Genome-wide identification and expression analysis of citrus fruitlet abscission-related polygalacturonase genes

Ting Ge¹ · Xue Huang¹ · Xiaoting Pan¹ · Jing Zhang¹ · Rangjin Xie¹

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Abstract

Polygalacturonases (PGs) encoded by a relatively large gene family are involved in plant organ abscission, but few data is available in citrus. Here, to explore the role of PGs in citrus fruitlet abscission (CFA), we have obtained 38 citrus PG (CitPG) members, based on the citrus genome sequences. The ORF length varied from 378 to 2418 bp, encoding proteins with theoretical pI and molecular mass ranging from 4.83 to 9.92 and from 13,951.71 to 85,542.28, respectively. Most *CitPGs* contained no less than 3 introns, suggesting a high probability of alternative splicing. Phylogenetic tree revealed that all PGs could be divided into three groups, in which 9 CitPGs, including CitPG2, CitPG3, CitPG10, CitPG24, CitPG27, CitPG29, CitPG30, CitPG33 and CitPG34 possessed a close relationship with abscission-associated PGs, indicating their role in CFA. Expression analysis further demonstrated that CitPG2, CitPG29 and CitPG34 might be involved in CFA, the expression levels of which could be induced by ethylene, inhibited by IAA and increased during CFA. The findings in this study have provided a foundation for future studies to elucidate the roles of *CitPGs* in CFA.

Keywords Citrus · Polygalacturonase · Fruitlet abscission · Gene expression · Cell wall

Introduction

Fruit shedding is a highly coordinated developmentally regulated event that enables fruit trees to discard infected, damaged or/and no longer needed fruits (Xie et al. 2013). In citrus, there are two successive but distinguishable abscission waves which are observed during fruit development (Iglesias et al. 2006; Xie et al. 2015a, b). The first wave is characterized by the shedding of massive flower and ovary from abscission zone A (AZ A) which lies at fruit pedicel, and the second one, namely “June drop”, discards a great number of growing fruitlets from abscission zone C (AZ C) which locates between calyx and ovary. With fruitlet development,

AZ A progressively becomes inoperative while AZ C is activated (Iglesias et al. 2006).

In citrus, shedding from AZ A is a major limiting factor in fruit yield, which is finely mediated by hormones including ethylene, ABA and auxin, etc. (Gómez-Cadenas et al. 2000; Iglesias et al. 2006; Xie et al. 2013, 2015a, b). Accumulating data have demonstrated that ethylene can promote fruit abscission. Exogenous application of aminoethoxyvinyl-glycine (AVG), an inhibitor of ethylene biosynthesis, obviously decreases citrus fruitlet abscission (CFA); on the contrary, 1-aminocyclo-propane-1-carboxylic acid (ACC), an ethylene precursor, effectively promotes CFA (Gómez-Cadenas et al. 2000; Iglesias et al. 2006). Similar results have also been reported in other fruit crops including apple (Stopar et al. 2007), mango (Hagemann et al. 2015), tomato (Zhang et al. 2015) and litchi (Li et al. 2015), plum (Meland and Kaiser 2016), etc. Compared to ethylene, auxin has opposite effect on CFA (Xie et al. 2015a, b). Treating the distal end of bean leaf explants with IAA could delay cell separation (Jacobs 1962), showing a more important role of auxin than ethylene in fruit abscission (Basu et al. 2013). Similarly, perturbations to polar auxin transport (PAT) with 2,3,5-triiodobenzoic acid blocks auxin through AZ tissues, obviously promoting fruit abscission (Else et al. 2004; Blanusa et al. 2005).

Ting Ge and Xue Huang have contributed equally to this work.

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✉ Rangjin Xie
xierangjin@163.com

¹ Citrus Research Institute, Southwest University, Chongqing 400716, China

Before fruit abscission, cell separation occurs in AZ, which is concomitant of intercellular space increase and middle lamella lysis which result from the degradation and/or remodeling of cell wall polymers, especially pectin in the middle lamella (Peng et al. 2013). Therefore, the genes involved in cell wall degradation have been widely studied with an aim to elucidate the molecular events underlying fruit abscission (Roongsattham et al. 2012; Peng et al. 2013). Of such genes, *polygalacturonases* (PGs) play a pivotal role in fruit abscission by depolymerizing the homogalacturonan backbone of pectin (Tucker et al. 1984; Bonghi et al. 1992; Roongsattham et al. 2012; Peng et al. 2013). Loss of an abscission-related PG function by T-DNA insertion could delay *Arabidopsis* floral organ shedding (González-Carranza et al. 2007). In tomato, three PGs including *TAPG1*, *TAPG2*, and *TAPG4* with different spatial and temporal expression profiles are all involved in fruit abscission (Kalaitzis et al. 1997). Similar cases are also observed in sweet cherry (Else et al. 2004; Blanus et al. 2005), citrus (Xie et al. 2015a, b, 2018), litchi (Li et al. 2015) and oil palm (Roongsattham et al. 2012). Even so, the role of PGs in CFA needs to be further investigated.

Recently, citrus genome sequences were released (<https://www.phytozome.net>), making genome-wide identification and analysis of PG genes performable. Here, we comprehensively investigated PG members including total number, gene structure, phylogenetic relationship, sequence conservation, and protein domain. After that, their expression patterns during CFA and under hormone treatments were also investigated.

Materials and methods

Materials and treatments

Seven-year-old trees (*Citrus sinensis* L. cv. 'Tarocco'), *Poncirus trifoliata* (L.) Raf. as rootstock, were used. At 7 days after anthesis, 1650 fruitlets were collected by manually cutting 4 cm below AZ A and then removed their ovaries. After that, the calyxes were treated with H₂O (as control), 1.5 mM 1-aminocyclo-propane-1-carboxylic acid (ACC) and 1.2 mM IAA, 550 calyxes for each treatment, and then put them in tissue culture vessels with MS media. After covered lids, samples were kept in a light chamber at 28 °C. AZ A tissues were collected by manually cutting ~1 mm at each side of the abscission fracture plane at 0 h, 6 h, 12 h, 18 h, 24 h after treatment, 100 fruitlets for each time point. Because all the calyxes treated by ACC were shed at 24 h, the AZ A tissues were not collected at this time point. All the samples were quick frozen using liquid nitrogen and stored at −80 °C until used. In parallel, the remaining 50 calyxes of each treatment (H₂O, ACC and IAA) were equally divided into 2 groups,

the abscission rate of which was measured with the following formation. Statistical significance was calculated with Student's *t* test.

Abscission rate

$$= \frac{[(\text{number of abscising calyxes}) / \text{total calyxes}]}{\times 100\%}.$$

Identification of PG genes and phylogenetic analysis

To identify PG genes from citrus (sweet orange and clementine) genomes (<https://www.phytozome.net>), a BLASTP search with *p* = 0.001 was performed using *Arabidopsis* PGs as seed sequences including At3g07970, At2g43860, At2g43890, At2g43880, At3g57510 and At2g41850. All the sequences containing typical PG domain (GH28) were obtained from the citrus genome database, which were referred to as CitPG1, CitPG2, ..., CitPGn, respectively. The functional and conserved domains were further testified by PROSITE program (<https://www.expasy.org/tools/scanprosite/>) (Apweiler et al. 2001). Additionally, a phylogenetic tree was built with CitPGs and some abscission-related PGs which were from *Arabidopsis*, soybean, grape, melon, papaya, pear, peach, tomato, oil palm, tobacco, kiwi, apple, litchi and *Brassica napus*, and downloaded from NCBI database (<https://www.ncbi.nlm.nih.gov/>). Phylogenetic tree was build using MEGA version 4.0 with NJ method at a bootstrap of 1000 replicates.

Sequence analysis

In order to characterize CitPGs, multiple sequence alignment was performed by ClustalX (Larkin et al. 2007) with default parameters. To obtain optimized alignment, the deduced amino acid sequences were adjusted manually using BioEdit (version 7.0.0) with default parameters [The National Resource for Biomedical Supercomputing (NRBSC): <https://www.nrbsc.org/>] (Nicholas et al. 1997). The genomic and cDNA sequences of each predicted gene were downloaded from the citrus genome database (<https://www.phytozome.net>), and then gene structure including intron distribution patterns, phases and intro-exon boundaries was analyzed using the GSDS web-based bioinformatics tool (<https://www.gsds.cbi.pku.edu.cn/>). The chromosomal position was provided by the Citrus Genome Database (<https://www.phytozome.net>).

Expression analysis

According to manufacturer's instructions, total RNA was extracted with RNAPure pure plant Kit (TIANGEN, China). About 1 µg total DNA-free RNA was used to synthesize first strand cDNA with PrimeScript 1st Strand cDNA Synthesis

Kit (TaKaRa, Japan). In this study, reverse transcription semi-quantitative polymerase chain reaction (RT-sqPCR) was used to identify the CFA-related *CitPGs* which were further testified by reverse transcription real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Citrus *actin* gene served as normalizer. With a Eppendorf Mastercycler (Serial No. 6333CO405507, Germany), RT-sqPCR was carried out in a 20 µl reaction volume containing 1 µl cDNA, 2 µl 10× PCR buffer, 0.4 µl of each primers (0.1 µM), 0.4 µl dNTP (2.5 mM), 0.1 µl Taq enzyme (5 U/µl) and 15.7 µl water, and the PCR program was 2 min at 94 °C, followed by 28 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C, with a final step at 72 °C for 2 min. PCR products were detected by electrophoresis in 1% agarose gel. RT-qPCR was performed using iCycler iQ5 real-time detection system (Bio-Rad) for 30 s at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C, with a final step at 72 °C for 1 min. The 10 µl reaction mixture containing 1 µl cDNA, 5 µl 2× SYBR Green PCR Master Mixture, 0.2 µl of each primers (10 µM) and 3.6 µl water. Each expression profile was independently verified in 3 biological replicates performed under identical conditions. Relative expression level was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). PCR primers used for RT-sqPCR and RT-qPCR were designed out of the conserved region and their amplification products varied from 130 to 200 bp in length. All primer sequences were listed in Table 1.

Results

Abscission rate

As shown in Fig. 1, the calyxes treated by ACC began to shed strikingly at 18 h, the abscission rate (AR) reaching 36.7%, significantly higher than 5% of the control. At 24 h, the ARs of ACC treatment and the control reached 100% and 90%, respectively. On contrary, the ARs of IAA treatment reached 0% and 12% at 18 h and 24 h, respectively, remarkably lower than that of ACC treatment as well as the control. Notably, the AR at 30 h after IAA treatment just reached 24%. Taken together, these results showed that ACC could prompt CFA and IAA had opposite effect.

Identification of PG genes in citrus

With *Arabidopsis* PGs as query sequence, BLAST search was performed at Phytozome (<https://www.phytozome.net>) with an aim to identify the PG genes in citrus. After protein domain and gene structure analysis, a total 38 PG members were identified. Of them, 2 members including

CitPG20 and 27 only belonged to clementine and 15 members specially belonged to sweet orange including CitPG8, 10, 22, 24–26, 28 and 31–38 (Fig. 2, Table S1), suggesting this gene family in sweet orange is more complex than that in clementine. ORF length varied from 378 (*CitPG24*) to 2418 bp (*CitPG19*), correspondingly encoding proteins with 125 and 805 amino acids. The theoretical pI and molecular mass of predicted proteins ranged from 4.83 (*CitPG11*) to 9.92 (*CitPG22*) and from 13,951.71 (*CitPG24*) to 85,542.28 (*CitPG19*), respectively, showing a significantly physiological and biochemical difference (Table 2).

Gene structure and chromosomal distribution

The gene structure of *CitPGs* was investigated by comparing full-length cDNAs with their corresponding genomic DNA sequences. The results showed that the number of introns varied from 1 (*CitPG24*) to 9 (*CitPG1* and *CitPG5*) (Fig. 2 and Table 2) with intron phase 0, 1 and 2. Thirty-five *CitPGs* contained no less than 3 introns, suggesting a high probability of alternative splicing.

Based on genomic locations (Table S1), a diagram was drawn by hand, to exhibit the distribution of CitPGs on sweet orange and clementine genomes (Fig. 3). On clementine genome, a total of 23 CitPGs unevenly distributed on scaffold 1, 2, 3, 4, 7 and 9, respectively. Briefly, CitPG15 existed on scaffold 1; CitPG13, CitPG16 and CitPG21 on scaffold2; CitPG23, CitPG29, CitPG14, CitPG18, CitPG4, and CitPG30 on scaffold 3; CitPG2, CitPG12 and CitPG17 on scaffold 4; CitPG1, CitPG5–7 and CitPG9 on scaffold 7; CitPG 3, CitPG 11, CitPG 19, CitPG 20 and CitPG27 on scaffold 9. Additionally, comparative analysis showed that the genome distribution was highly conserved between the sweet orange and clementine, for example the members on scaffold 7 of clementine were all arranged on scaffold00369 of sweet orange in the same order.

Phylogenetic analysis of CitPGs

Phylogenetic analysis showed that all PG proteins were clustered into 3 groups, referred to as G1, G2 and G3, respectively (Fig. 4). G1 contained 23 PG members, 14 from citrus and 9 from other plants. G2 consisted of 20 PGs, of which 16 and 4 members were belonged to citrus and other plants, respectively. Most notably, G3 were composed of 9 PGs, of which 8 members were from citrus. Further analysis revealed that CitPG33 and CitPG34 were closely related to the abscission-related CmPG1, similar cases were observed between CitPG10 and At2g43890, CitPG24 and At2g3880, CitPG3 and QRT2, CitPG27, 29–30 and LcPG1 as well as CitPG2 and GmPG11, indicating these CitPGs might be involved in organ abscission.

Table 1 The primers used for RT-qPCR and RT-sqPCR

No.	Gene names	Primer sequences	Gene IDs
1	CitPG01	Forward: 5'-GATGGCTCTACTTTAATCGG-3' Reverse: 5'-GACCACCCTAAGGTTCTGA-3'	Ciclev10027523m/Orange1.1g013309m
2	CitPG02	Forward: 5'-TGAAACGACTTTGACGAGG-3' Reverse: 5'-GATTTTGTGTTGGGGACG-3'	Ciclev10033386m/Orange1.1g043061m
3	CitPG03	Forward: 5'-TTGTTGTAGTGCCCCGTAG-3' Reverse: 5'-ACAGTGCCACCACCTTCG-3'	Ciclev10005317m/orange1.1g028660m
4	CitPG04	Forward: 5'-AGGCAGCATAGTATTTCAGG-3' Reverse: 5'-GGCAGATCCCACCATTTA-3'	Ciclev10024556m/Orange1.1g040748m
5	CitPG05	Forward: 5'-GCAGTGCCACAGCAAAGT-3' Reverse: 5'-CTTCCATTCTAGGACCGT-3'	Ciclev10026919m/Orange1.1g047411m
6	CitPG06	Forward: 5'-AACTTTGGTGCTGTGGGA-3' Reverse: 5'-CTCAATCTGAAGTTGGAGGT-3v	Ciclev10027137m/Orange1.1g039868m
7	CitPG07	Forward: 5'-GCAATCTGGGGATCAAGT-3v Reverse: 5'-GGTACAAGTAGCGGTGGA-3'	Ciclev10027074m/Orange1.1g041224m
8	CitPG08	Forward: 5'-AATCACTCGGGAATGGTC-3' Reverse: 5'-CTCCTGGTGTCTGTTGCA-3'	Ciclev10030577m/Orange1.1g042447m
9	CitPG09	Forward: 5'-AGCGATGTGCGATATTTG-3' Reverse: 5'-TTTCCTTAGCGACTCCA-3'	Ciclev10027366m/Orange1.1g047738m
10	CitPG10	Forward: 5'-TTCTAAACACGGACTACTGA-3' Reverse: 5'-TTAACCTCCAAACCTTCA-3'	Ciclev10004869m/orange1.1g038762m
11	CitPG11	Forward: 5'-GGGTGCTCAGATGTGGAT-3' Reverse: 5'-CAAATGACAAGTCGGAAACG-3'	Ciclev10004899m/Orange1.1g012057m
12	CitPG12	Forward: 5'-GTGTCTCCGATATTGTTGTG-3' Reverse: 5'-CTCCTGTTTGGTTGTTGC-3'	Ciclev10033716m/Orange1.1g046362m
13	CitPG13	Forward: 5'-TGTAAGATAGAGGGAGCAA-3' Reverse: 5'-TCCAGAATTTGTAGGAGC-3'	Ciclev10015111m/Orange1.1g040454m
14	CitPG14	Forward: 5'-TCAAAATCACCCACTCTTCC-3' Reverse: 5'-TTGTTGTGCCCCATAAACG-3'	Ciclev10023493m/Orange1.1g040962m
15	CitPG15	Forward: 5'-GCTTAGTAGGCATTGGAG-3' Reverse: 5'-CTTTATTAGCGGGTATTTTC-3'	Ciclev10010440m/Orange1.1g037736m
16	CitPG16	Forward: 5'-TGGCAGTTGGGTTTCATT-3' Reverse: 5'-CGTCTTGTACCACCGAGTT-3'	Ciclev10015423m/Orange1.1g015254m
17	CitPG17	Forward: 5'-GCTTTGAGGTTCTACGGG-3' Reverse: 5'-CACATCCTTGGAGTTTTC-3'	Ciclev10031220m/Orange1.1g009748m
18	CitPG18	Forward: 5'-TTGCTTCTCACTGGTGGA-3' Reverse: 5'-AAGACGAGGAGGATGTGG-3'	Ciclev10023712m/Orange1.1g043757m
19	CitPG19	Forward: 5'-TTCGTGGCAACCAACTCC-3' Reverse: 5'-CGTCAGGTTCTGCTCCATA-3'	Ciclev10004345m/Orange1.1g039959m
20	CitPG20	Forward: 5'-CACTTTACAGGCACAAG-3' Reverse: 5'-GATCAATGACAATGGGATTT-3'	Ciclev10006970m
21	CitPG21	Forward: 5'-GACCCTGCAAATCTCCTA-3' Reverse: 5'-TCAAATGTTCTCCTCCA-3'	Ciclev10017883m/Orange1.1g045893m
22	CitPG22	Forward: 5'-GTTTCAGCTCCTACTCCC-3' Reverse: 5'-TGCTTTCTATTCCATCCC-3'	Ciclev10030302m/Orange1.1g042417m
23	CitPG23	Forward: 5'-GGCACGCTTCTTTATGTTTC-3' Reverse: 5'-CTTCCCCTTCCATACGAT-3'	Ciclev10020162m/Orange1.1g013402m
24	CitPG24	Forward: 5'-CGATAAAGGATTTCCGAGGA-3' Reverse: 5'-CAGCGTGAAGTTACTGGTGAG-3'	Ciclev10003286m/Orange1.1g020062m
25	CitPG25	Forward: 5'-TGGCAGAGGTAGGGAGTT-3' Reverse: 5'-CAGATGAGGTCGGGTGTAA-3'	Ciclev10020035m/Orange1.1g012308m

Table 1 (continued)

No.	Gene names	Primer sequences	Gene IDs
26	CitPG26	Forward: 5'-GTTACGATTTTGGCTCCC-3' Reverse: 5'-GAACAACAAGGTTACGGATG-3'	Ciclev10008093m
27	CitPG27	Forward: 5'-GACGGCAAAACCTCCAAC-3' Reverse: 5'-AGGCCACTCTGATTCATCCT-3'	Ciclev10004864m
28	CitPG28	Forward: 5'-TCTTACCAGCCACCTCAC-3' Reverse: 5'-CACCTGTTACTACCACATCG-3'	Ciclev10004942m/Orange1.1g012788m
29	CitPG29	Forward: 5'-GAACTATTGATGGTCAGGGTG-3' Reverse: 5'-CGCTAAGATCGTTATGCC-3'	Ciclev10019941m/Orange1.1g011659m
30	CitPG30	Forward: 5'-GCAAGGAGACTAGACTCATTTG-3' Reverse: 5'-TCCCAGCAGGAACATACA-3'	Ciclev10019597m/Orange1.1g009075m
31	CitPG31	Forward: 5'-CCAGAAAATAGTCCCCACA-3' Reverse: 5'-CACCAAGGCTCCTAATGC-3'	Orange1.1g047599m
32	CitPG32	Forward: 5'-TTATGTAGCCGTTCTGTTC-3' Reverse: 5'-CCTTAAATGCCTGAGTATCA-3'	Orange1.1g041470m
33	CitPG33	Forward: 5'-CAGAACGGACTCAACAAAAG-3' Reverse: 5'-GCGATAATCAGATGGAGCA-3'	Orange1.1g041993m
34	CitPG34	Forward: 5'-ATTAGCATCCGTAGATAGCC-3' Reverse: 5'-CACAGTCATCACCAGTCCC-3'	Orange1.1g027251m
35	CitPG35	Forward: 5'-TGGCATTAGCATTGGAAGC-3' Reverse: 5'-GAACATTTTCGCATGACAGCA-3'	Orange1.1g043588m
36	CitPG36	Forward: 5'-GCCACAATAAATGTACCAAA-3' Reverse: 5'-CCTTCAAAGCTAAGCCAAT-3'	Orange1.1g036371m
37	CitPG37	Forward: 5'-CATCAAGACCGGCGATGA-3' Reverse: 5'-GCCTAATGCCTTCCCAATT-3'	Orange1.1g046693m
38	CitPG38	Forward: 5'-GAGCAGGATTACAGTTCAG-3' Reverse: 5'-TTTACCATCAAGAGTGCC-3'	Orange1.1g046411m
39	CitPG39	Forward: 5'-ACAGTCAGCAAACCTCACCTT-3' Reverse: 5'-CAATCGTCTCCGGTCTTAA-3'	Orange1.1g037377m
40	CitPG40	Forward: 5'-TTATGTGCCAACAGGGAG-3' Reverse: 5'-AAGAGTGCCACCAATGACT-3'	Orange1.1g041973m
41	CitPG41	Forward: 5'-GTTTCGTTCTTACTTTCG-3' Reverse: 5'-ACCAATACTGTGCCTTTT-3'	Orange1.1g047946m
42	CitPG42	Forward: 5'-TGGAAGATACCCGAATG-3' Reverse: 5'-GAACGGACAATATGCCTGA-3'	Orange1.1g047315m
43	CitPG43	Forward: 5'-GCCTCCAACGAATCAACA-3' Reverse: 5'-ATGGAAACACCGCTGACT-3'	Orange1.1g045402m
44	CitPG44	Forward: 5'-GCTCCAACAGATTACCGC-3' Reverse: 5'-TACAAGCCCACAACGAAG-3'	Orange1.1g048431m
45	CitPG45	Forward: 5'-AAACCATTGCAGCGATAT-3' Reverse: 5'-GATCACCAAGGCGATTGA-3'	Orange1.1g040317m
46	CitPG46	Forward: 5'-TGGACCATGTAGGCACAG-3' Reverse: 5'-TGGAGGAGGGCAACTTTT-3'	Orange1.1g047322m

Expression patterns of PG genes during CFA

Expression analysis revealed that *CitPG2*, *CitPG29* and *CitPG34* were related to CFA. Their expression levels were significantly increased during CFA and could be

up- and down-regulated by ACC and IAA, respectively (Fig. 5). With the exception of *CitPG2*, *CitPG29* and *CitPG34*, other *CitPGs* were expressed either at undetectable level or unchangeably, showing no direct relation to CFA.

Fig. 1 Effect of the IAA or 1-aminocyclo-propane-1-carboxylic acid (ACC) on citrus fruitlet abscission. Each point is mean $\pm$ SD of abscission rates of two sets of calyxes. Different lowercase letters at the same time point represent significant differences ($p < 0.05$)

Discussion

In citrus, the fruitlet abscission from AZ A is regulated by a strong hormonal component (Iglesias et al. 2006; Xie et al. 2013). More recently, it is widely accepted that ethylene plays a crucial role in promoting fruit abscission (Gómez-Cadenas et al. 2000; Iglesias et al. 2006; Stopar et al. 2007; Hagemann et al. 2015; Li et al. 2015). On the contrary, auxin can inhibit CFA (Xie et al. 2015a, b, 2018). In this study, the abscission rates were significantly increased by ACC and remarkably inhibited by IAA, which was consistent with the results reported previously (Iglesias et al. 2006; Xie et al. 2013, 2015a, b, 2018).

In plants, PGs are mainly responsible for degradation of pectin in middle lamella, which are encoded by a relatively large gene family (Rascio et al. 1987; Bowling and vaughn 2011; Roongsattham et al. 2012; Peng et al. 2013). To date, a lot of PG genes have been isolated from fruit crops

Fig. 2 Gene structures of 38 *CitPG* genes was built by GSDS web-based bioinformatics tool (<https://gsds.cbi.pku.edu.cn/>). Black box and line indicate exon and intron, respectively. The number on black line indicates the phases of corresponding introns

Table 2 The information of PG genes in citrus

Gene	Locus ID		ORF length (bp)	No. of introns	Prediction proteins		
	Clementina	Sweet orange			Length (aa)	PI	Mass (Da)
CitPG1	Ciclev10027523m	Orange1.lg013309m	1284	9	427	8.44	46,244.57
CitPG2	Ciclev10033386m	Orange1.lg043061m	1257	8	418	8.31	45,857.81
CitPG3	Ciclev10005317m	Orange1.lg028660m	1050	8	349	5.78	37,648.33
CitPG4	Ciclev10024556m	Orange1.lg040748m	1812	6	603	6.04	65,305.89
CitPG5	Ciclev10026919m	Orange1.lg047411m	1221	9	406	5.66	43,529.96
CitPG6	Ciclev10027137m	Orange1.lg039868m	1173	7	390	5.39	41,776.68
CitPG7	Ciclev10027074m	Orange1.lg041224m	1203	8	400	5.99	42,492.68
CitPG8	–	Orange1.lg042447m	1068	8	355	5.18	38,210.11
CitPG9	Ciclev10027366m	Orange1.lg047738m	1248	8	415	6.09	44,845.31
CitPG10	–	Orange1.lg037377m	1164	3	387	9.72	41,772.95
CitPG11	Ciclev10004899m	Orange1.lg012057m	1491	6	472	4.83	51,221.75
CitPG12	Ciclev10033716m	Orange1.lg046362m	1452	7	483	9.06	53,124.81
CitPG13	Ciclev10015111m	Orange1.lg040454m	1422	5	473	5.87	49,955.18
CitPG14	Ciclev10023493m	Orange1.lg040962m	1191	2	396	9.02	42,713.51
CitPG15	Ciclev10010440m	Orange1.lg037736m	1260	8	419	4.96	43,914.68
CitPG16	Ciclev10015423m	Orange1.lg015254m	1233	3	410	8.16	43,421.33
CitPG17	Ciclev10031220m	Orange1.lg009748m	1584	6	527	8.89	57,379.37
CitPG18	Ciclev10023712m	Orange1.lg043757m	1224	3	407	7.01	43,044.86
CitPG19	Ciclev10004345m	Orange1.lg039959m	2418	8	805	5.50	85,542.28
CitPG20	Ciclev10006970m	–	1143	3	380	6.52	41,222.92
CitPG21	Ciclev10017883m	Orange1.lg045893m	1014	2	337	8.73	36,706.86
CitPG22	–	Orange1.lg047322m	1029	3	343	9.92	37,142.96
CitPG23	Ciclev10020162m	Orange1.lg013402m	1332	4	443	8.34	48,127.63
CitPG24	–	Orange1.lg040317m	378	1	125	8.59	13,951.71
CitPG25	–	Orange1.lg048431m	1191	3	396	8.95	42,416.48
CitPG26	–	Orange1.lg045402m	1191	3	396	8.90	42,263.33
CitPG27	Ciclev10004864m	–	1446	5	481	7.51	52,870.36
CitPG28	–	Orange1.lg047315m	1254	5	417	8.91	44,578.90
CitPG29	Ciclev10019941m	Orange1.lg011659m	1443	5	480	5.71	52,912.12
CitPG30	Ciclev10019597m	Orange1.lg009075m	1635	6	544	8.50	59,867.26
CitPG31	–	Orange1.lg041973m	1179	3	392	9.02	41,932.09
CitPG32	–	Orange1.lg041470m	1185	6	342	7.29	42,851.26
CitPG33	–	Orange1.lg041993m	1176	3	391	8.86	41,468.99
CitPG34	–	Orange1.lg027251m	1194	2	397	5.19	41,472.60
CitPG35	–	Orange1.lg042768m	1194	3	397	8.49	42,335.32
CitPG36	–	Orange1.lg036371m	783	2	245	6.07	25,599.04
CitPG37	–	Orange1.lg046693m	672	4	223	7.51	23,248.48
CitPG38	–	Orange1.lg046411m	1179	3	392	9.87	41,879.09

including citrus (Liu et al. 2011), apple (Wu et al. 1993), pear (Hiwasa et al. 2003; Sekine et al. 2006), peach (Bonghi et al. 1992; Trainotti et al. 1993), and grape (Deng et al. 2007). Even so, investigating PG gene family by genome-wide analysis was barely reported in citrus. Here, a total of 38 putative *PG* genes were indentified in citrus, the number of which was almost equal to that (35 members) in oil palm

(Roongsattham et al. 2012), suggesting we have obtained the most of citrus *PG* members. As expected, there were some CitPG members specially belonging to clementine or sweet orange, which suggested that some new CitPGs remained to be discovered in other species, for example lemon and pomelo.

Fig. 3 Chromosomal locations of 38 *CitPG* genes. The scaffolds of clementina (C) and sweet orange (S) were indicated by black columns. The length of columns shows the relative position of PGs. 1 U is equal to 10 kb

In this study, the phylogenetic tree clustered 52 PG members, 38 from citrus and 14 from other plant species, into 3 groups, a little inconsistent with previously result which divided 28 PGs into 5 groups (Peng et al. 2013). This inconsistency may be explained by the difference in PG members and software which were used to construct phylogenetic tree in different lab. Of interest, further analysis found that the *CitPG*s in the same group possessed similar gene structure. For example, 8 out of 14 *CitPG*s in group G1 contained 3 introns, and others had 1, 2 or 4 introns with the exception of *CitPG5* and *CitPG9* containing 8 and 9 introns, respectively. This result suggested that the introns contribute to functional diversification.

Different groups were proposed of different biological functions in plants (Hadfield et al. 1998; Peng et al. 2013), and vice versa. Accumulating data have showed that *PG* genes are involved in organ abscission (González-Carranza et al. 2002, 2007; Roongsattham et al. 2012; Peng et al. 2013; Merelo et al. 2017). Over-expression of *PG* gene in apple caused premature leaf shedding (Atkinson et al. 2002), while virus-induced TAPG1 silencing inhibited tomato petiole abscission (Jiang et al. 2008). By expression analysis, Merelo et al. (2017) have identified 5 citrus *PG* members related to fruit abscission from AZ C, and all of them have a closed phylogenetic relationship with some abscission-associated PGs. In this study, phylogenetic analysis revealed that 9 *CitPG*s including *CitPG2*, *CitPG3*, *CitPG10*, *CitPG24*, *CitPG27*, *CitPG29*, *CitPG30*, *CitPG33* and *CitPG34* were close to the abscission-related PGs (Fig. 4), suggesting their roles in CFA, and other members might control other

physiological processes. According to gene ID, we found, as expected, that *CitPG2* (Ciclev10033386m), *CitPG10* (Ciclev10004869m), *CitPG27* (Ciclev10004864m) and *CitPG30* (Ciclev10019597m) were the same as the abscission-associated members that Merelo et al. (2017) reported. Another one, referred to as *CitPG43* (aCL675Contig4) by Merelo et al. (2017), was also related to fruit abscission, but did not know which one *CitPG* in this study corresponded to it because of its gene ID unavailable. However, based on the phylogenetic relationship, we inferred it should be *CitPG33* (Orange1.1g041993m) or *CitPG34* (Orange1.1g027251m). Further investigation revealed that both *CitPG33* and *CitPG34* were specific to sweet orange, so it was not surprised that Merelo et al. (2017) could not give it gene ID based on Clementine genome.

Mishra et al. (2008) reported that the cotton leaf abscission was initiated and promoted by ethylene through induction of at least cellulose and PG activities in its AZs, and this process of abscission could be inhibited by IAA. In litchi, a fruit abscission-related *PG* gene, *LcPG1*, could be up- or down-regulated by ethylene or 2, 4-dichlorophenoxyacetic acid (2, 4-D, a synthetic auxin) (Peng et al. 2013). In this study, expression analysis further revealed that 3 out of 38 *CitPG*s including *CitPG2*, *CitPG29* and *CitPG34* were related to CFA. The expression levels of these 3 *CitPG*s were significantly increased during CFA, and could be up- and down-regulated by ACC and IAA, respectively (Fig. 5). In our previous work, we founded that 7 *CitPG*s expressed up or down significantly during CFA, of which 6 members were remarkably related to CFA, i.e. Cs5g03170, Cs7g01690,

Fig. 4 Phylogenetic relationships between PGs. NJ tree constructed using MEGA version 4.0 with a bootstrap of 1000 replicates. The PGs used in NJ tree included 38 citrus PGs and 14 PGs from other plants, i.e. CmPG1(AF062465); CmPG2(AF062466); TAPG5(AAC28906); TAPG4(AAC28905); TAPG1(AAC28903); TAPG2(AAC28904); EgPG4(AFO53698); At2g43890; At2g43880; BnRDPG1(Q42399); PGAZAT(At2g41850); GmPG11(ABC70314); QRT2(At3g07970); LcPG1(JX855043). The bootstrap values low than 50 didn't show on the dendrogram

orange1.1t02526, orange1.1t02529, orange1.1t02530 and orange1.1t02532 (Xie et al. 2018). Of them, the Cs5g03170 (CitPG29) and Cs7g01690 (CitPG2) were further demonstrated to be related to CFA in this study. RNA-seq showed that the expression level of orange1.1t02526 (CitPG33) was very low during CFA (Xie et al. 2018), possibly due to this reason making it undetectable, here. However, its potential role in CFA was suggested above by phylogenetic analysis. Of interest, the orange1.1t02529, orange1.1t02530 and orange1.1t02532 in CsiDB (<https://citrus.hzau.edu.cn/orange/>) were all matched to the same sweet orange genome location (scaffold01797:5287...6399) in Phytozome (<https://www.phytozome.net>) by BLAST with RNA sequence. Taken together, the results obtained in this study were line with previous work reported by Xie et al. (2018). Merelo et al. (2017) have identified 5 CitPGs from AZ C involved in fruit abscission, of which, however, CitPG10 (Ciclev10004869m), CitPG27 (Ciclev10004864m) and CitPG30 (Ciclev10019597m) were not associated with CFA. Likewise, the CitPG29 related to CFA in this study was regarded as an abscission-unrelated member by Merelo et al. (2017). The inconsistency maybe resulted from different tissues, i.e. AZ A and C.

The CFA-related *CitPG* members seemed to be too few, but similar case was observed in oil palm, where only one (*EgPG4*) out of 35 putative non-redundant PG candidates were found to be related to abscission (Roongsattham et al. 2012). Other *CitPGs* were expressed in AZ A at either undetectable or unchangeable levels during CFA and under ethylene or IAA treatment, indicating they were not associated with CFA.

Conclusions

A total of 38 members were identified based on the citrus genome sequences, which were clustered into 3 groups according to phylogenetic analysis. Some CitPGs such as CitPG2, CitPG3, CitPG10, CitPG24, CitPG27, CitPG29, CitPG30, CitPG33 and CitPG34 were close to the abscission-related PGs. Expression analysis further revealed that the expression levels of *CitPG2*, *CitPG29* and *CitPG34* were paralleled with the abscission rates of citrus fruitlet, and could be induced by ethylene or inhibited by the IAA treatment. These results suggested that CitPG2, CitPG29 and CitPG34 might be involved in the process of CFA.

Fig. 5 The expression profiles of CitPG2, CitPG29 and CitPG34 under the IAA or 1-aminocyclo-propane-1-carboxylic acid (ACC) treatment. Values are means of three replicates $\pm$ SD. Different lowercase letters at the same time point represent significant differences ($p < 0.05$)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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