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Comparative Study of Gene Expression during Early Stages of Fruit Development

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ABSTRACT

In Egypt, Tomato production is very important and considers the first among vegetable production. Study of tomato fruit development is very important step toward improvement of fruit quality and quantity. The molecular changes during early stages of tomato fruit development still unclear and not well-studied. During Post-anthesis stages, e.g. stage 2, many molecular changes occur that set up the stage for fruit development process. In order to identify the molecular changes accompanies fruit development, we investigate the expression level of some genes/ gene orthologs in tomato in comparison to their expression level in Flowering tobacco not only in stage 2 fruit, but also in other types of tissue including vegetative, inflorescence, and all stages of fruit developed tissue.

Key words: Tomato, fruit development, fruit quality

Introduction

Tomato is one of the most widely grown vegetables in the world. It is grown on more than 5 million ha with a production of more than 152 million tons (UNCTAD.org). In Egypt, Tomato production is very important and considers the first among vegetable production. Study of tomato fruit development is very important step toward improvement of fruit quality and quantity. Fruit development has been well-studied in tomato (e.g., Gillaspy *et al.,* 1993; Tanksley, 2004; Lemaire- Chamley *et al.,* 2005; and Pesaresi *et al.,* 2014). For fruit to be, ovary undergoes significant and continuous developmental processes. These process has been divided into four stages (Gillaspy *et al.,* 1993; Tanksley, 2004): (1) ovary development prior to fertilization of the ovules, (2) fertilization followed by a period of cell division, (3) the cessation of cell division and the onset of cell expansion, and (4) ripening. Same developmental stages found in other angiosperm berries as well (e.g., Ollat *et al.,* 2002; Fei *et al.,* 2004). Stage 4 (ripening) in Tomato is the most studied stage of all. Many literatures studied the molecular changes that accompany ripening processes such as, cell wall softening, nutrient accumulation, and anthocyanin accumulation (e.g., Moore *et al.,* 2002; Adams-Phillips *et al.,* 2004; Eriksson *et al.,* 2004; Liu *et al.,* 2004; Barry *et al.,* 2005; Pesaresi *et al.,* 2014). On the other hand, the molecular changes during early stages of fruit development still unclear. Studies have shown that genes acting prior to fertilization (stage 1) influence fruit shape and size (Xiao *et al.,* 2009). During fertilization (stage 2), many ripening-related transcriptional changes in the pericarp were initiated (Gillaspy *et al.,* 1993; Liu *et al.,* 2002; Xiao *et al.,* 2009).

Depending on the pericarp (fruit wall) features, Many fruits fall into one of two categories: fleshy indehiscent (e.g., berries, in which the pericarp layers proliferate) or dry dehiscent (e.g., capsules, in which the pericarp becomes lignified). Some studies attempted to compare dry and fleshy fruits, one study use the comparison to establish analogies of the distinct biochemical and physiological processes that occur during final development steps using transcriptome data (Gómez *et al.,* 2014). Another comparative, but anatomical, study of dry and fleshy fruit development in Solanaceae showed that four corresponding stages can be identified in the development of capsules: (1) ovary development; (2) onset of cell division; (3) cessation of cell division accompanied by lignification; and (4) maturation as a final stage (Pabón-Mora and Litt, 2011). Prior to fertilization (during stage 1) ovary development is similar in capsules and berries, with little differentiation occurring in the ovary wall. During stage 2, cell proliferation is apparent in both species and the fruits grow in size, but the spatial domain of cell division is restricted in capsules relative to berries. During stage 3, capsules are characterized by extensive lignification rather than the cell expansion seen in berries, and during stage 4 the capsule dries and dehisces, in contrast to the ripening processes of the berry (Pabón-Mora and Litt, 2011). Thus, the changes that are responsible for the dramatic differences in structure between capsules and berries are largely initiated right after fertilization, stage 2.

To better understand the genetic control of fruit development. A comparative analysis of gene expression in closely related species, but with divergent fruit characteristics is a better approach to identify the molecular changes accompanies fruit development. This study uses a comparative approach to identify differences in some gene expression during stage 2 that direct capsule and berry development. *Nicotiana sylvestris* Speg. (flowering tobacco) represent species with the dry fruit (capsule), and *Solanum lycopersicum* L. cv. Micro-Tom (tomato) is an exemplar of species with a fleshy fruit; both are members in the solanaceae

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family, diploid, have numerous genetic and genomic resources available. Such an analysis can serve to identify candidate genes that may play a role in the differential processes involved in dry and fleshy fruit development. Expression profile (RT-PCR) was performed on all candidate genes during most stages of plant development and fruit development. QRT-PCR was used to analyze and compare the expression of candidate genes between Micro-Tom and flowering tobacco, during stage 2. The results were evaluated for patterns that might reflect functional processes associated with fruit type.

Materials and Methods

Plant growth and tissue collection:

Five plants each of *Solanum. lycopersicum* cv. Micro-Tom (tomato) and *Nicotiana. sylvestris* (flowering tobacco) were grown under 23˚C, and 12 hours light. The criteria described in Pabón-Mora and Litt (2011) to identify the four stages of fruit development were used. Stages are referred to by days postanthesis (DPA) after anthesis. Material representing each of the four developmental stages was collected as follows: stage 1, bud preanthesis), ovaries were isolated from the rest of the floral organs and harvested when buds reach 9cm, from flowering tobacco, and 0.8cm, from micro-tom; stages 2–4, flowers were tagged on the day of anthesis and carpels/fruits were collected afterword. Flowering tobacco carpels/fruits were collected at 4, 7, 16 DPA, whereas Micro-Tom carpels/fruits were collected at 2, 10, 50 DPA to represent stage 2, 3, and 4 respectively (Pabón-Mora and Litt, 2011). For RT-PCR, approximately 100 mg of vegetative stem, leaf, bud preanthesis, whole flower at anthesis, and fruit at stages 2, 3, and 4 were collected for each species. For qRT-PCR, ovaries/fruits at stage 2 were collected from three biological replicates.

Selection of candidate genes:

Gene expression levels for whole tomato and flowering tobacco fruits at the onset of stage 2 were compared (data not shown) using TOM2 oligo microarray (http://ted.bti.cornell.edu/). The TOM2 array contains long oligo probes (70 nucleotide) to approximately 12,000 unigenes, as defined by the Solanaceae Genomics Network (SGN, Mueller *et al.,* 2005; http://sgn.cornell.edu). A candidate five oligos were selected from microarray database (data not shown) for further analysis. Using their oligo sequence to blast against TOM2 microarray we were able to assist the full sequence of the unigenes in Tomato. Based on a BLAST search of each unigene in NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and SGN databases, Putative orthologs of each unigene were searched and used to design primers (Table 1). Primers were designed using primer express® software to amplify each candidate gene from *Nicotiana sylvestris* Speg. (flowering tobacco) and *Solanum lycopersicum* L. cv. Micro-Tom.

Reverse transcription PCR (RT-PCR) expression analyses:

Total RNA was prepared from approximately 100 mg of each of the 7 tissues collected for each species using the Trizol reagent (Invitrogen). Samples were DNAse treated using TurboDNAse (Ambion, Texas, USA). Total RNA was reverse transcribed using 2.0 µg of RNA with random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA). To evaluate expression levels, cDNA template was diluted to a standard concentration (20 ng/µL); 18S was used as a control. PCR was performed in 25µL reactions containing 2.5 µL of 10X buffer, 1 µL of 10mM dNTP mix, 5 unit Taq polymerase. 5 nmoles of each primer, and 30 ng of cDNA. Cycling parameters used for amplification are 94°for 3 minutes followed by 30 cycles of; 30s at 94, 30s at 57°, and 30s at 72°. The annealing temperature used to amplify AGL2 and WRKY6 was 55°C instead of 57°C, keeping the rest of parameters the same. The PCR was terminated by one cycle of 72°C at 5min. Products were visualized on a 1.2% agarose gel.

Quantitative Real-Time RT-PCR (*qRT-PCR*) *expression analyses*

To prepare template for qRT-PCR analyses, total RNA from stage 2 fruits collected from three plants per species was prepared using the RNAqueous Kit (Ambion) according to manufacturer's instruction. RNA Samples with 260/280 absorption ratio 2± 0.2 and a 260/230 ratio greater than 1.0, were used. cDNA for qRT-PCR was prepared using 2.0 µg of total RNA per sample, random hexamers and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA). cDNA was diluted to 10 ng/µL immediately prior to performing qRT-PCR. Each 20 µL reaction mixture consisted of 10 µL of 2x FastStart SYBR Green PCR Mastermix (Applied Biosystems), 300 uM of forward and reverse primers, 10 ng of cDNA template, and nuclease-free water**.** Having ABI-PRISM 7300 Real-Time PCR system (Applied Biosystems) we apply the default cycle parameters of 2min at 50°C, 10 min at 95°C, followed by 40 cycles of the following: 15sec at 95°C

and 1min at 60°C. Reactions were run in triplicate in three independent experiments at stage 2. The geometric mean of 18S gene expression was used as an internal control to normalize the variability in expression levels. Expression data then were analyzed using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen, 2001. The expression levels of the targets are normalized to the expression levels of the 18S gene. Flowering tobacco RQ were used as calibrators, so the expression ratios were generated by dividing each Micro-Tom RQ value by the RQ value of the flowering tobacco orthologs.

Results and Discussion

This work was designed to study the expression profile of some genes, which might play a role during fruit development, in a comparative view of both capsule *Nicotiana sylvestris* Speg. *(Ns)* and berry *Solanum lycopersicum (Sl)* species. The candidate genes were selected from microarray analysis results (data not shown) on the basis of differential expression at stage 2 of fruit development. A putative assessment of orthology was made based on BLAST searches of GenBank and SGN. Only one gene, from the selected genes, was published before in both *Sl* and *Ns.* Therefore we used the previously published names for that gene, *SlWRKY6* and *NsWRKY6*. The rest of the genes were published in only one of the two species. These genes were named by adding the prefixes "*Sl*" or "*Ns*" to the previously published name (e.g., *SlDET1*, *NsDET1; SlPDH, NsPDH; SlTAGL2, NsTAGL2 and SlPGIP, NsPGIP*) Gene names and abbreviations, SGN unigene numbers and GenBank IDs, are provided in Table 1.

Reverse transcription PCR **(**RT-PCR) expression analyses—the expression patterns of the 5 candidate genes were evaluated in 7 tissues including vegetative stem, leaf, bud preanthesis (stage1), whole flower at anthesis and fruit at stages 2, 3, and 4. Results are shown in Figure 1.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)—was performed at stage 2 of fruit development in both Micro-Tom and flowering tobacco to quantify the expression level for the 5 genes under investigation.

DEETIOLATED1 (*DET1*):

Arabidopsis *DET1* (*AtDET1)* is involved in the signal transduction cascade of light perception and morphogenesis. The tomato homolog of DET1 has been isolated from the mutant named high pigmented-2 (HP-2) (Mustilli *et al.,* 1999). Light-grown hp mutants display elevated levels of anthocyanins, are shorter and darker than wild-type plants, and have dark green immature fruits due to the overproduction of chlorophyll pigments. In contrast to det1 mutants, tomato hp-2 mutants do not display any visible phenotypes in the dark but only very weak phenotypes, such as partial chloroplast development (Mustilli *et al.,* 1999). Jones *et al.,* 2012 isolated *SlDET1* mutant alleles, which are allelic to *high pigment 2* (*hp2*). *SlDET1* found to affect both carotenoid and phenylpropanoid phytonutrients level in ripe fruit, whilst immature fruit mutant of *SlDET1* showed increased chlorophyll content, photosynthetic capacity and altered fruit morphology (Jones *et al.,* 2012). *SlDET1* expression in Micro-Tom was seen in the vegetative tissue as well as the flower and fruit (Figure 1) and there were no differences in expression level between different tissue types. On the other hand, DET1 homolog from tobacco have not been isolated or published yet. The putative ortholog in Nicotiana, *NsDET1,* was also expressed in both vegetative and inflorescence tissue, but was highly expressed in all flower and fruit development stages except stage 3, and 4 in which decreased dramatically (figure 1). DET1 transcriptional level was lower in tomato at stage 2 (figure 2) *NsDET1* expression profile shows that it may play roles during vegetative, flower and fruit development, but not well as important during later stages of dry fruit development and dehiscence.

Proline dehydrogenase (PDH):

PDH is the rate-limiting enzyme in proline degradation and serves important functions in the stress responses and development of plants by providing energy and metabolites from proline degradation. Ribarits *et al.,* 2007 isolated two proline dehydrogenases, *NtPDH1* and *NtPDH2,* from *Nicotiana tabacum*. Silencing of the *NtPDH* genes by RNA interference led to increased proline contents, decreased seed set, delayed seed germination and retarded seedling development pointing towards an important function of at least one of the two *NtPDH* genes during plant reproductive development.

Expression of putative ortholog of PDH in Tomato, *SlPDH,* noticed in vegetative and fruit tissue, the expression was less in bud preanthesis and flower at anthesis, then increased as the cell division increased at stage 2, 3 of fruit development (figure 1). At stage 4, expression decreased compared to earlier stages of fruit development. The finding of two publication; (1) Kimura et al. 2001, which isolated cytokinin-inducible tobacco *CIG1* gene that is very similar in sequence to *NtPDH*, and (2) Matsuo *et al.,* 2012 finding that cytokinins signal transduction pathway is more active during the cell division phase of tomato fruit development is pointing toward the possibility of PDH gene transcription level is influenced by cytokinin activity during tomato fruit

development. As cytokinin increased during stage 2 and 3 where the most cell division occur, *SlPDH* expression expected to increase. Similar to tomato, *NsPDH* expression noticed in all the tissue (figure 1), but was highly expressed at anthesis and at stages 2 (figure 1 and 2) which may or may not related to cytokinins level during flowering tobacco fruit development, which is not well studied in flowering tobacco yet. *NtPDH* Level of expression was also increased at stage 4 which may be a response to dehiscence occurring at that late stage. This is may be due to similar physiological changes occurred in highly desiccated mature pollen, in which both isolated *NtPDH* were strongly expressed (Ribarits *et al.,* 2007).

AGAMOUS-LIKE(AGL)2:

MADS box gene *TM29* was isolated from tomato Lycopersicon esculentum Mill (Ampomah-Dwamena 2002) and shared a high amino acid sequence homology to the Arabidopsis *SEPALLATA 1*, *2*, and *3* (*SEP1*, *2*, and *3*) genes. *TM29* functions in floral organ development, fruit development, and maintenance of floral meristem identity in tomato (Ampomah-Dwamena 2002). *TM29* showed similar expression profiles to *SEP1*, with accumulation of mRNA in the primordia of all four whorls of floral organs. In addition, *TM29* mRNA was detected in inflorescence meristem and shoot tip, but not in leave or root. Upon blasting of *TM29* in Genebank, *TAGL2* a transcription factor characterized from Solanum lycopersicum (Busi *et al.,* 2003) was the top blast. *TAGL2* expression level found to be induced during the tomato fruit development stage 1 (anthesis) and post anthesis, when active cell division occurs. Nicotiana sylvestris developmental protein SEPALLATA 1-like (LOC104210050) results from blasting with *TM29*, but no function was reported for that sequence. The putative ortholog of *TM29* in tomato and Nicotiana have been given the name *SlAGL2*nd *NsAGL2* respectively (Table 2). *SlAGL2* expression profile was not clear in stem tissue, was very low in leaf tissue, but was induced during pre-anthesise and stages 1, 2, and 3 of fruit development (figure 1), pointing toward functions in both floral organ development and fruit development, as supported in previous publication (Busi *et al.,* 2003 and Ampomah-Dwamena 2002). *SlAGL2* was 2x upregulated at stage 2 compared to flowering tobacco putative ortholog of SEP1, *NsAGL2* (figure 2). That's suggesting a more important role in early stages of fleshy fruit development compared to dry fruit. *NsAGL2* was induced starting from preanthesis and kept highly expressed until it diminished at stage 4 of fruit development as seeds mature (figure 1), which suggest its function in floral organ identity and important role in seeds and embryos development. These functions were assigned previously to Arabidopsis SEP genes (Ma *et al.,* 1991; Flanagan and Ma 1994).

WRKY transcription factors:

WRKY proteins constitute a large family of plant-specific transcription factors whose precise functions have yet to be elucidated. The first-described WRKY factor from *Arabidopsis*, was shown to be expressed in roots and flowers, (de Pater *et al*., 1996 and Robatzek *et al.,* 2001) but their function in flower remain unknown. AtWRKY6 found to be expressed in Arabidopsis senescent leaves and wounded mature leaves giving that it is involved in senescence and pathogen defense (Robatzek *et al.,* 2001). The role of WRKY transcription factors have been implicated primarily in plant defense and abiotic stresses (Chen *et al.,* 2012). However, one WRKY transcription factor in *Arabidopsis*, *TRANSPARENT TESTA GLABRA 2* (*TTG2*), seems to be involved in trichome and seed coat development (Johnson *et al.,* 2002). Many WRKY genes were isolated from Tobacco (Chen and Chen, 2000) and tomato (Liu *et al.,* 2014) and found to be induced by biotic and abiotic stress. The expression of the putative ortholog of WRKY6 in tomato, *SlWRKY6,* noticed in leaf tissue, preanthesis, stage 1, 2 and 3 of fruit development (figure 1). On the other hand, NsWRKY6 transcriptional level was not detected except in stage1, 2 and 4 of fruit developing, giving the implication that this gene may be involved in fruit development. A comparative view of WRKY6 gene expression at stage 2 fruit shows higher transcriptional level in Micro-Tom compared to flowering tobacco (figure 2), which may be due to more susceptibility of fleshy fruit to a biotic stress than dry fruit.

Polygalacturonase-inhibiting proteins (PGIP):

These type of proteins are extracellular plant protein inhibitors of endo-polygalacturonases (PGs) which play a role in cell wall disassembly, an important process in tomato ripening (Moore and Bennett, 1994; Cantu *et al.,* 2009). PG inhibitors isolated from tomato, PGIP (Stotz *et al.,* 1994), was predicted to mediate the rate at which tomato ripening occurs by regulating cell wall disassembly. However, evidence suggests that plant PG inhibitors play important role in limiting the activity of fungal and bacterial PGs and so limiting there colonization inside plant tissue (Janni *et al.,* 2013; Prabhu *et al.,* 2014). Thus, a role for tomato PGIP as a fungal inhibitor is likely. The putative ortholog of this gene in flowering tobacco (*NsPGIP*) is expressed in all tissue except stem, and its transcriptional level remain almost the same in different tissue type with somewhat higher expression at stage 2 (figure 1). *NsPGIP* transcriptional level noticed to be lower compared to their counterpart in Micro-Tom especially during ovary and fruit development stage 2 and 3(figure 1,2). Fungal

infection is likely to be a more prevalent problem in the development of fleshy fruits than dry; thus, a higher level of expression in tomato (figure 2) is consistent with a role for these genes in controlling fungal infection.

Table 1. First Column lists the top BLAST hit which used to name the gene, the species from which it was derived, and the GenBank accession number. Abbreviation names used in this paper for Micro-Tom and flowering tobacco are listed in columns 2 and 3. Fourth column lists the Unigene ID number from which the microarray probe for that gene was generated.

Top BLAST hit (species) GenBank accession number	Abbreviation in Micro-Tom (*Solanum lycopersicum*)	Abbreviation in flowering tobacco (*Nicotiana sylvestris*)	ID (SGN Unigene)
Tomato Deetiolated 1, tDET1 (*S. lycopersicon*) AJ224356.1	*SlDET1*	*NsDET1*	U223546
Proline dehydrogenase 2, PDH2 (*S. lycopersicum*) XM_004232063.1	*SlPDH*	*NsPDH*	U213163
Tomato Agamous-like 2, TAGL2 (*S. lycopersicum*) AY098738.2	*SlAGL2*	*NsAGL2*	U213617
WRKY transcription factor 6-like, *WRKY6* (*S. lycopersicum*) XM_004243438.1	*SlWRKY6*	*NsWRKY6*	U215688
Polygalacturonase Inhibitor Protein, PGIP (*S. lycopersicum*) L26529.1	*SlPGIP*	*NsPGIP*	U215373

Table 2. List of primers used for RT-PCR and qRT-PCR analysis.

Genes amplified	Primers used for amplification
SlDET1 and *NsDET1*	F: 5' GCTTTCTTGTATGATGATTTGCTTGCT 3' R: 5' TCTCCAGAATCTCTGATCTGAAGGATG 3'
SlPDH	F: 5' GCAAGAAATGCCTGGAACTTG 3' R: 5' CAATCGCTGGTTGAATAGCTGTA 3'
NsPDH	F: 5' GATACAGCTATTCAACCTGCAATTGA 3' R: 5' TCCAAAAATCATAGGGTCATCATCT 3'
SlAGL2	F: 5' CTCAATCTCAGGGTTTCTTCCAA 3' R: 5' TGGATCGTACCCAATTTGCA 3'
NsAGL2	F: 5' CTCAAACTCAGGGTTTTTTCCAA 3' R: 5' GCTGGATCATACCCTATTTGCAA 3'
SlWRKY6	F: 5' GTCTCGGTCAGGGCTCGAT 3' R: 5' CGCCATTGACAGCCATCTG 3'
NsWRKY6	F: 5' GCTCCCATGATCACAGATGGT 3' R: 5' TCCCTTCGCCATTTTTTGC 3'
SlPGIP and NsPGIP	F: 5' TGAATGTGAGTTATAATAGACTTTG 3' R: 5' TACATTCCGGCAACGGAGAGCCACA 3'
18S	F: 5' TGCATGGCCGTTCTTAGTTG 3' R: 5' GAGGTCTCGTTCGTTAACGGAAT 3'

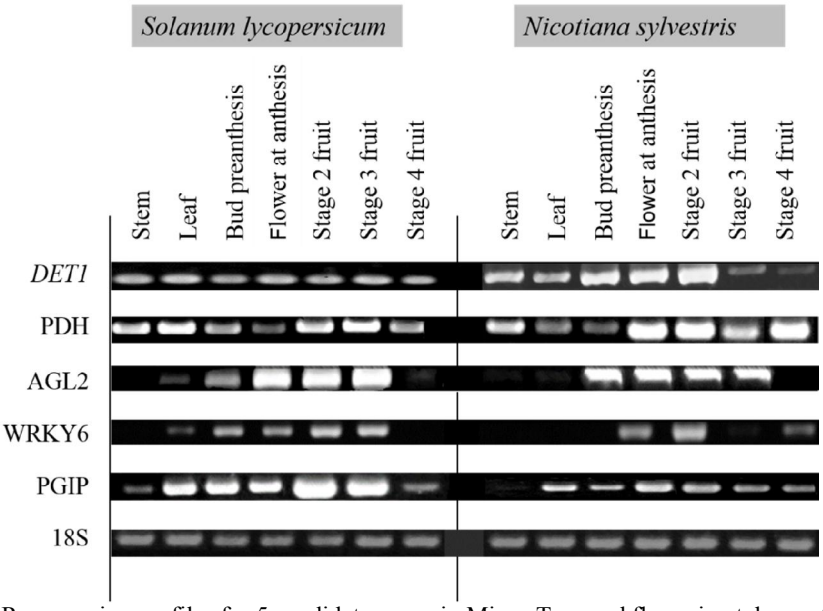

Fig. 1. RT-PCR expression profiles for 5 candidate genes in Micro-Tom and flowering tobacco tissues. Expression was tested in stem, leaf, bud preanthesis (stage 1), flower at anthesis, fruit at stages 2, 3 and 4 of development. 18S was used as control.

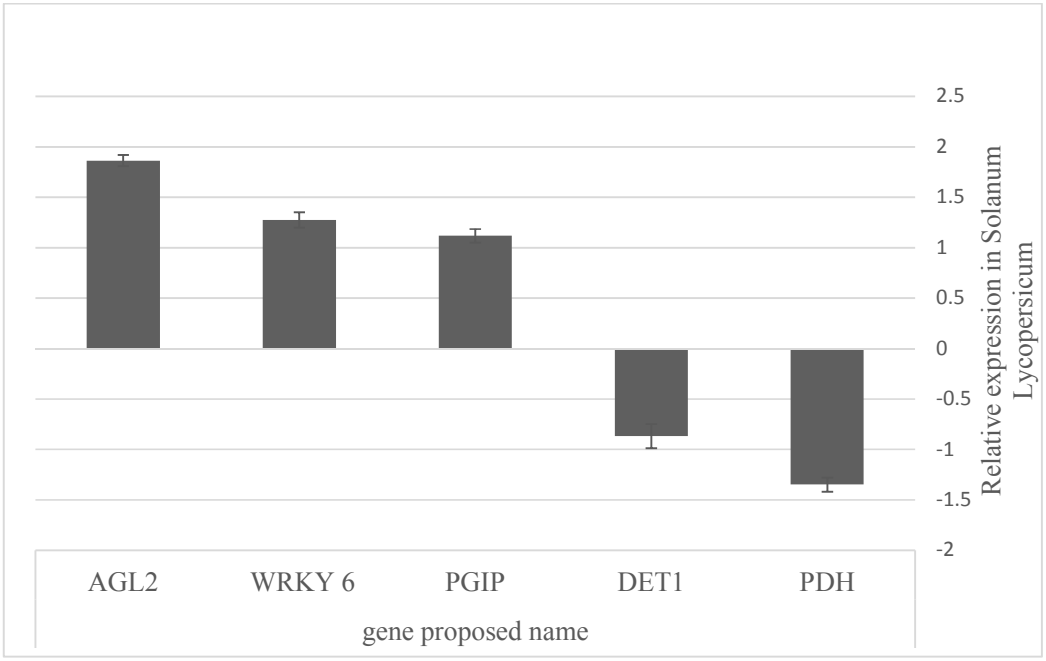

Fig. 2: qRT-PCR results showing expression of five genes at stage 2 of fruit development in Solanum lycopersicum relatively to their level of expression in *Nicotiana sylvestris* Speg. (flowering tobacco). Three biological replicates and three technical replicates were used to produce these results. Error bars represent standard errors.

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