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Polygalacturonase genes in tomato flower and leaf abscission zones- A novel trait for molecular breeding

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Abstract

Abscission of plant organs is a key process during plant life cycle and prerequisite factor involved in limiting the spread of disease, shedding of un-pollinated flowers and facilitates dispersal of seeds. In an agricultural context, abscission may become a major limiting factor for crop productivity. The organs abscise at a specific position called Abscission zone (AZ) and it is one of the prime traits to be manipulated during the crop improvement process towards the selection of reduced abscission lines. The tomato abscission polygalacturonase (TAPG) genes are abscission induced polygalacturonases and specifically induced in the AZ, which plays a major role in AZ separation. The current study had accentuated to identify the entire polygalacturonase gene families in tomato AZs, through AZ specific customized microarray. The results revealed that TAPG1, 2, 5, 7 and TPG6, PS2 genes were specifically induced and continuously overexpressed linearly along with abscission progression in tomato flower AZ. Similarly, the same set of genes were up-regulated upon abscission induction at the early hours (24 h) in the leaf AZ, indicating potential involvement in organ abscission. Our study provides new insights for the regulation of the early events in the process of tomato organ abscission and a novel trait for molecular breeding.

Key words: Microarray, polygalacturonase, gene expression, abscission, tomato, abscission-zone, breeding, RT-PCR.

Introduction

The cultivated tomato (*Solanum lycopersicum*), is the second most consumed vegetable worldwide and a well studied crop species in terms of genetics, genomics and breeding. Abscission is a natural process of plant development, in which organs such as leaves, flowers, fruits, and even branches, separate from the parent plant (Lewis *et al.*, 2006; Osborne, 1989). This process is required to recycle nutrients for continuous growth, to develop appropriate organs, survive diseases and facilitate reproduction (Addicott, 1982; Gonzalez-Carranza *et al.*, 2002; Lewis *et al.*, 2006; Sexton and Roberts, 1982). Since the era of crop domestication, a great emphasis has been put forth on the selection of genotypes/ plants exhibiting reduced abscission to improve crop quality and yield. For example, reduced seed shattering in rice (Li *et al.*, 2006), wheat (Tanno and Willcox, 2006), and maize (Doebley, 2004), and thinning in fruit tree species (Bangerth, 2000) were developed. In an agricultural perspective, both enhanced and delayed abscission is highly relevant for growers (Lewis *et al.*, 2006; Osborne, 1989). The abscission/separation process occurs in a predetermined region called abscission zone (AZ), composed of few layers of small and dense cytoplasmic cells, with small vacuoles and without any maturation characteristic, which resemble undifferentiated cells (van Nocker, 2009). Over the last two decades, abscission of Arabidopsis flower organs has served as a model for abscission research, even though Arabidopsis does not abscise its leaves or fruit, but only its floral organs (petals, sepals, and anthers) (Bleecker and Patterson, 1997; van Doorn and Stead, 1997). Further, recent advancement in the next generation sequencing (NGS) and microarray technologies enabled to obtain molecular insights of other abscission systems, such as olive fruit (Gil-Amado and Gomez-Jimenez, 2013; Parra *et al.*, 2013), melon fruit (Corbacho *et al.*, 2013), citrus leaves (Agusti *et al.*, 2008, 2009; Agusti *et al.*, 2012) and shoot tips (Zhang *et al.*, 2014), apple fruit and fruitlets (Botton *et al.*, 2011; Zhu *et al.*, 2011), litchi fruitlets (Li *et al.*, 2015) and rose petals (Singh, *et al.*, 2013).

Tomato serves as a model crop for studying fruit development (Klee and Giovannoni, 2011) and abscission (Abeles *et al.*, 1992), as it possesses a distinct joint-like structure in the AZ, comprising of 6-8 layers of cells including pedicel and the distal side of the flower pedicels (Mao *et al.*, 2000; Tabuchi and Arai, 2000). The physiology of tomato abscission was studied long ago (Roberts *et al.*, 2000; Sexton and Roberts, 1982), but the molecular mechanisms underlying the abscission process in this plant have only recently been elucidated (Liu *et al.*, 2014; Meir *et al.*, 2010, 2011; Nakano *et al.*, 2012; Nakano *et al.*, 2013, 2014; Wang *et al.*, 2013). In addition, the tomato genome sequence was published (Tomato Genome Consortium, 2012) recently, unraveling the genomic information of the crop. Significant progress has been made in understanding the coordinated action of several signaling components that regulate organ abscission. There have been several proposed models for the abscission process, classically divided into four phases, A to D (differentiation of cells, acquisition of the competence, execution of organ abscission, formation of a protective layer) (Estornell *et al.*, 2013; Patterson, 2001).

Over the years, numerous workers have reported correlations between changes in the expression of a range of cell wall-related enzymes and the events of abscission. The cell wall hydrolyzing enzymes includes expansins (Belfield *et al.*, 2005; Cho and Cosgrove, 2000), polygalacturonases (Gonzalez-Carranza *et al.*, 2002; Kalaitzis *et al.*, 1997), endoglucanases (del Campillo and Bennett, 1996; Mishra *et al.*, 2008), pectin methylesterases, pectate lyases (Sun and van Nocker, 2010) and xyloglucan endotransglucosylases (XTH) (Singh, *et al.*, 2011) which play a key role in the hydrolysis of middle lamella in the abscission zone cells leading to the cell separation. The increased activity of cellulases, CEL1, CEL2, CEL3 in the AZ was reported to be involved in the tomato flower pedicel and leaf abscission (Kuang *et al.*, 2012; Meir *et al.*, 2006; Roberts *et al.*, 2002; Roemer *et al.*, 2008). Among the cell wall hydrolyzing enzymes, polygalacturonases play a major role in the abscission process. In Arabidopsis, the polygalacturonase gene *ADPG1* is involved in the anther dehiscence, *ADPG2* involved in silique dehiscence, *QRT2* involved in flower abscission (Ogawa *et al.*, 2009). It was also shown that silencing the PG gene, PGAZAT delays floral organ abscission (Gonzalez-Carranza *et al.*, 2002).

Fewer studies have been carried to unravel the genes involved in abscission process in selected model systems. After the era of next-generation sequencing technologies like RNA-Seq, genome sequencing and microarrays had led to identify gene families and potential role in abscission systems. The abscission model includes Arabidopsis (Niederhuth *et al.*, 2013), Apple fruits (Zhu *et al.*, 2011), Melon fruits (Corbacho *et al.*, 2013), Citrus leaves (Agusti *et al.*, 2009). Recently we had elucidated complete transcriptome of tomato flower and leaf abscission systems (Sundaresan *et al.*, 2015). In the current study, we elucidated the tomato abscission polygalacturonase gene profiles using the microarray in tomato flower and leaf abscission systems and validated using RT-PCR technique.

Materials and methods

Plant material and abscission induction treatments: Tomato (*Solanum lycopersicum*, cv VF-36) inflorescences were harvested from 5 month old greenhouse grown plants between 08:00 and 10:00 a.m. Bunches of ex-plants containing at least 2 to 4 freshly open flowers were brought to the laboratory under high humidity conditions. Closed young flower buds and senesced flowers were removed. Pedicel abscission was evaluated by careful touching the distal side of the flower abscission zone (FAZ), and monitoring the abscised pedicels for calculating the percent of abscission at specified time intervals (0, 2, 4, 8, 14 h) after flower removal in explants. Further, the explants were maintained in conditions as described by Meir *et al.* (2010) and Sundaresan *et al.* (2018).

For leaf abscission experiments, plants from long day greenhouse having 6 to 8 true leaves were used. The leaves were debladed for 3rd and 4thleaf from cotyledon by leaving the subtended petiole of 2 cm from the abscission zone. The leaves were debladed for auxin depletion, and 48 h after leaf deblading, the plants were treated with ethylene (5 μL L^{-1}) for 24 h and transferred to the laboratory conditions. The ethylene air sample of 5 mLwas withdrawn from the chamber using gas-tight syringes and ethylene concentration was determined using Varian 3300 gas chromatograph equipped with a flame ionization detector and a C-5000 alumina packed column using helium as the carrier gas. Petiole abscission was observed by counting the number of detached petioles from leaf abscission zone (LAZ) at specified time intervals 0, 24, 48, 72, and 96 h after deblading and the same procedure was followed for the ethylene treated plants. For each study, 12 random plants were used for the experimentation and the experiment was replicated three times.

Sample collection of RNA extraction: The FAZ tissues were collected at each side of the abscission fracture around 1mm for each time point, excised less than 0.5 mm from each side of the visible AZ using a surgical scalpel. Leaf abscission zones (LAZ) tissues were collected by removing 1 mm of abscission fracture at the base of the petiole, which was still attached to the main stem at specified time intervals. For matured stem (MS) sections of about 7 mm long was collected from the basal internodal stem region, 5 cm above the ground level. The young stem (YS) section was harvested from the tender growing region of the shoot *i.e.*, 5 cm below the top expanding leaf. The young leaf (YL), *i.e.*, fully opened second leaf from the shoot apical meristem. The matured leaf (ML) was collected from the basal region of plant, which was fully matured, expanded and not turned yellow. The total root biomass (R) was harvested which includes tap root, secondary roots fibrous roots and washed with water until the soil adhering to the roots was completely removed. At least 40 segments of FAZ, LAZ; 10 samples of YL, ML, YS, MS and R were collected for each time point. All tissues collected were immediately frozen with liquid nitrogen and stored at -80°C till further use.

Gene expression profiling using the Agilent platform: RNA extraction, DNase treatment, and quality control: The tissue weighing 50 mg for each sample was frozen and homogenized using TOMY homogenizer (TOMY Micro Smash MS-100, Tomy Medico Ltd., Japan) and RNA was isolated using the Qiagen RNeasy Plant Mini kit (Qiagen, Hilden, Germany) following manufacturer's protocol with in-column DNase digestion treatment.The total RNA integrity was analyzed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, Palo Alto, CA).

Microarray labeling, hybridization, scanning, and data analysis: Individual time point tissue samples were labeled using Agilent Quick-Amp labeling Kit one-color (Agilent Technologies, USA) and cRNA was generated using the synthesized double-stranded cDNA as template. Cy3 CTP dye (Agilent) labeled cRNA was quantified using NanoDrop® ND-1000 and hybridized onto an AZ-specific microarray chip (Sundaresan *et al.*, 2015). The hybridized slides were scanned using Agilent Scanner and data was extracted and analyzed for gene expression patterns along with gene ontology using Agilent GeneSpring GX software. Statistical T-test *p*-value was calculated based on volcano plot using R programming.

cDNA- First-Strand Synthesis: Total RNA was converted to complimentary DNA (cDNA) using the Reverse Transcription System (Promega) using manufacturer protocol. Totally 2 µg of RNA was used for the cDNA construction from each samples using Oligo$(dT)_{15}$ primers. The synthesized cDNA was stored at -20°C till future use.

RT-PCR analysis: The cDNA was diluted to 1:20 concentration and normalized against *betatublin2* gene. The gene-specific primers were designed using IDT Primerquest tools and their respective annealing temperatures are listed in Table 1. The number of PCR cycles was optimized by the level of amplification

Table 1: List of Genes and primer Sequences used for semi quantitative RT-PCR analysis

Gene name	Transcript Identity	RT-PCR Primer sequence	Product size (bp)	Annealing temp (°C)
TAPG1	U 23053	F- GGGCTTGCAAGAACTCCAACAACA R- CATTGCTAGGCCTTGCCCAAGTTT	459	60
TAPG2	U 70480	F- TGCATCTCTATTGGCCCTGGAACT R- ACACCTTCAAGTGTTATGCCGCTG	428	62
TAPG4-	U 70481	F- TTGCCCTAAAGGAACTACGGCACT R- ACCAGAAGCTCTTCCTCCAGCATT	730	62
BETA TUBULIN2	TC 171630	F- AGGGCATTATACTGAAGGCGCTGA R- TCTGTATTGCTGTGAACCACGGGA	538	61

in end product for easy comparison under agarose gel. PCR was performed using Ampliqon Taq DNA Polymerase Master Mix and amplified in a peqSTAR 96 Universal Gradient thermal cycler (PEQLAB, Germany). The end PCR product was run on 0.8 % agarose gel and imaged using Image master VDS 1208 system.

Results

Flower and leaf abscission: The tomato flowers and leaves were removed to induce the abscission as the flowers and the leaf are the main source of auxin which inhibits abscission in the AZ (Fig. 1 A and B). Leaf petioles did not abscise upto 24 h after deblading, whereas the flower pedicels started to abscise 8 h after flower removal and 100 % of the pedicels abscised completely in 14 h after flower removal (Fig. 1C). Ethylene treatment applied for 24 h had no effect on leaf abscission in control (without deblading) plants during at least 96 h after application (data not shown). Notably, in debladed plants, the ethylene effect on petiole abscission was already pronounced after 24 h of treatment. Thus, 50 % of the petioles abscised after 48 h in response to ethylene, whereas in untreated debladed plants, only 10 % of the petioles abscised (data not shown). After 96 h, almost 100 % of the petioles in the ethylene-treated plants abscised (Fig. 1C). These results indicate that ethylene is effective in inducing abscission only in debladed plants.

Expression pattern of polygalacturonase genes in tomato FAZ and LAZ: Our results show that all the tomato abscission polygalacturonase genes (TAPG1, 2, 4) specifically expressed in the FAZ and LAZ (Fig. 2) and not expressed in the non abscission zone tissues (NAZ) of flower pedicel (FNAZ) and leaf petiole (LNAZ) except TAPG1 with low expression in the leaf NAZ at the final stage of abscission (Fig. 3). In the FAZ, the *TAPG1, 2, 4, 5* and PG7 its expression was low at 0 h but increased gradually up to 14 h after flower removal upon abscission progression (Fig 2). Similarly, the *TAPG1, 2, 4, 5* and *PG7* its expression was low at 0 h but increased gradually up to 72 h after leaf deblading and declined after that upon abscission progression (Fig 2, 3A, and 4) except PG7, where the expression declined by 42 h after leaf deblading. Contrasting to *TAPGs*, the wound-inducible PG / Pg related to germination (XOPG1) gene is downregulated during abscission progression in both the systems (Fig. 2 F and L).To conclude, *TAPG1, 2, 4, 5* and PG7 was highly expressed in the FAZs and LAZs in parallel with the abscission progression (Fig. 2).The RT-PCR results (Fig 3) confirms the microarray results (Fig. 2).

Expression pattern of polygalacturonase genes in other tissues: The expression profile of the *TAPGs* was also analyzed in various tissues such as young leaves (YL), old leaves (OL), young shoots (YS), old shoots (OS), roots (R) (Fig. 3). None of the TAPG1, 2, 4 was expressed in any of the above tissue samples which clearly demonstrates the specificity of these enzymes and potential role in abscission systems.

Discussion

The understanding of abscission mechanisum is of great importance to control seed, fruit drop and harvesting practices. Thus, advances made on model plants and crops are of major importance since they may provide potential candidate genes for further biotechnological applications. Tomato is a very convenient model crop to study the abscission process as it develops a distinct AZ in the middle of flower pedicels and at base of leaf petioles. It is one of the oldest crop plants, in which the genetic linkage map was constructed and currently we have high density

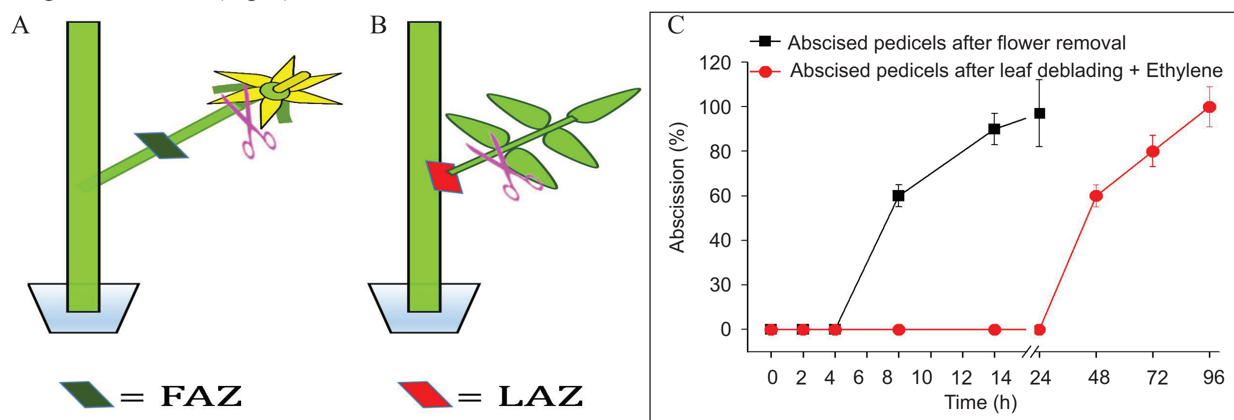

Fig.1. Schematic illustration of flower (A) and leaf (B) explants of tomato (*Solanum lycopersicum* cv. VF-36) held in DDW, before flower removal and leaf deblading, respectively, and their relative position of cuts indicated by the scissors; Schematic presentation of the FAZ and LAZ tissue sampling for RNA extraction (Sundaresan *et al.* 2015). Ethylene pretreatment was performed by exposing the explants after leaf deblading to ethylene (10 ppm) for 24 h in a closed chamber at 20°C. (C) Effect of flower removal or leaf deblading + ethylene (10 ppm for 24 h) on the kinetics of pedicel/ petiole abscission. The results are means of four replicates (30 flowers or leaves each) ± SE.

molecular map (with most of the wild species), molecular markers more than 2200 with average distance less than 1cM of different types including RFLPs, AFLPs, SSRs, CAPS, RGAs, ESTs, and COSs mapped on the complete 12 chromosomes and also QTL of agriculturally important traits had been mapped (Tomato Genome Consortium, 2012).

Our data demonstrate an important role for *TAPGs* in tomato flower and leaf abscission. It is well established that the activation of abscission machinery involves high coordination of gene networks which involves various alterations in the TF networks (Estornell *et al.*, 2013), cell wall remodeling enzymes, protein modifications, and synthesis of defense proteins at the last stage of abscission process. Gene encoding the tomato polygalacturonases (*TAPGs*) was isolated from the tomato (Kalaitzis *et al.*, 1995;

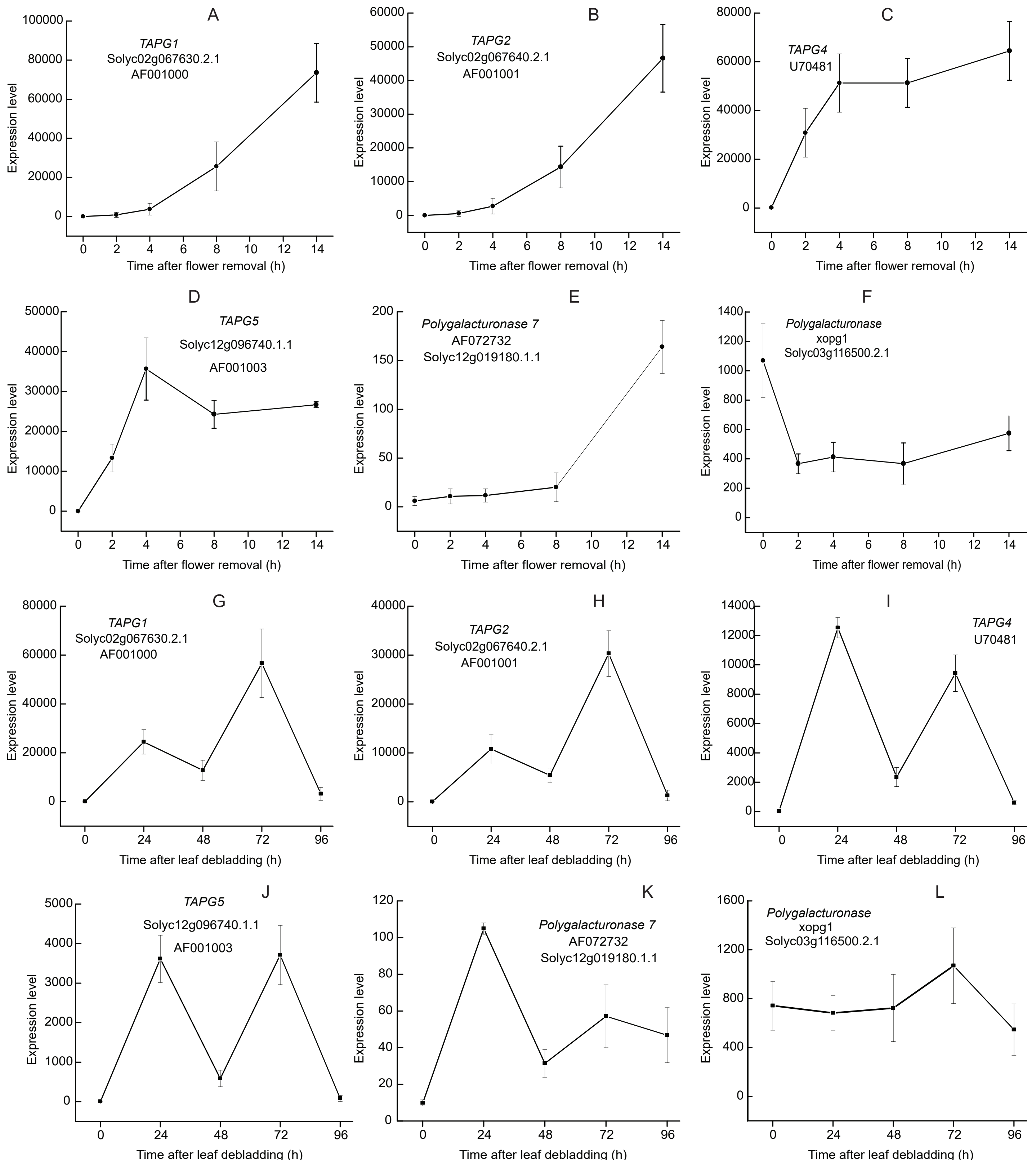

Fig. 2. Microarray expression profiles of tomato abscission polygalacturonase genes in the FAZ (A - F) and LAZ (G – L)at various time points following abscission induction (A-I). The assay included members of the tomato abscission polygalacturonase family genes TAPG1, 2, 4, 5, 7 and XOPG1.

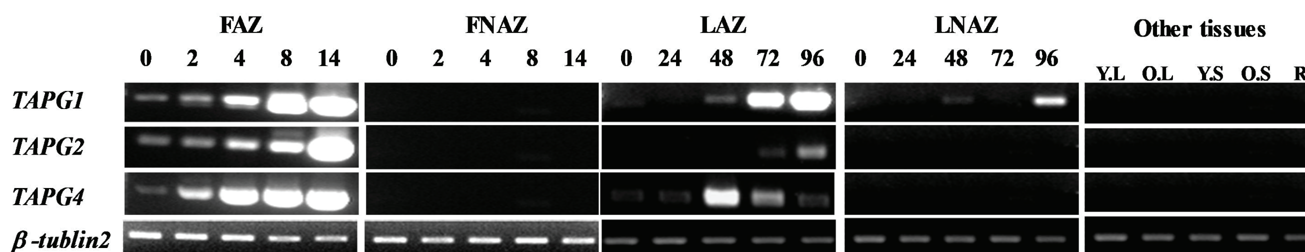

Fig. 3. The RT-PCR results of tomato abscission polygalacturonase genes in the FAZ, FNAZ, LAZ, LNAZ at various time points following abscission induction. The expression levels were also determined at the young leaf (Y.L), old Leaf (O.L), young shoots (Y.S), old shoots (O.S), roots (R). The FAZ and FNAZ results have been already published in our earlier manuscript (Meir *et al.*, 2010). Similar results were obtained for three biological replicates.

Kalaitzis *et al.*, 1997). It has been shown that the three tomato abscission polygalacturonases TAPG1, TAPG2, and TAPG4 are involved in the tomato fruit abscission (Kalaitzis *et al.*, 1997) and we have also shown there temporal and spatial expression pattern in the tomato flower abscission zone during abscission process (Jiang *et al.*, 2008; Meir *et al.*, 2010). Silencing the Tomato abscission polygalacturonases (TAPG1) gene delayed the tomato petiole abscission and also shown that the increased break strength is needed in the abscission zone in silenced plants even after ethylene treatment (Jiang *et al.*, 2008). The plants transformed with an antisense construct of *KD1* (Ma *et al.*, 2015) and *THyPRP* is driven by the abscission specific *TAPG4* promoter strongly inhibited both pedicel and petiole abscission (Sundaresan *et al.*, 2018). This clearly shows the potential role of TAPG4 in delaying abscission process. However, there has been no definitive demonstration of a role for a TAPG in tomato leaf abscission. This is the first time we report the involvement and temporal expression pattern in the tomato leaf abscission zone (LAZ), LNAZ, roots, shoots, leaves (Fig 2 and 3). The current microarray results of FAZ data (Fig 2 and 3) were in accordance with our previous microarray results (Meir *et al.*, 2010; Sundaresan *et al.*, 2015). None of the *TAPG1, 2, 4* was expressed in any of the above tissue samples,which clearly shows that the *TAPGs* are confined to abscission zone and their expression patterns are specific with abscission progression (Fig 3). The joint-less trait, where no abscission zones are formed on leaves, flowers, or fruit, would reduce product loss through dropping. Similarly, we can use the impaired PG/ TAPG Locus, a crossbreed of impaired QTLs can be utilized for the breeding to obtain fewer abscission cultivars. Moreover, the TAPG4 and TAPG5 genes were induced immediately and expressed at higher levels upon abscission induction, these gene promoters can be potentially incorporated in molecular breeding, by creating stable transformed genetic engineered plants to have less abscission prone lines.

Considerable research interest has therefore been dedicated to identify the endogenous and environmental factors that trigger the abscission process and regulate the rate at which it proceeds.The microarray results for the FAZ and LAZ allowed us to establish a clear sequence of events occurring during the acquisition of the tissue sensitivity during the early stage of abscission process and association of altered expression levels of genes related to cell wall hydrolyzing enzymes at early and late stages of abscission process. The analyses revealed that the FAZ and LAZ share both similar expression patterns during the execution of organ abscission. Our study provides new insights for the regulation of the late events in the process of tomato organ abscission and valuable information for the molecular breeding for reduced abscission.

Sequence deposition: The microarray data for the WT (cv. 'VF-36') FAZ (12 arrays) and the LAZ (12 arrays) samples were submitted in Gene Expression Omnibus database (NCBI-GEO) under the accession id GSE45355; GSE45356 and approved for the public.

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