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In silico Analysis of Polyamine Rich Transgenic Tomato Fruit Transcriptome for Salicylic Acid Biosynthesis

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EFFECT of higher levels of polyamines (PAs), spermidine (SPD) and spermine (SPM), were evaluated on the salicylic acid biosynthesis genes. It has been previously reported that higher steady state levels of a pathogenesis protein pR1b1, a salicylic acid (SA) regulated protein, are present in tomato fruits with higher levels of anabolic biogenic amines, spermidine (SPD) and spermine (SPM) resulting from the expression of yeast ***S-adenosylmethionine decarboxylase*** (*SAMDC*), under a fruit specific E8 promoter in ripening tomato fruit. Based on this observation, we hypothesized that high SPD/SPM fruit would enhance the expression of SA-biosynthesis genes leading to enhanced SA production. In plants, SA is synthesized either through *phenylalanine ammonia-lyase* (*PAL*) or *isochorismate synthase* (ICS) pathway. We identified ten putative genes for the SA biosynthesis pathways in tomato and determined the relative abundance of their transcript based on RNAseq transcriptomic analyses of mature green and turning stage tomato fruits from WT and two isogenic independent SAMDC-transgenic homozygous lines with higher levels of SPD/SPM in ripening fruits. We show that at MG stage the transgenic fruits exhibited higher steady state levels of transcripts of *CS*, *PAL1-7, ICS, IPL/PRXR1, CS,* and *PAL 4-10,* but lower levels for *CM1*, *CM2, EPS1,* and *PBS3-2*. At the turning stage of tomato fruit ripening the steady state levels of only *CS* and *PBS3-2* were upregulated whereas the transcript levels of *CM1*, *CM2*, *PaL1-4AAO*, *PBS3-1*and *EPS1* were down regulated. Taken together these results suggest that SPD/SPM play role in the SA biosynthesis and higher levels of various genes especially in in PAL pathway likely increased the SA levels in the transgenic fruits.

Keywords: IC, PAL, Polyamines, RNASeq, Salicylic acid Biosynthesis, *Solanum lycopersicum* L.

Introduction

Salicylic acid (SA) is a signaling molecule that plays a significant role in both local and systemic acquired resistance (SAR) to both biotrophic and hemi-biotrophic pathogens by inducing a hypersensitive response in plant pathogens (Raskin et al., 1992). This SA response in plants acts as a secondary messenger and activates pathogen resistance (Wildermuth et al., 2001). Exogenous SA treatment has been shown to activate expression of pathogenesis-related (PR) genes in several plants and disease resistance (van Verk et al., 2011; Tuna et al., 2007). Furthermore, a crosstalk between SA and many other plant hormones methyl jasmonate and ethylene has been reported (van Verk et al., 2011). Goyal et al. (2016) have reported to cause an accumulation of a salicylic acid (SA) regulated PRIb1, a

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pathogenesis related protein, in transgenic tomato with enhanced levels of SPD/SPM in fruits. These authors have further shown PR1b1 increase was not associated with production of ethylene or methyl jasmonate. However, the levels of SA were high in transgenic fruits with high SPD/SPM and these authors suggested that PAs increased levels of SA that in turn increased expression of PR1b1 protein.

Often, salicylic acid is implicated in the plant response and adaptation to several abiotic stresses including salt, drought heavy metals (Metwally et al., 2003; Faraz et al., 2020). Exogenous application of SA increases activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), which are reported to be the major components of plant defense against both biotic and abiotic stresses. Also, SA has been shown to impact plant growth, reproduction, and bio-productivity including plant size increase, number of flowers, and leaf area (Najafabadi et al., 2013).

Tomato (*Solanum lycopersicum* L.) has spread globally and became agronomically number two significant horticulture crops after potato (Knapp, 2002; Daunay et al., 2008; Gebhardt, 2016). Tomato is the only domesticated species from 12 supplementary wild lineages included in section *Lycopersicum*; it is characterized with their shiny yellow flowers; pinnatifid or pinnate and non-spiny leaves (Peralta et al., 2006; Bai & Lindhout, 2007; Knapp & Peralta, 2016). Efforts for improvement and conservation of *S. lycopersicum* variation are vital to integrate valuable traits, such as fruit flavor, nutritive value, shelf life and tolerance to biotic and abiotic stresses (Klee & Tieman, 2013; Wang et al., 2017).

SA biosynthesis is regulated in plants through either isochorismate (IC) or phenylalanine ammonia-lyase (PAL) pathways with chorismate being necessary for both pathways (van Verk et al., 2011, Dempsey & Klessig, 2017). PAL changes phenylalanine (Phe) to transcinnamic acid (TCA) in the PAL pathway. TCA is converted to SA through intermediates, depending on the plant species; ortho-coumaric acid or benzoic acid (BA). BA may be converted to SA via BA2- hydroxylase. Several plant species have recognized genes encoding *isochorismate synthase (ICS*), which transforms chorismate to isochorismate (Barry & Giovannoni, 2007). Following its synthesis, *A. thaliana ICS1* gene product is exported to the chloroplast stroma, where SA synthesis takes place (Dempsey & Klessig, 2017). On the same context of IC pathway, no plant gene identical to bacterial isochorismate pyruvate lyase (IPL), which converts IC to SA and pyruvate, has been identified. However, Zhou et al. (2018), have identified a putative peroxidase enzyme, named PRXR1, that converts IC to SA, thus has been considered a potential IPL. Torrens-Spence et al., 2019 are addressing that plants convert IC to SA. PBS3 and EPS1 are two enzymes that were found to complete SA biosynthesis from isochorismate in *Arabidopsis* through a metabolic pathway diverse from that of bacteria.

Polyamines (PAs), including putrescine (Put), spermidine (Spd), and spermine (Spm), are low molecular weight organic polycations present in all living organisms and are involved in many metabolic processes in plants, including morphogenesis, cell division, proliferation, organogenesis, and programmed cell death (PCD) to defend against many stresses that face plants (Kaur-Sawhney & Galston, 2003; Cassol & Mattoo, 2003; Casero & Marton, 2007; Nambeesan et al., 2008; Handa & Matto, 2010; Handa et al., 2011, 2014). They are essential for all cells and have been considered as endogenous growth regulators (Galston & Kaur- Sawhney, 1995; Srivastava & Handa, 2005). PAs have also been shown to prolong the shelf- life of tomato fruit (Mehta et al., 2002; Nambeesan et al., 2010) and to increase longevity in several organisms, including tomato fruit (Eisenberg et al., 2009; Handa et al., 2018). They also engage in transcriptional as well as translational gene regulation (Veress et al., 2000; Kasukabe et al., 2004; Yoshida et al., 2004; Alcázar et al., 2006; Igarashi & Kashiwagi, 2006; Mattoo & Handa, 2008; Nambeesan et al., 2008; Handa & Mattoo, 2010; Handa et al., 2018). Leaf and fruit senescence are associated with a fall in rates of PAs in many plant systems. The main catabolism of PAs is polyamine oxidation, which results in the production of H_2O_2 which induces the activity of antioxidant enzymes which helps plants to tolerate abiotic stress (Szepesi et al., 2011).

Shelf life of fruit is normally determined by the rate of change of this metabolic activity leading to fruit softening. Earlier investigations have shown the advantage of PAs enriched tomato homozygous independent genotypes (556HO

and 579HO) in slow aging (delay fruit ripening) (Mehta et al., 2002) which is associated with increased fruit shelf life and fruit quality attributes (Handa & Matto, 2010; Handa et al., 2011, 2014).

The aim of this study is to determine whether enriched and accumulated PAs play a role in the SA biosynthesis. To address this question, we have identified putative genes for the SA biosynthesis pathways in tomato. In this manuscript we report the relative abundance of transcripts of these genes using RNAseq transcriptome analyses of WT and two isogenic independent SAMDC-transgenic homozygous lines with higher levels of SPD/SPM in ripening fruits. We show that response of fruit maturation and ripening genes to elevated PAs levels is different. In general, genes of PAL pathways were up regulated at MG stage in high PAs fruits However a few genes were also up regulated only in the ripe stage of transgenic fruits. Based on these results we suggest that SPD/SPM play a role in the SA biosynthesis likely by increasing the expression of all PAL genes, thus contributing to the increased SA levels in the transgenic fruits.

Materials and Methods

Plant material and fruit collection

Wild Type (WT) tomato **(***Solanum lycopersicum* L. cv. Ohio 8245), and its isogenic transgenic lines (556HO and 579HO) homozygous for expressing yeast SAMdc gene (*ySAMdc*) under *Sl*E8, a fruit-ripening promoter (Mehta et al., 2002), were used in this study. Tomato plants were grown at the greenhouse facility of Horticulture and Landscape Department, Purdue University, Indiana, USA under normal practices. All plants were grown in glasshouse on high porosity potting mix (52 Mix, Conard Fafard Inc., MA USA), supplied with 16hrs day/8 h night photoperiod and 23°C day/18°C night temperature conditions. Flowers were tagged at anthesis stage and fruit development recorded as day post anthesis (DPA). Mature green (MG) fruit were characterized after 37-39 DPA The Breaker (BR) stage was approximately 42 DPA and by ripening-associated color change from green to pink. Fruit of subsequent ripening stage were defined in "days post-breaker", as ripening stage of four days after breaker (BR4). The fruits of WT, 556HO, and 579HO genotypes were harvested at two stages of fruit development MG and BR4 and stored at -80°C until used.

RNA sequencing (RNASeq) experiment and normalization of read counts

Total RNA was extracted from pericarp tissues of tomato fruits as described by Eggermont et al., (1996). Total RNA was pre-treated with RNase-free Dnase (Promega, www.promega.com) and cDNA was synthesized using Superscript II reverse transcriptase kit as previously described (Mehta et al., 2002). Quality control of sequencing was made by using FastQC. Based on the result from FastQC, all samples were of good quality (quality score> 30). RNAseq reads were aligned to *Solanum lycopersicum* L. genome using Tophat version 2.1.1 with default parameters (Trapnell et al., 2009). Gene counts were calculated by HTSeq (Anders et al., 2015) using parameter "htseq-count -s no" (For stranded= no, the data is not from a strand- specific assay, a read is considered overlapping with a feature regardless of whether it is mapped to the same or the opposite strand as the feature). The annotation information used in this step was downloaded from (https://solgenomics.net/organism/Solanum_lycopersicum/genome). *S. lycopersicum* L. Genomics Network. The number of mapped reads for a particular gene were scored as 'counts' and used for downstream analysis, including differential gene expression. The HTSeq files for tomato at MG and BR4 fruits from WT, 556HO and 579Ho were normalized in reads per kilo base mRNA per million bases mapped reads (RPKM) as previously described by Mortazavi et al. (2008) and Dillies et al. (2013). Finally processed data file(s) included read counts and gene function annotation were employed to identify and analyze expression patterns for gene expression profiles of for SA biosynthesis pathways ibn plants. Orthologues of *A. thaliana PRXR1*, *PBS3*, and *EPS1* genes in *S. lycopersicum* were identified according to The *Arabidopsis* Information resource (TAIR) of genetic and molecular biology data for the model higher plant *Arabidopsis thaliana* (http://arabidopsis.org) and extracted from tomato RNASeq transcriptomic data.

Statistical analyses

A Microsoft Excel add-in statistical package XLSTAT (2014.3.05) was used for calculation and level of significance was determined by T-TEST, standard deviation (SDEV), and standard error (SERROR) to investigate the differential gene(s) expression between WT and genetically modified tomato genotypes. R software (R Development Core Team, 2016) was used to generate HeatMap

application and the correlation matrix between the three studied genotypes; WT, 556HO and 579HO. Heatmap visualization to investigate differential gene expression by using "tidyr" and "ggplot2" packages at R software version (R DEVELOPMENT CORE TEAM 2012). To create Box-and-whisker plots (Koutecký, 2015). Six stages of tomato (WT-MG, WT- BR-4, 556-MG, 556-BR-4, 579-MG, and 579-BR-4) were graphed. Estimation of the statistical significance was set via analysis of variance (ANOVA) and the Tukey HSD provided by R (function ANOVA).

Results and Discussion

Identification of gene transcripts for SA biosynthesis in tomato

Based on tomato genome sequence and gene ontology (GO) database, 16 significant gene transcripts of SA metabolic pathway have been identified in *Solanum lycopersicum* L. (http://solgenomics.net/) and represented in fruit RNASeq transcriptome (Table 1). Identified genes included peroxidases (*PRXR1*), GH3-family proteins (*PBS3*) and benzoyl transferases (*EPS1*) that were detectable in the three biological replicates of each developmental ripening stage. The presented study has newly detected and investigated the orthologues of *A. thaliana PRXR1*, *PBS3*, and *EPS1* genes in *S. lycopersicum* filling in the gap between synthesis of chorismic acid and SA biosynthesis as earlier evidenced in *A. thaliana* (Zhou et al., 2018; Torrens-Spence et al., 2019). Figure 1 represents the SA biosynthetic pathway, both IC and by the PAL pathway in plants (Dempsey & Klessig, 2017). The protein coding gene codes for BA2H was not detected in MG and BR4 fruit developmental stages.

TABLE 1. Differential gene expression of SA metabolic pathway homologues during ripening of enhanced PAs tomato fruits.

Gene_description	Gene_ID	Gene_length
(CS) Chorismate synthase	Solyc04g049350.2	7691.00
(ICS) Isochorismate synthase	Solyc06g071030.2	8935.00
(PAL 1)Phenylalanine ammonia-lyase	Solyc10g086180.1	3237.00
(PAL 2)Phenylalanine ammonia-lyase	Solyc09g007900.2	3186.00
(PAL 3)Phenylalanine ammonia-lyase	Solyc09g007910.2	8203.00
(PAL 4)Phenylalanine ammonia-lyase	Solyc09g007920.2	5042.00
(PAL 5)Phenylalanine ammonia-lyase	Solyc03g036470.1	690.00
(PAL 6)Phenylalanine ammonia-lyase	Solyc03g036480.1	309.00
(PAL 7)Phenylalanine ammonia-lyase	Solyc03g042560.1	1469.00
(CM) Chorismate mutase 1	Solyc02g088460.2	4217.00
(CM)Chorismate mutase 2	Solyc11g017240.1	3251.00
(AAO)Aldehyde oxidase	Solyc11g071620.1	7591.00
Tomato PRXR1 Orthologes in TAIR database		
AT4G21960.1 (Homologous gene in *A. thaliana*)		
IPL/PRXR1-homologue1	Solyc02g080530.2	1693.00
IPL/PRXR1-homologue2	Solyc02g032830.1	1484.00
Tomato PBS3 Orthologes in TAIR database		
AT5G13320 (Homologous gene in *A. thaliana*)		
(PBS3-1) GH3 family protein	Solyc10g009610.1	4243.00
(PBS3-2) GH3 family protein	Solyc10g011660.2	4072.00
(PBS3-3) GH3 family protein	Solyc10g009620.1	2525.00
(PBS3-4) Auxin-responsive GH3-like	Solyc12g005310.1	2003.00
Tomato EPS1 Orthologes in TAIR database		
AT5G67160 (Homologous gene in A. thaliana)		
(EPS1-1) N-hydroxycinnamoyl/benzoyltransferase-like protein	Solyc01g107070.2	957
(EPS1-2) N-hydroxycinnamoyl/benzoyltransferase 3	Solyc02g093180.2	1957
(EPS1-3) N-hydroxycinnamoyl/benzoyltransferase 4	Solyc01g107050.2	823
(EPS1-4) Hydroxycinnamoyl transferase)	Solyc08g005890.2	2157

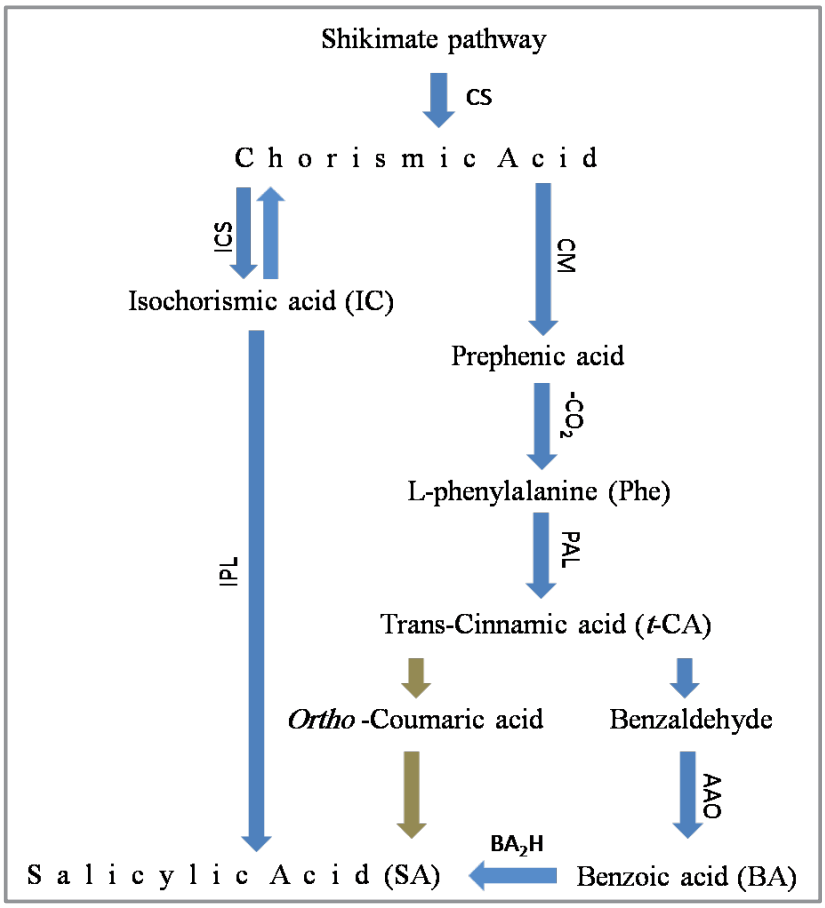
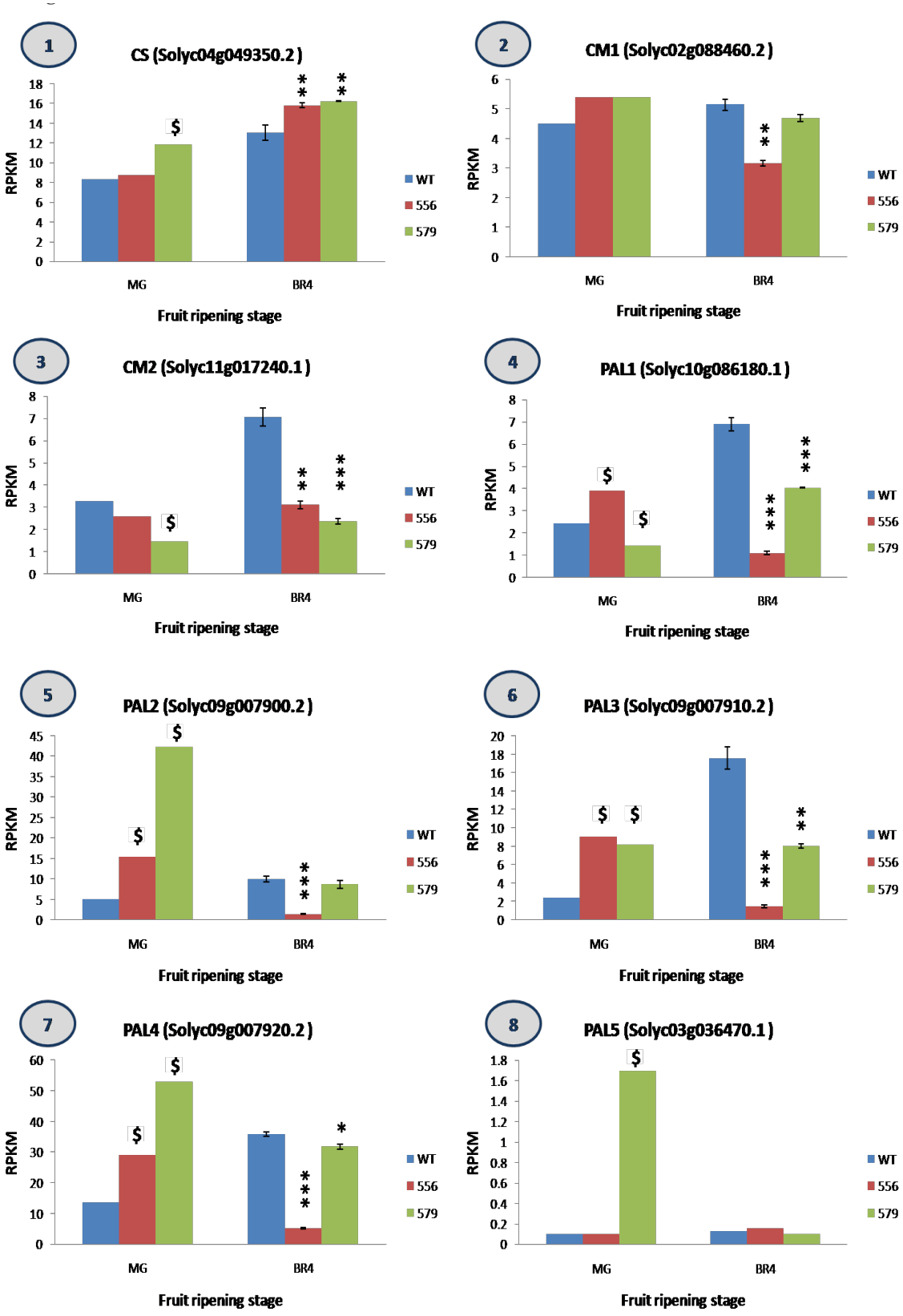


Fig. 1. Schematic representation of A) proposed (Chen et al., 2009) and B) known (Dempsey & Klessig, 2017) steps of SA biosynthesis in plants.

The 556HO and 579HO ripe tomato fruits have been previously quantified for PAs levels and shown to accumulate about 3-fold higher spermidine (Spd) and spermine (Spm) with concomitant decrease in putrescine (Mehta et al, 2002). Figure 2 illustrates the RPKM values to calculate and compare the expression of each gene involved in SA biosynthesis pathway: *CS, ICS, CM1, CM2, PAL1, PAL2, PAL3, PAL4, PAL5, PAL6, PAL7, AAO, IPL/PRXR1-H, PBS3-1, PBS3-2* and *EPS3*.

The transcript level of CS levels was up-regulated at MG stage of 579HO genotype, while CS has shown the same behavior for both transgenic genotypes as compared to the WT (Fig. 2, Panel 1). The transcript levels of *CM1* were down-regulated at BR4 stage in 556HO (Fig. 2, Panel 2), whereas *CM2* was down-regulated in MG stage in 579HO and the same behavior was detected in both genotypes as compared to the WT in BR4 stage (Fig. 2, Panel 3). Latter demonstration may give a rise to the inhibitory effect of chorismic acid accumulation on the activity of CM to produce prephenic acid, precursor of phenylalanine amino acid. These suggest that increase PAs likely promote higher accumulation of chorismic acid in 556HO and 579HO genotypes.

Among the various PAL homologues, the seven PAL genes were expressed during fruit ripening. However, their expression based on steady state transcript accumulation varied significantly. *RPKM* values were higher in *PAL2, PAL4* and *PRXR1* than that for the other genes. Transcript levels of *PAL1, PAL2, PAL3* and PAL4 (Fig. 2, Panels 4-10) were significantly up-regulated in MG stage and down-regulated in BR4 stage of 556HO lines as compared to the WT fruit of the corresponding stages. PAL gene and its homologues were up- regulated at developmental MG stage.


1
CS (Solyc04g049350.2)
2
CM1 (Solyc02g088460.2)
3
CM2 (Solyc11g017240.1)
4
PAL1 (Solyc10g086180.1)
5
PAL2 (Solyc09g007900.2)
6
PAL3 (Solyc09g007910.2)
7
PAL4 (Solyc09g007920.2)
8
PAL5 (Solyc03g036470.1)
RPKM
MG
BR4
Fruit ripening stage
WT
556
579

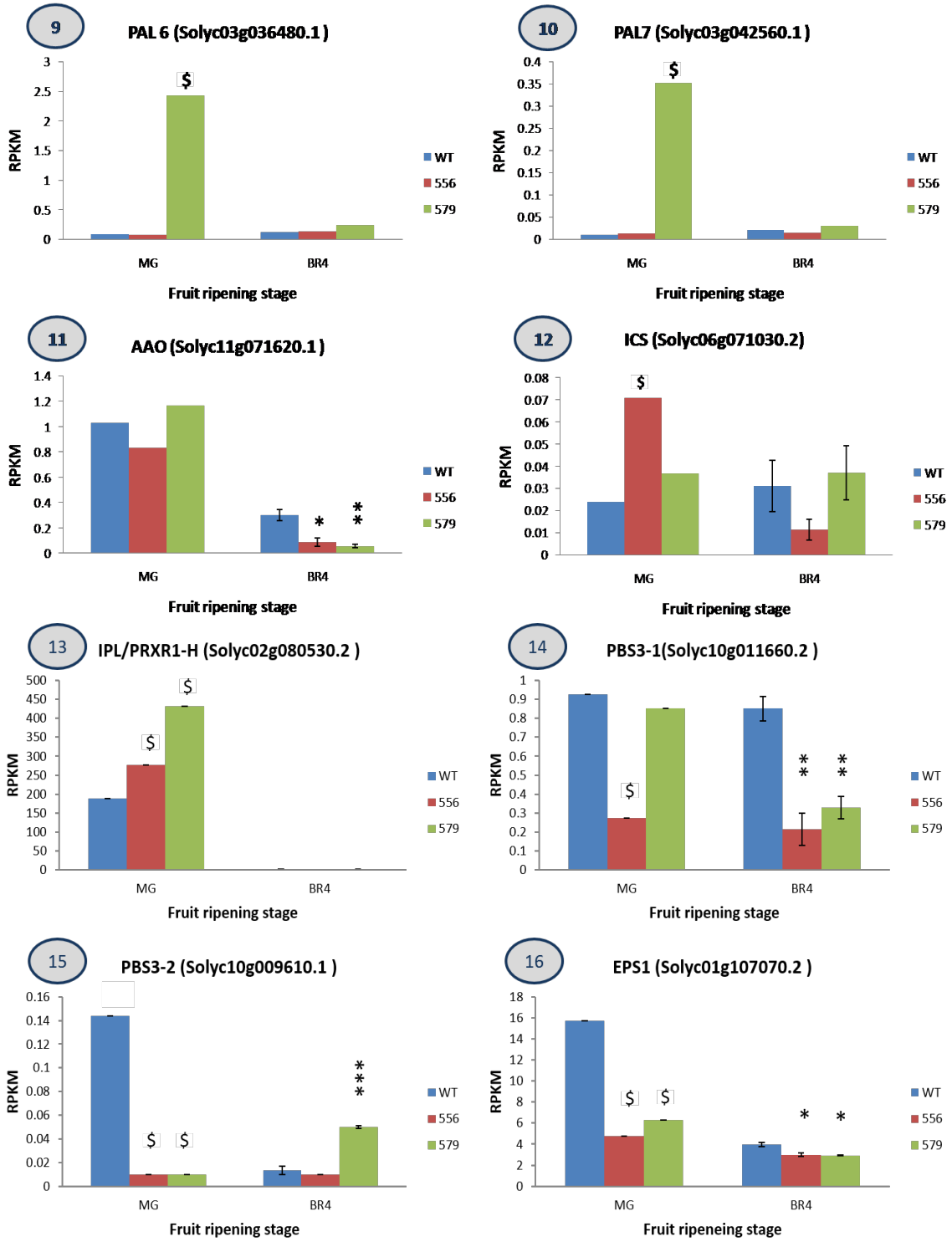


Fig. 2. Changes in the steady state transcript levels of ICS, PAL1, PAL2, PAL3, PAL4, PAL5, PAL6, PAL7, CM1, CM2, CCS, AAO, IPL/PRXR1-H, PBS3-1, PBS3-2 and EPS3 [RNASeq experiments were performed during ripening of WT (cv. Ohio 8245), independent transgenic tomato fruits enhanced in spermidine/spermine levels (556HO and 579HO). X axis represents the analyzed growing stages (MG and BR4) of tomato fruits. Y axis values indicates the normalized read counts of differentially expressed genes per Kb per million reads (RPKM) of three independent biological replicates. The data were shown as mean ± s.e.m; *, P< 0.05, **, P< 0.005,*, P< 0.0005. $ refers to significant reading at MG stage].**

Due to the phytohormonal action of SA, it was reasonable to be up-regulated at the development stage and then declined in ripening stage. This is because ethylene growth hormone is forefront cause and responsible for ripening enhancement, especially under genetic modifications by specific expression of y*SAM*dc at ripening stages (Mehta et al., 2002). *PAL5, PAL6* and *PAL7* did not show a significant increase in their transcript level in MG stage of genotype 556HO compared to the WT but exhibited a significant up-regulation in the same stage in genotype 579HO compared to the WT. In BR4 ripening stage, they did not show a significant change in their transcript levels in both genotypes compared to the WT. (Fig. 2, Panels 8-10). AAO transcript levels were significantly down-regulated in both 556HO and 579HO genotype at BR4 of 556HO stage (Fig. 2, Panel 11). On the other hand, the *ICS* was highly significant up- regulated during MG stage of 556HO genotype compared to WT-MG fruit. The *ICS* exhibited the highest accumulation in MG stage of 556HO genotype. However, the expression of *ICS* was significantly down-regulated in BR4 stage of the same genotype, 556HO (Fig. 2, Panel 12). Moreover, IPL/PRXR1 levels were up-regulated in MG stage of both genotypes and were not expressed at all during BR4 in either WT or the transgenic lines (Fig. 2, Panel 13). This down- regulation as the fruit matures might be due to the advanced ripening stage of tomato (Baldassarre et al., 2015), as peroxidase has been previously reported to be expressed in the pericarp of the mature green fruit and is, down-regulated at the later maturity stages (breaker stage) of fruit development. The rapid decline in the mRNA levels of peroxidase might be due to the loss of an inducing signal that function to sustain peroxidase expression during the mature green stages of fruit development (Sherf & Kolattukudy, 1993).

PBS3-1 was down-regulated in both ripening stages of both genotypes but the level of decrease was much more pronounced in 556HO (Fig. 2, Panel 14). Its homolog, *PBS3-2* however, was down-regulated in MG of both genotypes, compared to the WT but was significantly up-regulated during BR4 stage of genotype 579HO (Fig. 2, Panel 15). EPS3 transcript levels were down-regulated in both ripening stages in both genotypes compared to the WT corresponding stages (Fig. 2, Panel 16). Hereby, these results suggested a pronounced role of PAs in up-regulation and favoring the gene expression profile of SA biosynthesis through IC pathway during MG and BR4 stages of fruit ripening of both genotypes, especially the protein product of IPL/PRXR1-homologue1 (*Solyc02g080530.2*) and PBS3 (*Solyc10g009610.1*) (Zhou et al., 2018). WRKY transcription factors were previously shown to bind to *ICS1* promoter and activate gene expression thus favoring the ICS pathway of SA production (van Verk et al., 2011).

Transcriptional changes in SA biosynthetic pathway genes involved in SA biosynthetic pathway revealed unique and notable up-regulation of *PAL5* (*Solyc03g036470.1*), *PAL6* (*Solyc03g036480.1*), and *PAL7* (*Solyc03g042560.1*) genes at MG developmental stage of 579HO genotype (Fig. 3). The differential gene expression was not consistent in some readings between the PAs isogenic lines.

Dissection of gene expression alteration of SA biosynthetic pathway genes among enriched PAs genetically modified tomato genotypes

Transcriptionally induced salicylate biosynthesis genes were grouped in a Box plot according to their RPKM values (Fig. 4) and the percentage of salicylate biosynthesis genes was determined in each case. In BR4 ripening stage, the WT had the highest percentage of differentially expressed salicylate biosynthesis related genes, followed by 579HO, while 556HO showed the lowest percentage of expressed genes (Fig. 4A). In MG developmental and ripening stage, all 3 genotypes showed an increase in percentage of SA biosynthetic genes compared to the percentage in BR4 stage, with 579HO exhibiting the highest induction of SA biosynthetic genes in MG stage compared to both 556HO and WT in the same ripening stage (Fig. 4B).

These results have highlighted the conventional impact of engineered PAs in prompting a modification in the gene expression profiles of SA biosynthetic genes. Remarkably, the analysis of changes in fold expression of SA biosynthesis genes has revealed clear differences between the two transgenic lines. The latter finding could be attributed to the position in which the construct expressing yeast SAMdc gene (*ySAMdc*) under *Sl*E8 promoter was genetically incorporated (Mehta et al., 2002). Bioinformatics studies revealing the correlation

coefficient and clustering analyses might be of interest and provide a comprehensive understanding about the interaction between PAs and genes responsible for SA biosynthesis pathway. The effect of individual PAs on SA biosynthesis and on PA metabolism and vice versa has been previously studied in *Arabidopsis* plants by exogenous application of PAs or SA using SA mutants (Tajti et al., 2019). Synthesis of polyamine and its catabolism beside the pronounced changes in SA synthesis was upregulated by polyamines treatments *in vitro*, especially after SPD or SPM (Tajti et al., 2019). Each PA was found to have a distinct role. To increase the strength of our RNASeq data, a correlation matrix of transcriptome profiling was constructed for genes predicted to be involved in SA biosynthesis pathway (Fig. 5). Transcript levels of IPL/PRXR1-homologue1 (Haem-peroxidase, *Solyc02g080530.2*) were found to be significantly elevated (~2.5 fold) in MG ripening stage of 579HO compared to the corresponding stage of both the WT and 556HO (Fig. 5). Latter finding has most likely suggesting that SA biosynthesis via IC pathway has higher preference than the PAL pathway. Also, the correlation matrix has sub-clustered 556HO and 579HO together and away from WT genotype at MG stage. To reveal a more comprehensive picture regarding SA biosynthesis in plants and its regulatory elements under PAs enrichment, studying the different stimuli affecting the expression rates of WRKY 46 transcription factor and PR-induced molecular marker genes need to be further studied and understood.

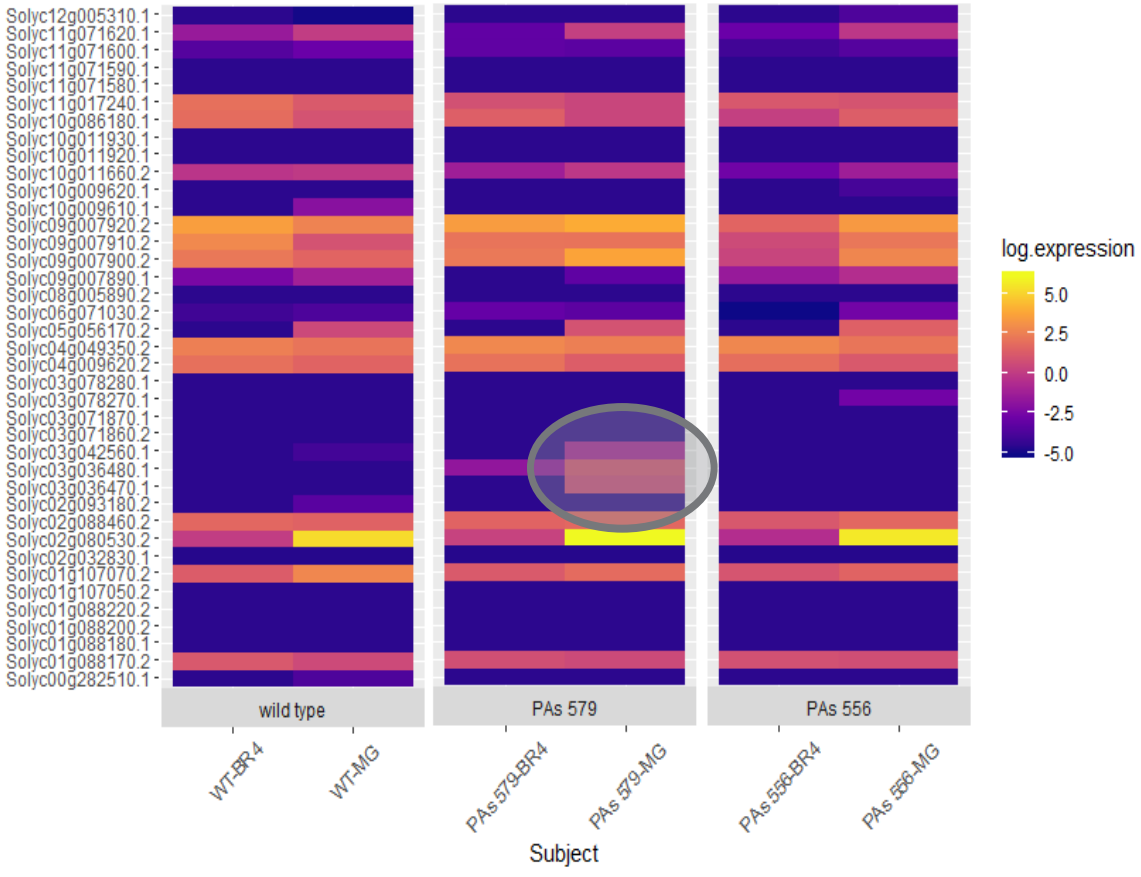


Fig. 3. Heatmap of differentially expressed genes responsible for SA biosynthetic pathway in PAs enriched homozygous cell lines 556HO and 579HO as well as in WT, at two ripening stages of tomato fruits [Transcriptional analysis of expressed genes was represented as RPKM-fold changes compared to the relevant WT developmental stage by a color scale. Blue and yellow indicate statistically decreased and increased transcript levels, respectively. Grey shaded region in 579HO genotype refers to up-regulated notable and unique genes; *PAL5 (Solyc03g036470.1), PAL6 (Solyc03g036480.1), and PAL7 (Solyc03g042560.1)*].

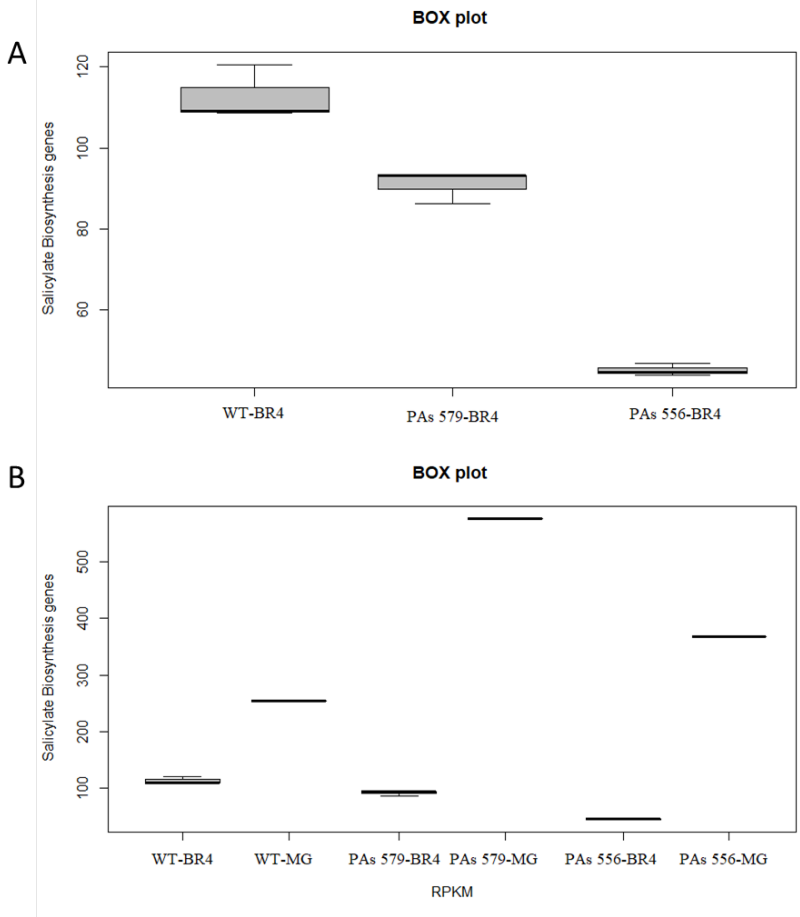


Fig. 4. Box plot representing WT, 556HO and 579HO age (BR4) (A) and in the 3 genotypes in 2 fruit ripening stages (BR4 and MG) (B).

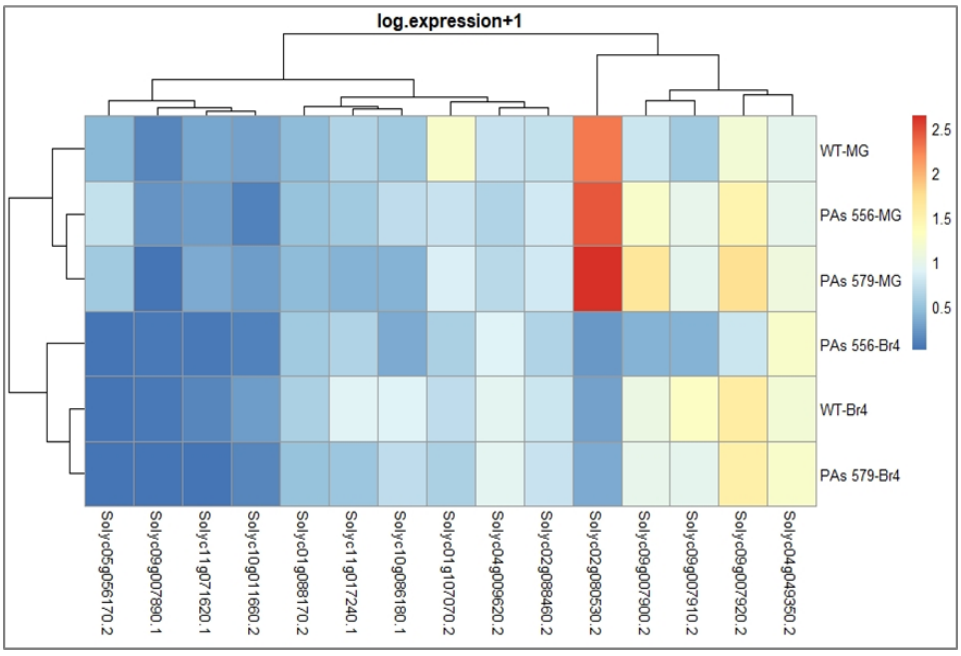


Fig. 5. Correlation matrix between the studied genotypes (WT, 556HO, and 579HO) of tomato transcriptome profiling of identified putative genes to be involved in SA pathway in plants.

Conclusions and Future Perspectives

Figure 6 summarizes the effects of higher PAs on expression of the SA biosynthesis genes in WT, 556HO, and 579HO genotypes at the two stages of fruit development. These results show that a PAs influence expression of most genes in the ripening tomato fruit. Result show that transgenic fruits at MG stage exhibited higher steady state levels of transcripts of *CS*, *PAL1-7, ICS, IPL/PRXR1, CS,* and *PAL 4-10,* but lower levels for *CM1*, *CM2, EPS1,* and *PBS3-2*. The pattern at the turning stage of fruit ripening was dramatically different as only *CS* and *PBS3-2* were upregulated whereas the transcript levels of *CM1*, *CM2*, *PaL1-4AAO*, *PBS3-1*and *EPS1* were down regulated. Taken together these results suggest that SPD/SPM play differential role in the SA biosynthesis during fruit ripening and likely increased the SA levels in the transgenic fruits.

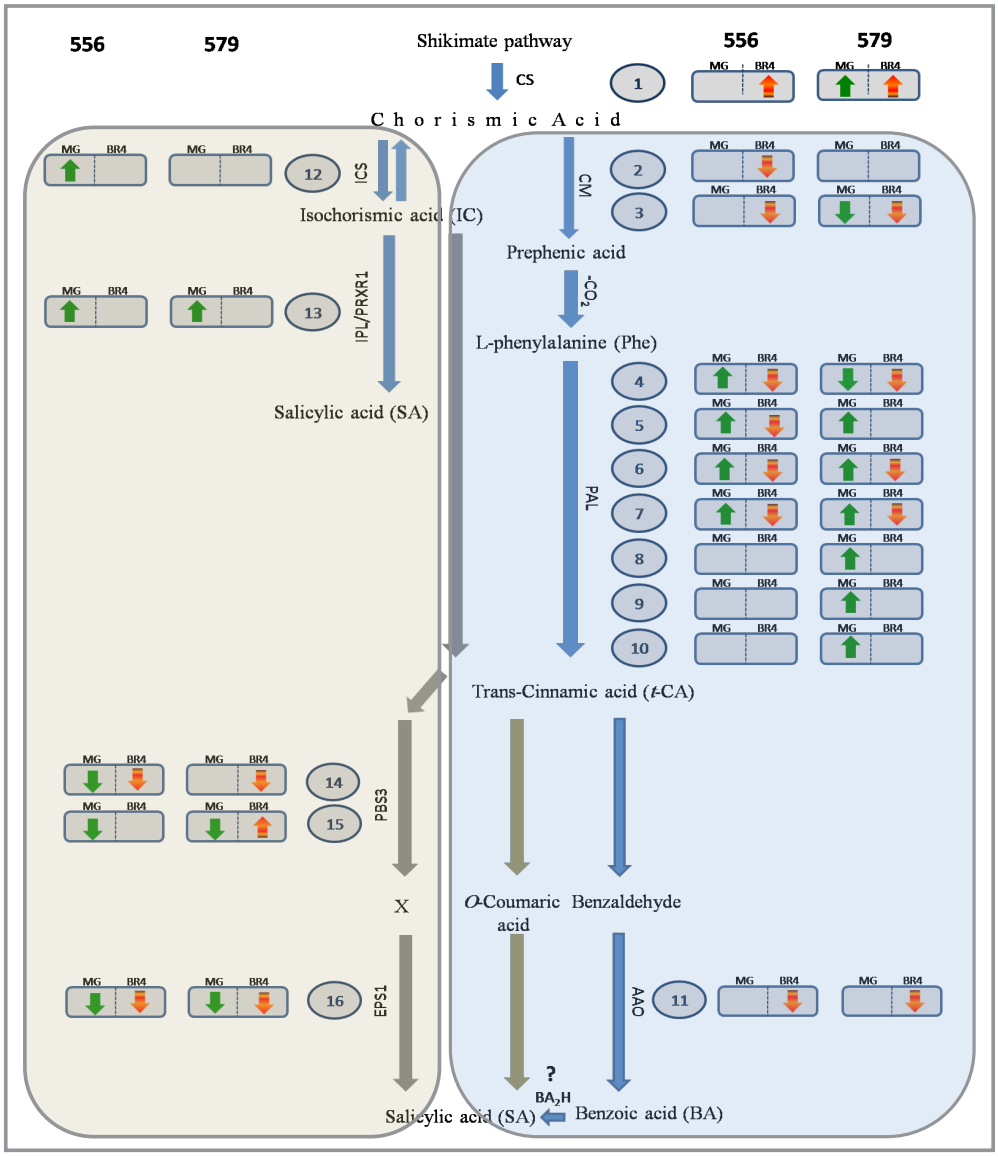


Fig. 6. Tentative schematic representation showing differential gene expression of SA genes in WT, 556HO, and 579HO genotypes in two stages of fruit development and ripening stages in RPKM values [Up-and downward green arrow heads refers to up- or down- regulation of significant gene expression reads at the specified fruit stage. Up-and downward greenish-orange arrow heads refers to up- or down- regulation of significant gene expression read at certain stage. Blue shaded inset refers to known steps of SA biosynthesis in plants shown by Dempsey & Klessig, (2017). Green shaded inset refers to proposed steps according to this study and Chen et al. (2009)].

Higher polyamines have been shown to alter metabolome in Tomato (Mattoo et al., 2006). A nMR based metabolome investigation showed that higher polyamines increased levels of glutamine, asparagine, choline, citrate, fumarate, malate, and an unidentified compound A in the red transgenic fruit, while the levels of valine, aspartic acid, sucrose, and glucose exhibited significant decreases in thgh PAs fruits. In other metabolome investigation using the WT and high PAs fruits grown under different mulches, Fatima et al. (2016) has shown that both environmental and genetic background plays significant role in determining the metabolic levels of various metabolites. This study showed that high endogenous spermidine and spermine content caused strong effect on the levels of amino acids, Krebs cycle, intermediates and energy molecules (ADP / ATP) in ripening fruits of plants grown under different agroecosystem environments. Effects of Hairy vetch on metabolome was similar to that of PAs. Effect of the deficiency of ethylene or methyl jasmonate were unique under all growth conditions and generally were opposite of high polyamine genotype results.

Conclusions and Future Perspectives

Collectively, these results affirmed that interactions between metabolite pathways and growth environments can greatly affect the outcome of fruit metabolome (Fatima et al., 2016). It has been shown previously that expression of SAMDC was carried under a fruit ripening promoter. THE SAMDCD transgenic fruits were found to contain about 3-fold higher levels of SPD and SPM and fruits exhibited slight attenuation of ripening (Mehta et al., 2002). These findings might be attributed to promoted production of amino acids (like, phenylalanine) in elevated endogenous Spd and Spn PAs (Mattoo et al., 2006; Fatima et al., 2016). The present study confirms a similar effect PAs on SA biosynthesis pathway during ripening. We are presently confirming the levels of SA in high PAs fruits to provide the support to hypothesis that PAs upregulate SA biosynthesis and pathogenesis related PR1B1 protein.

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تحليل الترنسكريبتوم لإنزيمات الأيض لعملية التخليق الحيوى لحمض الساليسيلك عبر تراكم متعدد الأمينات المهندسة وراثيا أثناء نضج ثمار الطماطم

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مما لا شك فيه أن الهرمونات النباتية (Phytohormones) تلعب دورا رئيسيا و بارزا فى أغلب العمليات الحيوية داخل الخلية النباتية. و تأتى مركبات "متعدد الأمينات" (Polyamines) من أهم تلك الهرمونات النباتية التى تشكل أهمية لا غنى عنها فى العديد من العمليات البيولوجية كنمو الخلايا و تمايزها و تنظيم عمليتى نسخ و ترجمة الجينات النباتية. كما يظهر أثرها واضحا جليا فى تأخير شيخوخة النباتات. كما تشارك مركبات " متعدد الأمينات " فى إطالة عمر نبات الطماطم (على وجه الخصوص) بتقليل مقدار التقدم فى العمر و تأخير النضج أثناء مراحل تنمية و نضج الثمار.

يلعب حمض الساليسيليك SA دورا هاما فى تحمل النبات لظروف الاجهاد الحيوية وغير الاحيائيه. و ينتج عن ذلك مقاومة الامراض المحلية و المكتسبة فى النبات و يشارك أيضا في التخفيف من عوامل الإجهاد اللااحيائي مثل سميه المعادن ، والجفاف ، والملوحة العالية ، و البرودة والحرارة. من منطلق هذا فقد استهدفت هذه الدراسة إلى تعيين و فهم و تحليل مدى التفاضل فى التعبير الجينى للجينات المسؤولة عن عملية التخليق الحيوى لحمض الساليسيلك(SA)على مستوى النسخ الجينى (Transcription) لتلك الجينات. و لقد أجريت هذه الدراسة على نبات الطماطم المهندس وراثيا بتراكم متعدد الأمينات بصفة موجهة أثناء مراحل نضج ثمار الطماطم. و للكشف و الوقوف على مدى تأثر أو تغير عملية النسخ على مدى التفاضل فى التعبير الجينى للجينات المسؤولة عن عملية التخليق الحيوى لحمض الساليسيلك أثناء تراكم مركبات متعدد الأمينات فقد تم عمل تحليل الترنسكريبتوم (Transcriptome) للمراحل المختلفة لنضج ثمار الطماطم المهندسة و غير المهندسة وراثيا.

و قد خلصت النتائج إلى وجود تغير مؤثر من الناحية الإحصائية فى مستوى التعبير الجينى لمتماثلات (Homologues) جينات كلا من *PAL/IC* فى مراحل النضج المختلفة على مستوى تحليل الترنسكريبتوم.