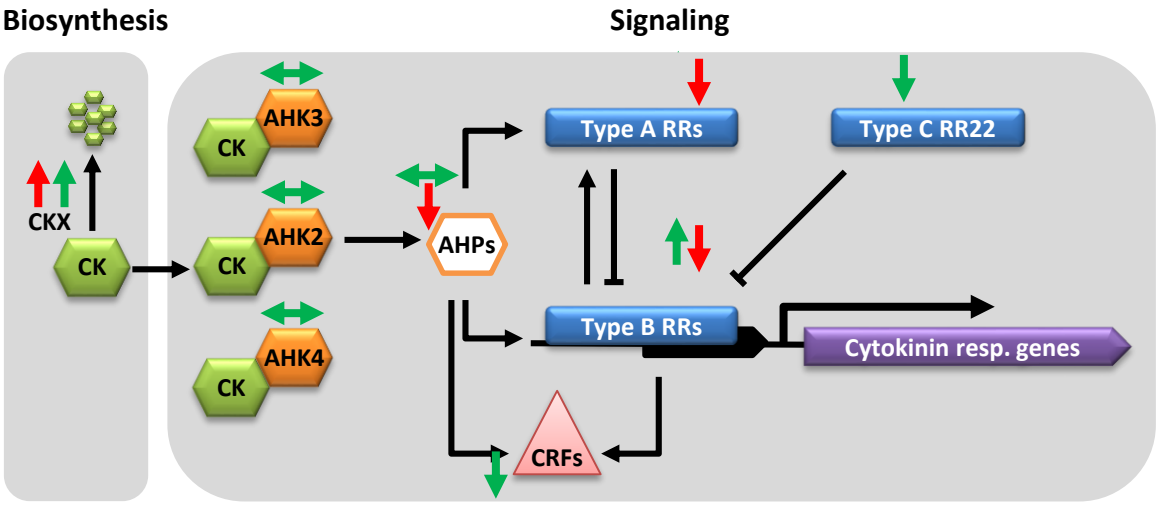

Figure 5.5: Consensus effects of SPD and SPM on cytokinin metabolism and signaling cascade.

Description is same as in legends to Figure 5.1. *AHK2,* histidine kinase receptor 2; *AHK3,* histidine kinase receptor 3; *AHK4,* histidine kinase receptor 4; *AHPs,* histidine-containing phosphotransfer protein; *RRs,* response regulators; *CK,* cytokinin; *CKX,* cytokinin dehydrogenase; *CRFs,* cytokinin response (*resp.*) factors.

How do transgenic plants engineered for PA levels fare in terms of the CK signaling cascade just described? In *E8:ySAMdc* tomato fruits, response regulators *AHP1* protein, type-A (*TRR3*) and type-B (*RR8*), were downregulated, supporting an antagonistic effect of higher PAs on CK signaling (Kolotilin et al., 2011). On the other hand, *35S:AtSPMS-9 Arabidopsis* leaves were downregulated for *CRF8* transcripts but type-B (*ARR1*) and type-A response regulators (*ARR3*, *5* and *7*) were upregulated (Gonzalez et al., 2011). A type-C response regulator *ARR22* was downregulated in SPM-accumulating transgenic *Arabidopsis* leaves. Further confirmation of these results would help establish if the SPM and SPD are involved in CK signaling pathway. At present it seems that CKs favor PUT biosynthesis and inhibit SPD and SPM accumulation by stimulating ADC and PAO activities and also possibly by increasing their conjugation. Also, PUT does not appear to affect CK biosynthesis or signaling whereas SPD and SPM do so by stimulating the catabolism of CK by upregulating CK dehydrogenases. However, SPD inhibits while SPM promotes expression of genes involved in CK signaling.

5.8 PA-Abscisic acid crosstalk

ABA, a sesquiterpenoid, is another plant hormone involved in the development and stress responses of plants, which include seed germination, lateral root formation, leaf and fruit size development, and stomatal closure in response to drought stress (Kanno et al., 2010; Nitsch et al., 2012; Raghavendra et al., 2010). ABA and ethylene synergistically regulate fruit ripening (Kumar et al., 2014; Lohani et al., 2004; McAtee et al., 2013; Sun et al., 2012a; Zaharah et al., 2013; Zhang et al., 2009b). The first committed, rate-limiting step in ABA biosynthesis is the cleavage of plastid-localized 9-*cis*-violaxanthin and 9-*cis*-neoxanthin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin (Handa et al., 2014 and references therein). Short-chain dehydrogenase reductase catalyzes the conversion of xanthoxin into abscisic aldehyde, which is converted into ABA by ABA aldehyde oxidase (AAO) (Figure 1.2). ABA is catabolized by ABA-8'-hydroxylases

into 8'-OH-ABA, followed by isomerization to phaseic acid. Additionally, glucosyltransferase glucosylates ABA to its storage and transport form.

Changes in ABA and PA levels have been studied during seed maturation and germination, and fruit growth and ripening, as well as in response to low-temperature, water-stressed and UV-B conditions to understand interactions between them (Antolín et al., 2008; Bagni et al., 1980; Cvikrová et al., 1998; Fromm, 1997; Gomez-Jimenez et al., 2001; Hurng et al., 1994; Lee et al., 1995b; Martinez-Madrid et al., 2002; Martinez-Madrid et al., 1996; Nayyar et al., 2005; Puga-Hermida et al., 2003; Rakitin et al., 2008; Serrano et al., 1995; Valero et al., 1998; Yokota et al., 1994; Yoshikawa et al., 2007). Under water stress conditions, transcript levels of *ADC*, *SpdSyn* and *SpmSyn* were upregulated in *Arabidopsis* and impaired in ABA-insensitive (*abi1-1*) and ABA-deficient (*aba2-3*) *Arabidopsis* mutants (Alcazar et al., 2006a). *ADC2* transcripts and activity were reduced in *NCED3* knockout *Arabidopsis* mutant *nc3-2* (Urano et al., 2009). ABA treatment stimulated expression of *SAM1* and *SAM3*, encoding *S*-adenosyl-L-methionine synthetase, in tomato (Espartero et al., 1994), *SPDS3* transcripts in *Arabidopsis* (Hanzawa et al., 2002), and enhanced PUT and SPM, but not SPD, levels in drought-tolerant cv. *Populus popularis* compared to drought-susceptible cv. Italica (Chen et al., 2002). ABA and stressors such as high salt and dehydration upregulated expression of *AtADC2* leading to accumulation of PUT, a decrease in SPD, and unaltered SPM levels (Urano et al., 2003). The chilling-tolerant rice seedlings had increased PUT but not SPD and SPM levels upon chilling (Lee et al., 1997). Thus, both ABA and PUT have been implicated in reducing plant damage from low-temperature stress in tomato plants (Jiang et al., 2012). In wheat, the ABA treatment enhanced PUT and SPM but decreased SPD (Kovacs et al., 2010; Rakitin et al., 2009). In leaf discs from drought-tolerant grapevine, ABA enhanced expression of PA synthesizing (*ADC*, *ODC* and *SAMdc*) as well as catabolizing enzymes (DAO and PAO) resulting in a net 2- to 4-fold accumulation of PUT and SPM but reduction in SPD levels, whereas leaf discs from drought-susceptible grapevine were higher in only DAO and PAO transcripts (Toumi et al., 2010). Presence of ABA-response elements (ABREs) in promoter

regions of *ADC2*, *SAMdc1*, *SAMdc2*, *SPDS1* and *SpmSyn* in *Arabidopsis* suggest that ABA-response elements may regulate PA biosynthesis (Alcazar et al., 2006b).

Reciprocal complementation tests on *adc* (PUT-deficient) and *aba2-3* (ABA-defective) mutants suggested that PUT positively regulates expression of *NCED3* in response to cold stress (Cuevas et al., 2008; Cuevas et al., 2009). High PUT level in transgenic *35S:AtADC2 Arabidopsis* downregulated the CYP707A gene that encodes ABA-catabolizing enzyme ABA-8'-hydroxylase (Alcazar et al., 2005). Together, these add to findings mentioned earlier that PUT and ABA positively regulate each other's biosynthesis under abiotic stress (Alcazar et al., 2010). However, SPD and SPM act antagonistically to PUT and inhibit ABA synthesis and induce its hydroxylation. For example, SPM reduced polyethylene glycol (PEG)-induced osmotic stress by modifying ABA and antioxidant levels in soybean pods and seeds (Radhakrishnan and Lee, 2013a, b); SPM accumulation in *35S:AtSPM-9* and *35S:AtSAMdc1 Arabidopsis* leaves inhibited expression of *ALDEDHYE OXIDASE* (*AAO2*); and enhanced levels of SPD and SPM in transgenic *E8:ySAMdc* tomato fruits, decreased *AAO4* transcripts and increased ABA 8-hydroxylase 3 transcripts. Application of SPD did not alter free or bound titers of ABA in radish seedlings under normal growth condition (Choudhary et al., 2012b) or under chromium stress (Choudhary et al., 2012a), but reduced copper-induced accumulation of bound ABA in radish seedlings (Choudhary et al., 2012b).

The ABA signaling network is well characterized and has been elegantly described elsewhere (Cutler et al., 2010; Raghavendra et al., 2010; Umezawa et al., 2010; Weiner et al., 2010). ABA binds to cytosol- and nucleus-localized PYRABACTIN RESISTANCE (PYR) / PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family of ABA receptors, causing inhibition of protein phosphatase 2Cs (PP2Cs), the negative regulators of ABA signaling. Upon release from PP2C-mediated repression, the SNF1-related kinase 2 (SnRK2) phosphorylates bZIP-like transcription factors including ABA-INSENSITIVE 5 (ABI5) and ABA-RESPONSIVE ELEMENT BINDING FACTOR 2 (ABF2/AREB1) and ABF4/AREB2 (Shukla and Mattoo, 2008). After phosphorylation these factors bind with *cis*-regulatory ABA-response elements (ABRE, ACGTGT) and regulate expression of abiotic

stress-responsive genes (Figure 5.6). SnRK2 also phosphorylates and regulates activity of other target proteins including anion channel SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1), inward rectifying K^+-CHANNEL IN *ARABIDOPSIS* THALIANA (KAT1), and reactive oxygen species (ROS)-producing RESPIRATORY BURST OXIDASE HOMOLOG F (RbohF). The constitutive expression of *NtPYL4* receptor resulted in a 2.6-fold increase in total PAs in tobacco hairy roots compared to control roots (Lackman et al., 2011), but higher SPM levels in *35S:AtSPMS-9 Arabidopsis* leaves did not show any specific trend in regulation of *PYL* genes (Gonzalez et al., 2011). Accumulation of higher PAs in *35S:AtSPMS-9 Arabidopsis* leaves or *E8:ySAMdc* tomato fruits resulted in 2- to 3-fold increase in transcript levels of *PP2C* proteins, suggesting that higher PAs inhibit both ABA accumulation and ABA signaling by altering expression of PP2C proteins (Gonzalez et al., 2011; Kolotilin et al., 2011). PUT was shown to inhibit ABA degradation but ABA signaling genes in *35S:AtADC2 Arabidopsis* leaves were not altered, suggesting that PUT may not play a major role in ABA signaling (Alcazar et al., 2005).

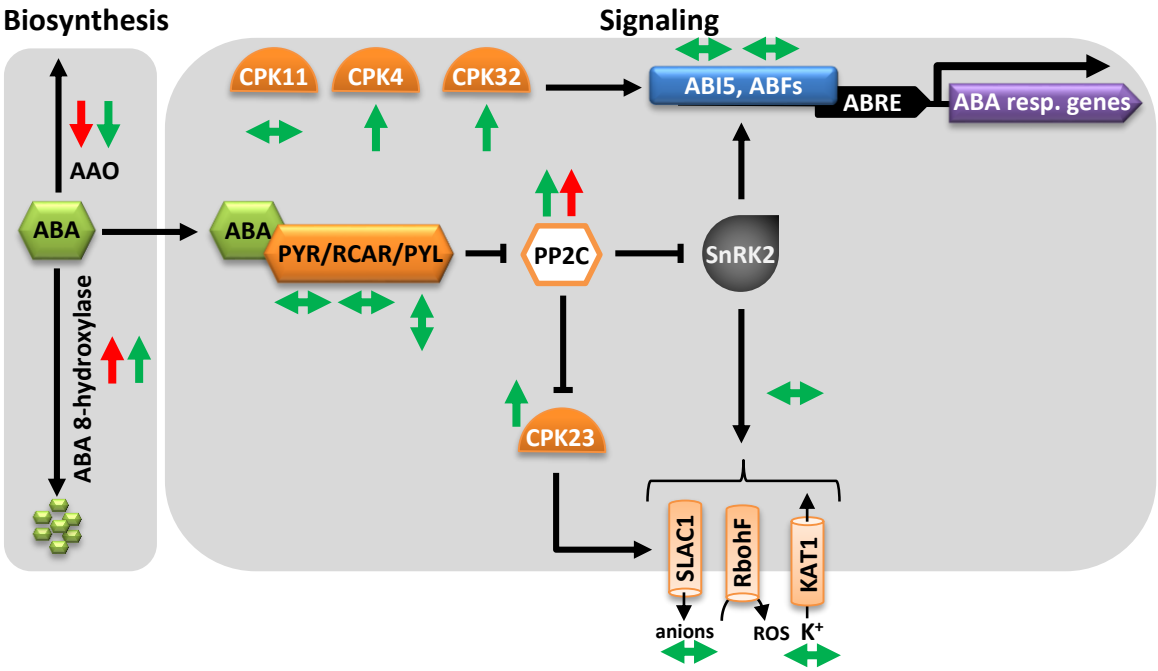

Figure 5.6: Consensus effects of SPD and SPM on ABA metabolism and signaling cascade.

Description is same as in legends to Figure 5.1. *AAO,* abscisic acid aldehyde oxidase; ABA, abscisic acid; *ABFs,* ABA responsive element-binding factors; *ABI5,* ABA insensitive 5; *ABRE,* ABA response element; *CPK4,* calcium-dependent protein kinase 4; *CPK11,* calcium-dependent protein kinase 11; *CPK23,* calcium-dependent protein kinase 23; *CPK32,* calcium-dependent protein kinase 32; *KAT1,* K+ channel in *Arabidopsis thaliana*; *PP2C,* protein phosphatase 2C; *PYL,* PYR1-like; *PYR,* pyrabactin resistance; *RCAR,* regulatory component of ABA receptor; *RbohF,* ROS-producing respiratory burst oxidase homologue F; *SLAC1,* slow anion channel-associated 1; *SnRK2,* SNF1-related kinase 2.

5.9 PA-Salicylic Acid crosstalk

SA is an immunity signaling molecule in plant response to pathogens (Rojo et al., 2003; van Wees et al., 2000), low temperature (Lei et al., 2010; Zhang et al., 2013), high temperature (Kaplan et al., 2004; Widiastuti et al., 2013), high salinity (Jayakannan et al., 2013; Mutlu and Atici, 2013; Singh and Gautam, 2013; Tufail et al., 2013), heavy metals (Idrees et al., 2013; Mostofa and Fujita, 2013; Pandey et al., 2013), and water deficit (de Agostini et al., 2013; Marcinska et al., 2013). SA also delays ripening by inhibiting ethylene biosynthesis (Fan et al., 1996; Li et al., 1992). SA biosynthesis is derived via the shikimate pathway intermediate chorismate, which is ultimately converted into SA via either the isochorismate pathway or phenylalanine pathway. However, under stress conditions, plants synthesize SA mainly from the chloroplast-localized isochorismate pathway (Dempsey et al., 2011). Several enzymes in plants have been characterized that catalyze modification of SA through glycosylation, methylation, amino acid conjugation, or hydroxylation (Dempsey et al., 2011; Garcion and Métraux, 2007; Lee et al., 1995a; Sendon et al., 2011; Verpoorte and Memelink, 2002).

Exogenous application of SA was shown to enhance accumulation of free PUT, SPD, and SPM in *Arabidopsis*, maize leaves, tomato fruits, bamboo shoots, asparagus, citrus, and callus cultures of carrots (Luo et al., 2012; Sudha and Ravishankar, 2003; Wei et al., 2011; Zhang et al., 2011; Zheng and Zhang, 2004). SPD also enhanced accumulation of 2-*O*-β-D-glucosyl salicylic acid, a conjugated form of SA in TMV-resistant tobacco plants (Lazzarato et al., 2009). Transgenic *Arabidopsis* plants (*35S:AtSPMS-9*, *35S:AtSAMdc1*) or tomato fruits (*E8:ySAMdc*) with enhanced SPD/SPM levels showed about 2-fold or higher increase in transcript levels of SAM-dependent carboxyl methyltransferase (*SAMT*), an enzyme that catalyzes methylation of SA into methyl salicylate (MeSA). The *35S:AtSPMS-9 Arabidopsis* leaves had higher transcripts of GH3-like phytohormone amino acid synthetase (*GH3.5*), an enzyme that catalyzes conjugation of SA to aspartic acid, producing salicyloyl-*L*-aspartic acid (SA-Asp). The tobacco leaf disc specifically responded to SPM by enhancing SALICYLIC ACID-INDUCED PROTEIN KINASE (SIPK, an orthologue of

MPK6) and WOUND-INDUCED PROTEIN KINASE (WIPK) via mitochondrial dysfunction (Mitsuya et al., 2009; Takahashi et al., 2003). The SIPK, WIPK, and their homologuess have been implicated in defense signaling against osmotic stress (Droillard et al., 2000; Hoyos and Zhang, 2000; Ichimura et al., 2000; Mikołajczyk et al., 2000), low temperature (Ichimura et al., 2000; Jonak et al., 1996), ozone treatment (Samuel et al., 2000), wounding (Bogre et al., 1997; Ichimura et al., 2000), and pathogen invasion (Cardinale et al., 2000; Desikan et al., 2001; Ligterink et al., 1997; Nühse et al., 2000; Zhang and Klessig, 1998). Collectively, these results indicate mutual positive regulation of SA conjugates and PAs biosynthesis.

NON-EXPRESSOR OF PATHOGENESIS-RELATED GENE 1 (NPR1) is an ankryin-repeat-containing protein that interacts with TGA transcription factors to promote transcription of SA-responsive defense genes (Wu et al., 2012c). NPR3 and NPR4, paralogues of NPR1, are CUL3 ligase adaptors and function as SA receptors (Fu et al., 2012). Under physiological conditions, NPR3 and NP4 stimulate NPR1 degradation to inhibit SA signal transduction (Figure 5.7). Pathogen infection induces SA binding with NPR3 and NPR4 in a concentration-dependent manner and releases NPR1 from repression (Kaltdorf and Naseem, 2013). SPM-accumulating *Arabidopsis* plants (*35S:AtSPMS-9*) had 2-fold higher levels of both *NPR3* and *NPR4,* suggesting that SPM inhibits SA signaling by enhancing degradation of NPR1 by CUL3-NPR3 and CUL3-NPR4. Although both SA and SPM have been reported to enhance plants' tolerance against pathogens (Raju et al., 2009), their crosstalk during plant–pathogen interactions remains to be determined.

Spd-induced response against infection seems to be mediated by SA (Lazzarato et al., 2009), whereas a response elicited by SPM seems independent of SA signaling pathway (Hiraga et al., 2000; Mitsuya et al., 2007; Takahashi et al., 2003; Takahashi et al., 2004; Uehara et al., 2005; Yamakawa et al., 1998). PUT may not regulate SA biosynthesis or signaling, as was ascertained by studies showing that PUT-accumulating *Arabidopsis* plants (*35S:AtADC2*) did not show any differential expression in SA biosynthetic or signaling genes.

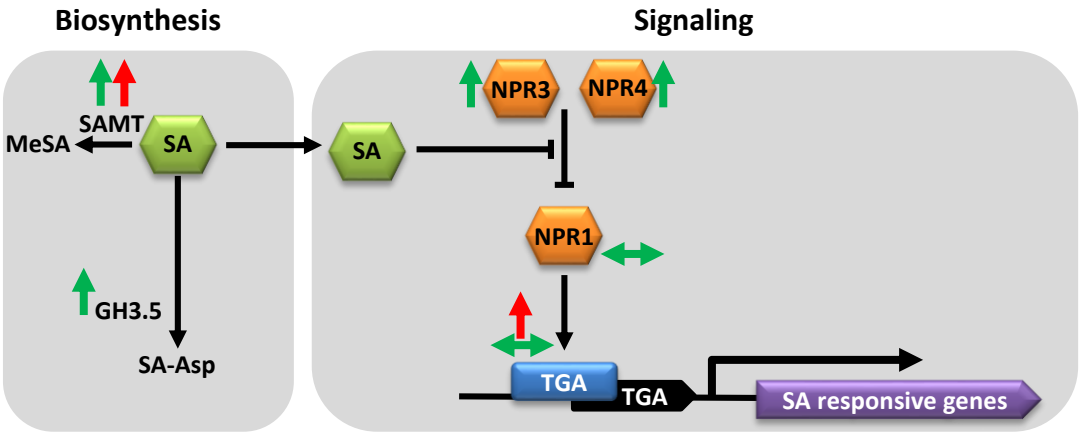

Figure 5.7: Consensus effects of SPD and SPM on SA metabolism and signaling cascade.

Description is same as in legends to Figure 5.1. *GH3.5,* GH3-like phytohormone amino acid synthetase; *MeSA,* methyl salicylate; *NPR1*, non-expressor of pathogenesis-related gene 1; *NPR3,* NPR1-like protein 3; *NPR4,* NPR1-like protein 4; SA, salicylic acid; SA-Asp, Salicyloyl-l-aspartic acid; *SAMT,* SAM-dependent carboxyl methyltransferase; *TGA,* TGA transcription factor.

5.10 PA-Brassinosteroid crosstalk

Brassinosteroids (BRs) are C27, C28, and C29 steroidal hormones that, in recent years, have been associated with plant cell elongation, vascular differentiation, root growth, responses to light, resistance to stresses, and senescence (Clouse and Sasse, 1998; Kim and Wang, 2010). Their biosynthetic pathways were first elucidated using cultured *Catharanthus roseus* cells, but identification of genes and enzymatic characterization have been intensively investigated using the *dwarf* mutants defective in BR biosynthesis or perception (Fujioka and Yokota, 2003). More than 50 naturally occurring BRs have been identified. BRs are derived from membrane-associated sterol campesterol through multiple C-6 oxidation steps whereas campesterol is synthesized mainly from isopentenyl disphosphate (Bajguz, 2012; Choudhary et al., 2012c; Fujioka and Yokota, 2003; Shimada et al., 2001). BRs have been shown to play essential roles in a wide range of physiological and developmental processes and now considered as plant hormones (Kim and Wang, 2010). Exogenous applications of PAs or 24-epibrassinolide (EBL, an active form of BRs) have been implicated in enhancing stress tolerance against drought, chilling, and salt stresses in rice, tomato, and *Adiantum capillus-veneris* plants, respectively (Farooq et al., 2010; Jiang et al., 2012; Sharma et al., 2014). EBL enhanced accumulation of PUT but inhibited production of higher PAs (SPD/SPM) in radish seedling with or without Cu or Cr stress, and supplementing EBL with PUT further enhanced accumulation of PUT and SPD (Choudhary et al., 2011). However, enhanced PAs in *Arabidopsis* plants (*35S:AtADC2*, *35S:AtSAMdc1*, *35S:AtSPMS-9*) and tomato fruits (*E8:ySAMdc*) were not found to have corresponding changes in any of the known BR biosynthesis gene transcripts.

The brassinosteroid signal transduction pathway is given in Figure 5.8. In *Arabidopsis*, BR binds with BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor and induces its auto-phosphorylation and dissociation from BRI1 KINASE INHIBITOR (BKI1). Active BRI1 forms a heterodimer with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and phosphorylates BR-SIGNALING KINASE 1 (BSK1) and CONSTITUTIVE DIFFERENTIAL

GROWTH 1 (CDG1) that, in turn, phosphorylates BRI1-SUPPRESSOR 1 (BSU1). BSU1 deactivates BRASSINOSTEROID INSENSITIVE 2 (BIN2) through dephosphorylation, releasing BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS SUPPRESSOR 1 (BES1, also named BZR2) from repression. BZR1 and BES1 then regulate transcription of BRs-responsive gene either directly or through transcription factors. BZR1 and BES1 are also activated by PROTEIN PHOSPHATASE 2A (PP2A), which is itself negatively regulated by SUPPRESSOR OF BR1 (SBI1) via methylation (Choudhary et al., 2012c; He et al., 2005; Kim and Wang, 2010; Zhu et al., 2013). Transgenic *Arabidopsis* plants deficient in SPM (*spms-2*) showed 1.6-fold upregulation of BSU1 transcripts whereas tomato fruits with higher SPD and SPM showed 2.9-fold upregulation of BES1/BZR1 homologue protein 2 (Solyc02g063010) at B stage and then 2.5-fold decrease thereafter at R stage. Transcript levels of genes encoding BR signal transduction proteins remained unaltered in PA-accumulating transgenic *Arabidopsis* plants (*35S:AtADC2*, *35S:AtSAMdc1*, *35S:AtSPMS-9*). More studies are needed for deeper insight in our understanding of the role(s) of PAs in BR biosynthesis and/or signaling.

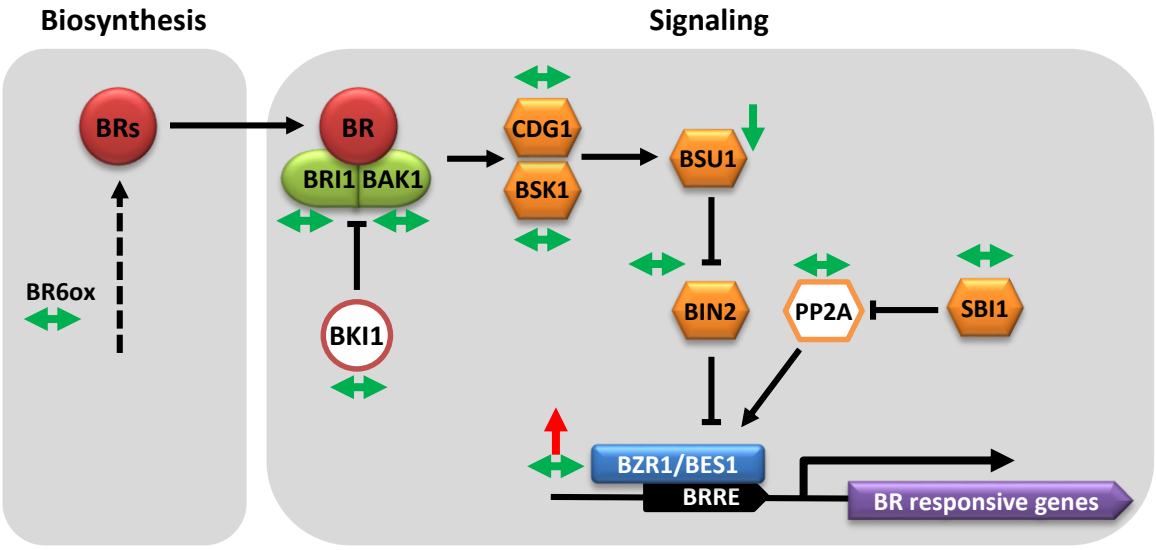

Figure 5.8: Consensus effects of SPD and SPM on brassinosteroid metabolism and signaling cascade.

Description is same as in legends to Figure 5.1. *BAK1,* BRI1-associated receptor kinase 1; *BES1,* BRI1-EMS suppressor 1/BZR2; *BIN2,* brassinosteroid insensitive 2; *BKI1,* BRI1 kinase inhibitor; *BR,* brassinosteroid; *BR6ox,* BR-6-oxidase; *BRI1,* brassinosteroid insensitive 1; *BRRE,* brassinosteroid response element; *BSK1,* BR-signaling kinase 1; *BSU1,* BRI1-suppressor 1; *BZR1,* brassinazol resistant 1; *CDG1,* constitutive differential growth 1; *PP2A,* protein phosphatase 2A; *SBI1,* suppressor of BR1.

5.11 Concluding remarks and perspective

Plants are sessile organisms that have acquired the ability to adapt to a continuously changing environment. A wealth of information has emerged during the twentieth century showing that plants accomplish growth and propagation of their species by activating specific signaling pathways which allow them to survive under optimal to harsh environmental conditions. Perception of the environmental clues is largely controlled by a few plant hormones: auxin, cytokinin, gibberellins, ABA, ethylene, SA, brassinosteroids, and jasmonates. However, it is now recognized that the plant response to environmental conditions is complex and orchestrated by a network of the equally complex crosstalk among plant hormones (Garay-Arroyo et al., 2012). PAs are emerging as important plant growth and development regulators (Handa and Mattoo, 2010). They play essential roles in both physiological and developmental processes (Nambeesan et al., 2008). We recently proposed that PAs act as 'rejuvenator molecules' and restore to an aging cell the metabolism of the younger cell in plants (Handa and Mattoo, 2010; Mattoo et al., 2010a). SPD extends the shelf life of tomato fruit and retards seasonal senescence of plants (Nambeesan et al., 2010) is an observation in tune with a similar role proposed for SPD in other organisms (Eisenberg et al., 2009). Although many plant cellular processes are also regulated by one or another plant hormone, little is understood about crosstalk among plant hormones and biogenic amines. As collated here, the transcriptomic data suggest a complex relationship among the three PAs, and their role(s) in the biosynthesis and signaling pathways of plant hormones. However, these analyses only provide an initial evaluation of the interactions among PAs and other plant hormones, based on a limited number of genes and plant systems analyzed. Our models provide a simplistic road map that can be further modified and revised as more transcriptome, proteome, and metabolome data become available.

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