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Overexpression of the *Rubus idaeus* Polygalacturonases Gene *RiPG2* Accelerates Fruit Softening in *Solanum lycopersicum*

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Abstract: The high susceptibility of raspberries to softening restricts the development of the raspberry industry. The primary causes of fruit softening are the breakdown of components linked to the cell wall and the destruction of the cell wall structure itself. Polygalacturonase (PG), a key enzyme that catalyzes pectin degradation, plays a critical role in fruit softening. However, there are currently limited studies on the mechanism of *PG* genes in raspberry fruit softening. In this study, a *PG* gene, *RiPG2*, was isolated from raspberry (*Rubus idaeus* L.). 'Polka' fruits and tomato plants overexpressing *RiPG2* were obtained by *Agrobacterium tumefaciens*-mediated leaf disc transformation to elucidate the role of *RiPG2* in fruit softening. The total length of the *RiPG2* gene is 1185 bp, and the gene encodes a total of 394 amino acids. The GFP fusion protein was expressed at the chloroplast under laser confocal microscopy, indicating that the RiPG2 protein is localized to the chloroplasts. Phenotypic analysis revealed that the fruit firmness of three strains was considerably less than that of controls, but PG enzyme activity was increased. Overexpression of *RiPG2* altered the content of cell wall components, with an increase in water-soluble pectin (WSP) and ion-bound pectin (ISP) but a decrease in protopectin, cellulose, hemicellulose, and covalently bound pectin (CSP). In addition, *RiPG2* positively regulated the expression of cell wall metabolism-related genes such as *SlEXP1*, *SlTBG4*, *SlXTH5*, and *SlPL*. These results suggest that the *RiPG2* gene regulates the structure and composition of the cell wall and acts synergistically with other cell wall metabolism-related genes to promote fruit softening. This study provides a new candidate gene for molecular breeding to improve raspberry firmness.

Keywords: fruit maturity; polygalacturonase; pectin degradation; raspberry

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1. Introduction

The cell wall plays an important role in plant growth and development. In addition to serving as a barrier against pathogen attack, cell walls can also support and connect cells and determine the mechanical strength of plant structures [1,2]. Cellulose, hemicellulose, and pectin are the main components of the plant cell wall, which are linked to each other in a spatial network by various bonds [3]. Pectin is a branched-chain anionic polysaccharide composed of GalA units linked by α-1,4 glycosidic linkages. There are three main types of pectin: homogalacturonan (HG), rhamnogalacturonan-I (RGI), and rhamnogalacturonan-II (RGII). Among them, HG is abundant in the middle layer of cell walls and is thought to be critical for intercellular adhesion [4,5]. Pectinases are responsible for the breakdown of pectin that occurs during the ripening process. Polygalacturonases (PGs), pectate lyases (PLs), β-galactosidases (β-GALs), and xyloglucan endo-transglycosylase/hydrolases (XTHs) are some common pectinases [6]. PGs are one of the most abundant mature inducible enzymes that belong to the glycosyl hydrolase family 28 (GH28) and contain four conserved domains [7]. PG is widely used in the food, beverage, textile, and paper

industries. It extracts and clarifies fruit juices, reduces viscosity, and releases nutrients, a property that has led to its use in improving feed absorption and textile degumming [8]. In previous reports, there have also been many studies on the reduction of PG enzyme activity by post-harvest treatment of fruit to improve fruit quality [9,10].

PGs were first discovered in pathogenic fungi, and with the progress of research, studies of this gene have been conducted in various plants [11,12]. The function of PGs is to cleave the chain of homogalacturonan, resulting in decreased intercellular adhesion and disintegration of the cell wall structure [13]. Therefore, PGs can participate in many processes of plant growth and development, such as regulating seed coat permeability, affecting plant morphology, and participating in organ abortion, pollen development, and pollen tube elongation [14–16]. In addition, PGs are closely related to fruit softening. Transcriptome analysis of sugar apple (*Annona squamosa* L.) at different storage stages revealed that four PG genes were up-regulated within 6–12 days of storage, and PG enzyme activity increased rapidly during storage. This reflects the catalytic effect of PGs on fruit softening and development [17]. Among the 43 *FcPG* genes in fig (*Ficus carica* L.), *FcPG9*, *10*, *11*, *12,* and *13* were clustered on chromosomes. In addition, they were tightly clustered with the PG gene, which plays a fruit-softening role in four Rosaceae fruit trees. The four *FcPG* genes in clade B also exhibit similar tight clustering. In addition, transient overexpression of *FcPG12* resulted in fruit firmness that was lower than the control (empty plasmid injection) after three days, while *FcPG12* expression levels and PG enzyme activity were higher than those of the control [18]. Similar functional validation also exists in papaya (*Carica papaya* L.), where transient expression of *cpPG1* in papaya results in flesh softening [19]. In pear (*Pyrus* spp.), 28 out of 61 members of the PG gene family were expressed during the process of increased fruit softening. Using a combination of phylogenetic and correlation analysis, *PbrPG6* was selected for the construction of a silencing vector to validate its role in fruit softening. Silencing of *PbrPG6* inhibited PG enzyme activity and maintained fruit firmness [20]. In addition, members of the PG gene family have been studied in kiwifruit (*Actinidia chinensis*) and peach (*Prunus persica* L.) at various stages of fruit softening in previous reports [21,22]. In terms of gene editing, CRISPR was used to edit FaPG1, which can improve fruit firmness and prolong shelf life in strawberries (*Fragaria ananassa*) [23]. Candelas et al., have also performed similar studies [24]. Inhibition of the expression of *PpPG21* and *22* by VIGS significantly reduced the activity of the PG enzyme and preserved fruit firmness at the later shelf-life stage in melting flesh (MF) peaches [25].

Raspberry (*Rubus idaeus* L.), also known as the "golden fruit", is a small berry with important economic value [26]. Raspberry fruits are rich in anthocyanidins, ellagic acid, flavonoids, and other nutrients, with high nutritional and medicinal values [27,28]. However, like most soft fruits, raspberries often exhibit rapid softening and ripening, resulting in a short post-harvest shelf life and difficult storage and transportation [29,30]. As a highly perishable soft fruit, the postharvest loss of fresh raspberries is of great concern to growers and consumers. Therefore, understanding the softening process during fruit ripening is a high priority to improve the management of this fruit [31]. However, current reports on raspberry fruit softening mainly focus on the inhibitory effects of physical and chemical methods on the softening of raspberry fruit stored at low temperatures after harvest. Physical methods, such as electron beam irradiation, UV-C irradiation, and modified atmosphere packaging, and chemical methods, such as $CaCl_2$ and pectin methylesterase (PME) treatments, were able to delay softening to some extent [27,32,33]. However, there are fewer studies on raspberry softening at the molecular level.

In this study, a *PG* gene, *RiPG2*, was cloned from raspberry and stably transferred into the model plant tomato (*Solanum lycopersicum*). To verify the role of *RiPG2* in fruit softening, firmness, expression level of the *RiPG2* gene, and PG enzyme activity were measured in transgenic tomatoes and control groups (wild-type tomatoes and *S. lycopersicum* plants transformed with an empty vector). The degradation of cell walls is accompanied by changes in their component content. Therefore, the cell wall components of transgenic and control tomato fruits were also measured, including water-soluble pectin (WSP), ion-bound

pectin (ISP), covalently bound pectin (CSP), protopectin, cellulose, and hemicellulose [34]. In addition, cell wall-loosening proteins, such as expansins (EXP), are also involved in the degradation of the cell wall [35]. Therefore, four additional genes encoding pectinase and cell wall-loosening proteins were selected for fluorescence quantification to analyze the synergistic effects between them. This study confirmed the catalytic role of *RiPG2* in raspberry fruit softening and provided a new idea to improve the firmness of raspberry fruit by knocking out the *PG* gene using genetic engineering methods.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

Materials were taken from the raspberry 'Polka' planted at the Xiangyang Farm of Northeast Agricultural University. Raspberry plants are planted 2 m apart in rows and 1 m apart from each other [36]. In June 2022, raspberry roots, stems, leaves, and four periods of fruits were collected. After treatment with liquid nitrogen, these plant materials were stored at −80 °C for use. The tomato 'Micro-Tom' was used for genetic transformation and functional verification experiments.

2.2. Isolation and Cloning of RiPG2

Total RNA was extracted from raspberry fruits using the OminiPlant RNA Kit (Kangweishiji, Beijing, China) and used as a template for first-strand cDNA synthesis using TransScript®First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). High-purity RNA and cDNA were obtained by 1.0% agarose gel electrophoresis evaluation [37]. The amplification primer was designed in *RiPG2* conserved sequence using Premier 5.0 software (Supplementary Table S1) and sent to the organization (Huadaliuhe, Beijing, China) for synthesis. The sequence of *RiPG2* was amplified using the cDNA obtained by reverse transcription as a template. Purified DNA fragments were obtained from the DNA Clean-up Kit (Kangweishiji, Beijing, China) and then ligated with the pEASY®-T5 vector (TransGen Biotech, Beijing, China) [38]. Sequencing was performed after overnight incubation on resistant LB medium containing kana (per 100 mL medium: 1 g peptone + 0.5 g yeast powder + 1 g NaCl + 50 μL kana + 1.5 g agar). The concentration of kanamycin (kana) was 100 mg mL^{-1}.

2.3. RiPG2 Bioinformatics Analysis

RiPG2 sequence was translated into amino acid sequence using DNAMAN5.2. PG protein sequences in other species with high homology to the amino acid sequence of RiPG2 were identified by BLAST in NCBI and subjected to multiple sequence comparison. The primary structure and structural domains of the RiPG2 protein were predicted using ExPASy (https://web.expasy.org/protparam/, accessed on 29 September 2023) and the SMART protein domain analysis website (http://smart.emblheidelberg.de/, accessed on 29 September 2023), respectively. A homologous phylogenetic tree of RiPG2 was constructed using MEGA11.0 (https://megasoftware.net/, accessed on 29 September 2023).

2.4. Subcellular Localization of RiPG2 Protein

RiPG2 without stop codon was amplified by PCR using the primer with *BamH*I or *Sal*I digestion sites (site-F and site-R, Supplementary Table S1). The coding region was cloned into the pCAMBIA1300s GFP vector by homologous recombination. The p35S: *RiPG2*-GFP and p35S: GFP (control) vectors were transformed into *Agrobacterium tumefaciens* strain GV3101. They were injected into the leaves of five weeks old *Nicotiana benthamiana* according to the method published by Sparks IA [39]. The injected tobacco was incubated under low light for 48–72 h. A small disk was placed on the slide by gently pressing a punch on the leaf. The slide was observed under a laser confocal microscope. Compare the expression of the control protein and the RiPG2-GFP fusion protein to determine the location of the RiPG2 protein in the cells [40].

2.5. Quantitative Real-Time PCR (qPCR) Analysis of RiPG2

Total RNA was extracted from three stages of control and transgenic tomato fruits using the OminiPlant RNA Kit (Kangweishiji, Beijing, China). The RNA was evaluated by agarose gel electrophoresis. Afterward, total RNA from each sample was reverse transcribed using TransScript®First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) to synthesize the first strand of cDNA. The conservative region of the *RiPG2* nucleic acid sequence was identified through sequence alignment, and the sequence with high specificity was selected to design qRT-PCR primers (Supplementary Table S1). Using cDNA as a template, specific primers and BlazeTaqTM SYBR®Green qPCR Mix 2.0 (GeneBio, Guangzhou, China) were used for qRT-PCR. The PCR reaction procedure was as follows: pre-denaturation at 94 °C for 30 s; then followed by denaturation at 95 °C for 5 s; annealing at 54 °C for 40 s; extension at 72 °C for 30 s in one cycle, repeated 34 times; and finally, holding at 72 °C for 10 min. The quantitative study used the 18 s gene as a reference gene. The quantitative data obtained were analyzed using the $2^{-\Delta\Delta CT}$ method [41].

2.6. Overexpression Vector Construction and Genetic Transformation of Tomatoes

Primers containing *BamH*I (forward) and *Sal*I (reverse) enzyme sites were designed, and restriction sites were added to the *RiPG2* cDNA by PCR. The pCAMBIA1300s vector was cleaved by the same restriction endonuclease site. The PCR product was linked to the pCAMBIA1300s linear vector through homologous recombination. Then, the connecting product was converted into *Agrobacterium tumefaciens* GV3101.

The appropriate amount of tomato 'Micro-Tom' seeds were disinfected with 75% alcohol and 3% NaClO. After drying on sterile filter paper, the seeds were placed in sowing medium (2.289 g L^{-1} MS + 15 g L^{-1} sucrose + 7.5 g L^{-1} agar). After two true leaves were grown, tomato genetic transformation was performed using the *A. tumefaciens*-mediated leaf disc method proposed by Fillatti et al. [42]. The true leaves were cut into 0.5 cm squares as explants and placed on preculture medium (4.4 g L^{-1} MS + 30 g L^{-1} sucrose + 7.5 g L^{-1} agar + 1 mg L^{-1} zeatin + 0.1 mg L^{-1} IAA) for two days in the dark. The explants were then inoculated with *Agrobacterium tumefaciens GV3101* carrying the construct *RiPG2*: pCAMBIA1300s and OD_{600} = 0.5. The explants were cocultured for 2 days and then transferred to the selected culture medium (4.4 g L^{-1} MS + 30 g L^{-1} sucrose + 7.5 g L^{-1} agar + 2 mg L^{-1} zeatin + 0.1 mg L^{-1} IAA + 10 mg L^{-1} kana + 400 mg L^{-1} tim). The regenerated buds were cultured in shoot elongation medium (4.4 g L^{-1} MS + 30 g L^{-1} sucrose + 7.5 g L^{-1} agar + 0.2 mg L^{-1} zeatin + 0.1 mg L^{-1} IAA + 10 mg L^{-1} kana + 400 mg L^{-1} tim) and rooting medium (4.4 g L^{-1} MS + 30 g L^{-1} sucrose + 7.5 g L^{-1} agar + 1 mg L^{-1} IBA + 10 mg L^{-1} kana + 400 mg L^{-1} tim). Finally, they were transplanted into a nutrient dish and cultured in a culture box. To obtain pure and concordant lines, seeds from positive seedlings were collected and screened up to the T3 generation [43]. Note that Kana acts as a screen. Tim is timentin, which acts to inhibit *Agrobacterium*.

2.7. Fruit Firmness Determination

The Texture Analyzer TA. Touch (Baosheng, Shanghai, China) was used to analyze fruit firmness in freshly harvested fruits. The instrument parameters were as follows: speed test 1 mm/s, target mode distance 6 mm, and trigger force 5 g [23].

2.8. PG Enzyme Activity Measurement

The Polygalacturonase (PG) Activity Assay Kit (Yuanju, Shanghai, China) was used to determine the PG enzyme activity. The extraction solution was added into centrifuge tubes containing tissue in a ratio of mass (g) to volume (mL) of 1:10. After homogenizing in an ice bath, centrifuge tubes were centrifuged at 10,000 rpm for 10 min at a low temperature [44]. The supernatant was removed and stored on ice for testing. Samples from the assay and control tubes were processed according to the instructions, and the absorbance values of the solutions were measured at 540 nm using a spectrophotometer (Yuanju, Shanghai, China). Finally, the PG enzyme activity was calculated using the following formula:

2500 × (ΔA + 0.001) ÷ W. ΔA: Δ assay − Δ control, where W is the sample fresh weight, g, and the unit used is mg/h/g FW.

2.9. Cell Wall Substance Content Measurement

ISP was determined by the ISP Assay Kit (GraceBio, Suzhou, China). CSP was detected by CSP Assay Kit (GraceBio, Suzhou, China). Cellulose contents from fruits were obtained by the Cellulose Content (CLL) Assay Kit (GraceBio, Suzhou, China). The test solution was extracted according to the manufacturer's instructions, and absorbance was measured at 520 nm using an Ultraviolet UV-3600i Plus (Shimadzu, Kyoto, Japan) spectrometer. Hemicellulose, protopectin, and WSP contents were determined using the method of Wang et al. [45]. The test solution was extracted and incubated at room temperature for 15 min. Absorbance was measured at 520 nm using an Ultraviolet UV-3600i Plus (Shimadzu) spectrometer.

2.10. Analysis of Other Cell Wall Gene Expression

Specific primers were designed (Supplementary Table S1) by Shi et al. [46] to perform qPCR experiments on several key enzyme genes involved in cell wall degradation: pectin hydrolysis-related genes (*SlTBG4* and *SlPL*) and cellulose and hemicellulose hydrolysis-related genes (*SlEXP1* and *SlXTH5*). The specific test methods are consistent with those described in Section 2.5.

2.11. Statistical Analysis

SPSS19.0 and the Student's *t*-test were used for statistical analysis. The mean value ± SD of at least three replicates is shown. $p \leq 0.05$ indicates a significant difference compared to the control.

3. Results

3.1. Primary Structure and Property Analysis of RiPG2 Protein

The gene sequence of *RiPG2* was 1182 bp in length and encoded a total of 394 amino acids (Figure S1). The software prediction results showed that the theoretical molecular mass (MW) of the RiPG2 protein was 42.58 kDa and the theoretical isoelectric point (pI) was 9.28. This protein was hydrophilic, with an average hydrophilicity coefficient of −0.31 (Figure S2).

PG proteins from 11 species whose amino acid sequences were highly similar to the *RiPG2* protein sequence were found on the NCBI website and compared by multiple sequence analysis. The amino acid sequences of conserved domains of the *RiPG2* protein were basically the same as those of other PG proteins. The conserved domains of PG proteins consist of four conserved functional domains (Figure 1A). In addition, according to the phylogenetic tree, *RiPG2* was most closely related to *Fragaria vesca* (XP_011458024.1) (Figure 1B).

3.2. Subcellular Localization of RiPG2 Protein

Subcellular localization is often used to determine the location of a protein. Chloroplast spontaneous fluorescence was used to indicate the position of chloroplasts. Under laser confocal microscopy, green fluorescent proteins (GFP) were expressed in the nucleus, plasma membrane, and cytoplasm of the cells. However, the expression of the 35S:RiPG2-GFP fusion protein can only be observed in chloroplasts. This indicated that the RiPG2 protein was located in the chloroplast (Figure 2).

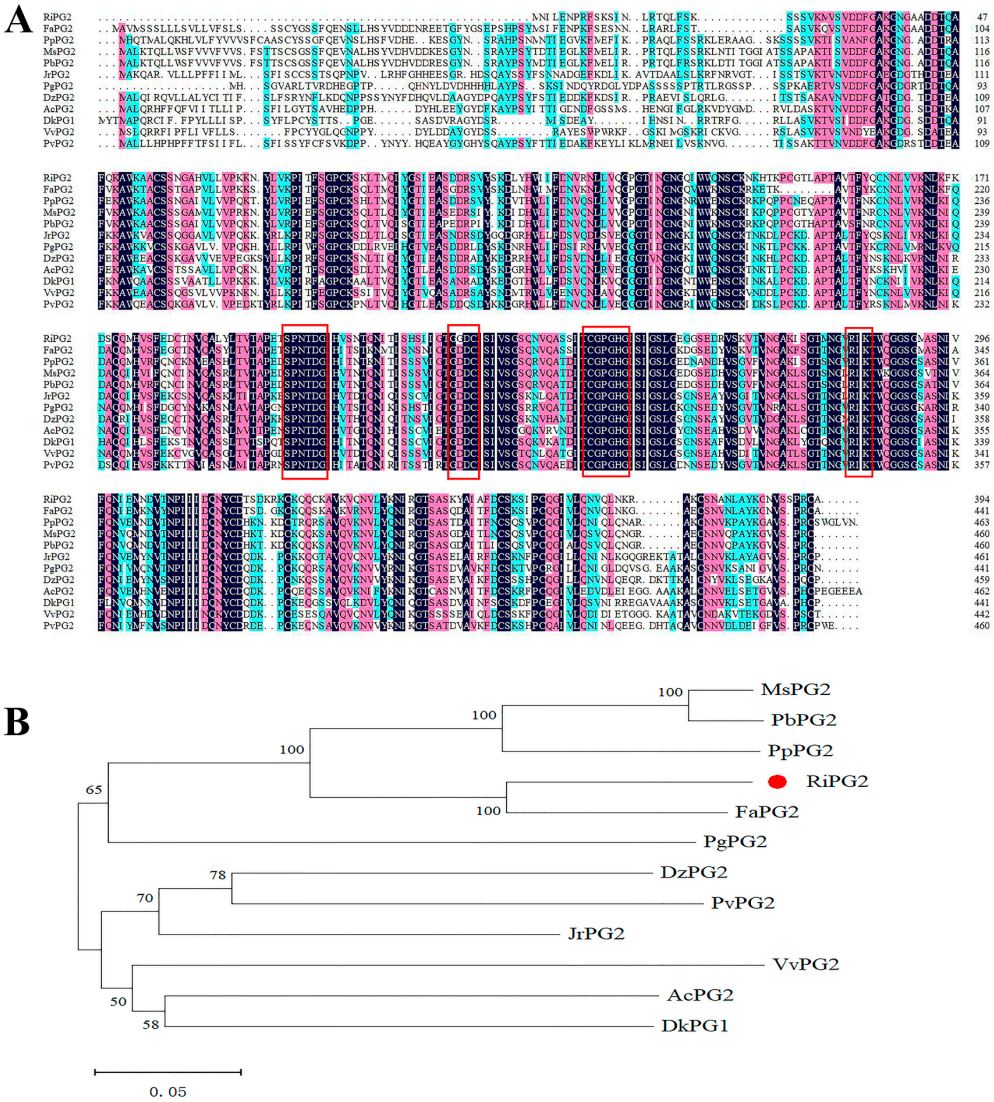

Figure 1. Comparison of amino acid sequences and phylogenetic relationships analysis of PG proteins from raspberry and other 11 plant species. (**A**) Multiple sequence comparisons between PG proteins. The four conserved domains of the protein are shown in the red boxes. Identical amino acids are shown in dark blue. Red color indicates amino acid similarity of 75% or more, and green color indicates amino acid similarity of 50% or more. (**B**) Phylogenetic tree analysis of RiPG2 (marked by the red circle) and other plant PG proteins. The accession numbers are as follows: FaPG2 (XP_011458024.1, *Fragaria vesca*), PpPG2 (XP_020426071.1, *Prunus persica*), MsPG2 (XP_050106357.1, *Malus sylvestris*), PbPG2 (XP_009369438.1, *Pyrus x bretschneideri*), JrPG2 (XP_018834442.2, *Juglans regia*), PgPG2 (XP_031396109.1, *Punica granatum*), DzPG2 (XP_022766694.1, *Durio zibethinus*), AcPG2 (XP_057488003.1, *Actinidia chinensis*), DkPG1 (ACJ06506.1, *Diospyros kaki*), VvPG2 (XP_002282772.1, *Vitis vinifera*), and PvPG2 (XP_031270986.1, *Pistacia vera*).

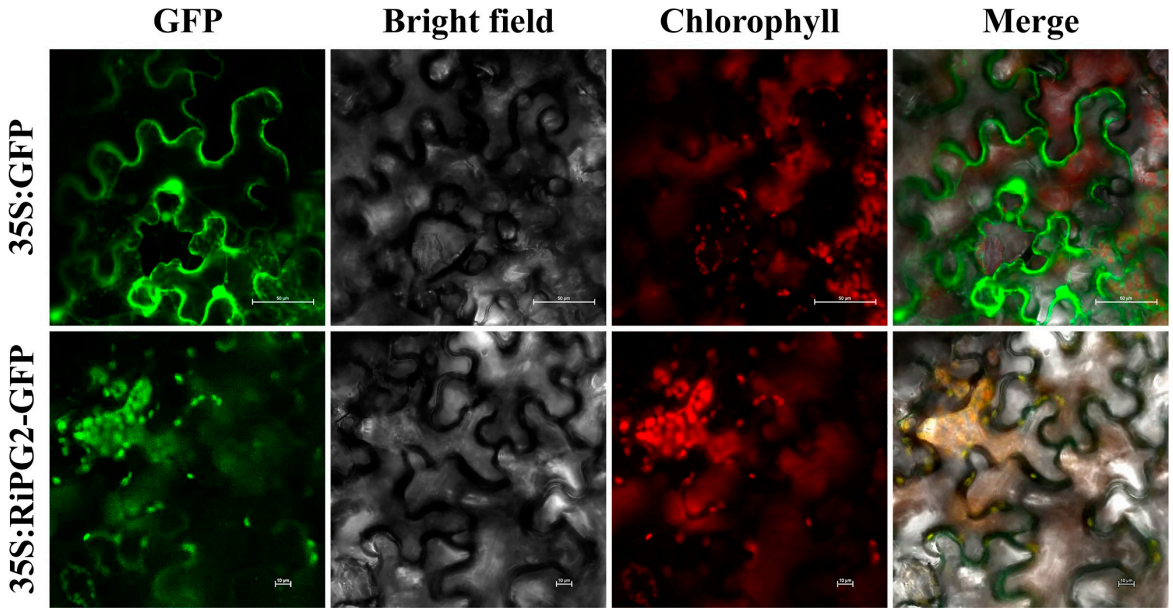

Figure 2. Subcellular localization of the RiPG2 protein. The GFP-tagged pCAMBIA1300s vector and pCAMBIA1300s:RiPG2 expression vector were injected into 5–6 week old tobacco leaves, which were incubated in low light for two days before being sectioned and observed under a fluorescence microscope.

3.3. Expression of the RiPG2 Gene in Raspberry Fruit

The expression levels of *RiPG2* in different tissues of raspberries were analyzed using qRT-PCR. The results showed that the gene was expressed in all tested tissues. The expression of the *RiPG2* gene increased significantly with fruit ripening, with the highest expression found in the red fruit stage. Except at the green fruit stage, the expression level of the *RiPG2* gene in fruits was significantly higher than that in the other three raspberry tissues (Figure 3).

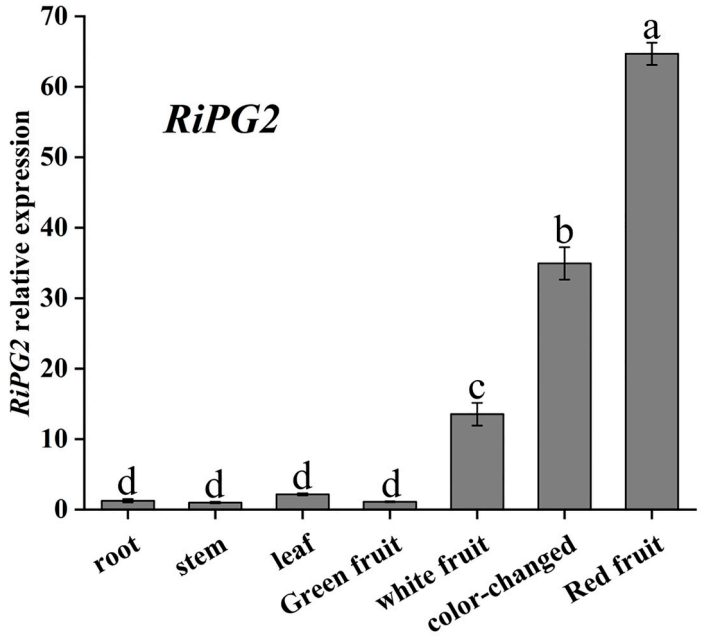

Figure 3. *RiPG2* gene expression in raspberry. Root expression level was used as a control. The data shown in the graph are the average of three replicates. Letters (a–d) indicate significant within-group variability ($p \leq 0.05$). The error bars in the bar graph indicate the standard deviation.

3.4. Phenotypic Observation and Quantitative Analysis of Transgenic Tomatoes

To verify the function of *RiPG2*, the *RiPG2* gene was transferred into tomatoes, and transgenic strains were obtained through verification (S1, S2, and S3). Using WT and unloading lines (UL, *S. lycopersicum* plants transformed with an empty vector) as controls, no significant change in the phenotype of the transgenic plants was observed (Figure 4A). Transcription level analysis of the leaves of the transgenic strains using qRT-PCR revealed that the expression levels of the transgenic strains (S1, S2, and S3) were significantly up-regulated compared to the WT and UL strains (Figure 4B). Agarose gel electrophoresis confirmed the presence of the target fragment in all three transformed lines (S1, S2, and S3) (Figure 4C).

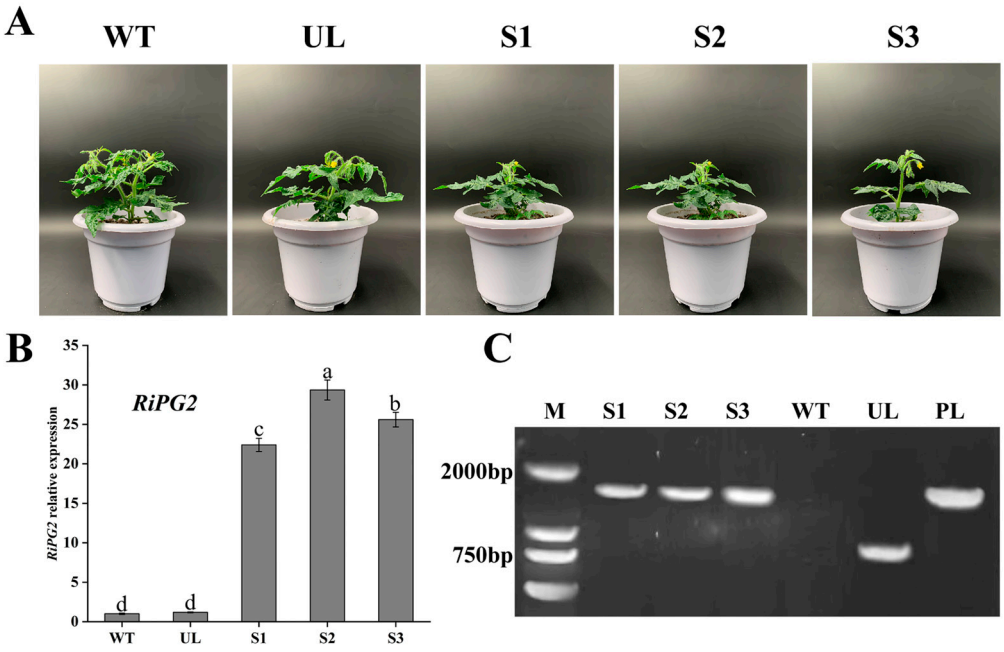

Figure 4. The phenotype and gene expression analysis of transgenic tomato lines. (**A**) Phenotypes of tomato plants overexpressing the *RiPG2* gene (S1, S2, and S3) and controls. (**B**) The transcription level of *RiPG2* in tomato lines overexpressing the *RiPG2* gene (S1, S2, and S3) and controls. (**C**) RT-PCR and electrophoretic verification of positive tomato plants. The data shown in the graph are the average of three replicates. Letters (a–d) indicate significant within-group variability ($p \leq 0.05$). The error bars in the bar graph indicate the standard deviation.

3.5. Overexpression of RiPG2 Promotes Tomato Fruit Softening

Afterwards, tomato fruits were collected, and the phenotypes were observed by the naked eye on the 35th, 40th, and 45th days after flowering. The results showed that the overexpression of the *RiPG2* gene had no significant effect on the size and shape of tomato fruits (Figure 5A). To further investigate the changes in firmness-related physiological indices of transgenic tomato fruits, the content of cell wall material, fruit firmness, and related enzyme activities were measured.

The expression level of the *RiPG2* gene in the three transgenic tomato lines (S1, S2 and S3) gradually increased with the ripening of tomato fruits. Among the three transgenic lines, S2 had higher expression levels at 35 and 40 days after flowering, while S3 had the highest expression level at 45 days after flowering (Figure 5B). As the fruit matured, the firmness of the transgenic tomato fruits decreased gradually. During the developmental phase, significant differences in fruit firmness were observed between the transgenic, WT, and UL lines (Figure 5C). The variation trend of firmness was consistent with the expression of the *RiPG2* gene, which further proved that the *RiPG2* gene promoted tomato fruit softening. In addition, the PG enzyme activity of transgenic strains was consistently higher than that of WT and UL strains throughout the entire fruit development process (Figure 5D). The

above results indicated that overexpression of *RiPG2* enhanced PG enzyme activity, thereby promoting cell wall hydrolysis and accelerating fruit softening.

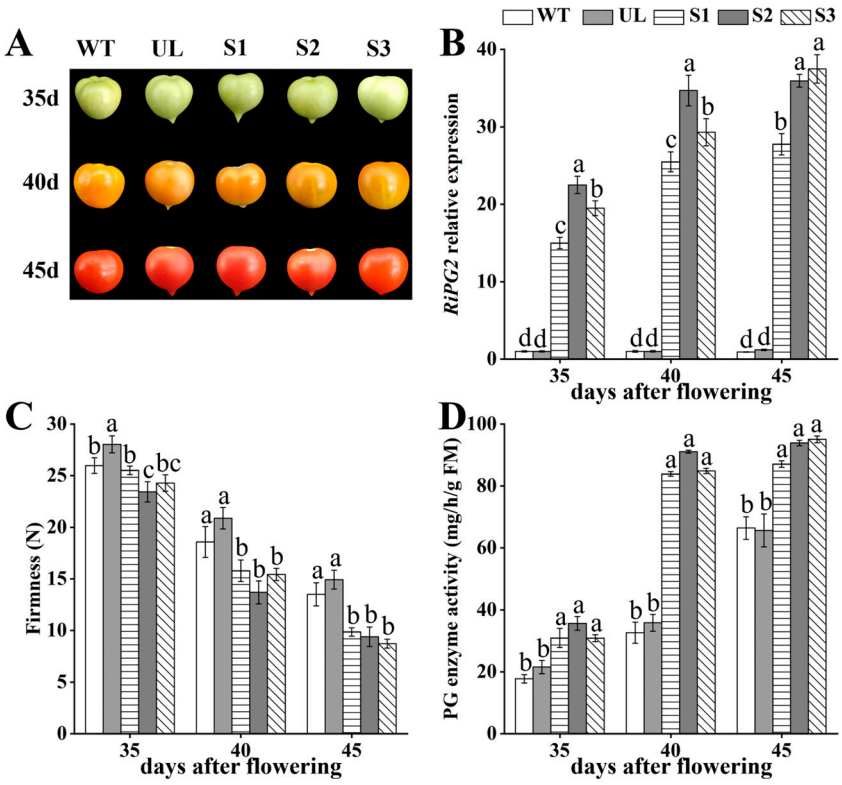

Figure 5. Phenotype, firmness, and activity of polygalacturonase (PG) in tomato fruits overexpressing *RiPG2*. (**A**) The phenotype of fruits, (**B**) the transcription levels of *RiPG2*, (**C**) the fruit firmness, and (**D**) the PG enzyme activity of tomato plants overexpressing the *RiPG2* gene (S1, S2, S3) and controls at 35, 40, and 45 days after flowering. The scale was 1 cm. The data shown in the graph are the average of three replicates. Letters (a–d) indicate significant within-group variability ($p \leq 0.05$). The error bars in the bar graph indicate the standard deviation.

3.6. Changes in Cell Wall Substances

The changes in fruit firmness are closely related to the changes in pectin substance. Therefore, the pectin content of transgenic tomato fruits at different stages was measured. The results showed that with the increase in fruit ripeness, the WSP and ISP contents of WT, UL, and transgenic strains increased gradually. The difference was that the WSP content of transgenic strains was significantly higher than that of WT and UL strains at each developmental stage (Figure 6A), while the significant difference in ISP contents between transgenic strains and controls was reflected on the 40th and 45th days after flowering (Figure 6B). On the contrary, the protopectin content decreased with fruit ripening. Compared to WT and UL strains, a significant decrease in protopectin content occurred in transgenic fruits (Figure 6C). Meanwhile, the results in the figure also reflected the trend that fruit ripening was accompanied by a decrease in CSP, cellulose, and hemicellulose contents. The CSP content of the transgenic lines has an obvious difference from that of the control at 35 and 40 days after flowering (Figure 6D). At 35 and 40 days after flowering, the CSP contents of the transgenic lines and the control were significantly different (Figure 6D).

However, the contents of cellulose and hemicellulose in the fruits of the three transgenic lines were particularly significant in the later stages of growth and development (Figure 6E,F). In this study, it was found that there were remarkable differences in the contents of cellulose, ISP, and hemicellulose between three transgenic strains. This may be related to the regulation of the *RiPG2* gene, but the specific regulatory mechanism still needs further research.

The above data suggests that the overexpression of *RiPG2* can increase softening during fruit ripening by affecting the contents of cell wall components. Among them, the contents of soluble pectin and ion-bound pectin were negatively correlated with tomato fruit firmness, while the contents of cellulose, hemicellulose, CSP, and protopectin were positively correlated with fruit firmness.

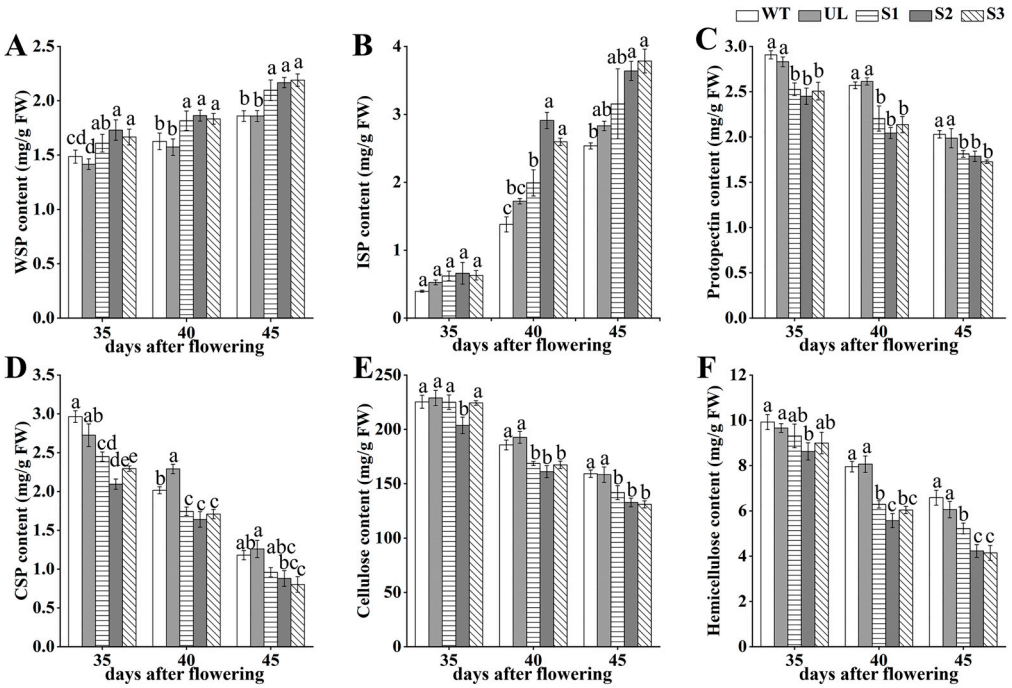

Figure 6. Contents of cell wall substances in the tomato fruits overexpressing *RiPG2*. The contents of (**A**) WSP, (**B**) ISP, (**C**) protopectin, (**D**) CSP, (**E**) cellulose, and (**F**) hemicellulose in WT, UL, and transgenic tomato lines (S1, S2, and S3) at 35, 40, and 45 days after flowering. The data shown in the graph are the average of three replicates. Letters (a–e) indicate significant within-group variability ($p \leq 0.05$). The error bars in the bar graph indicate the standard deviation.

3.7. Expression Levels Analysis of the Genes Related to Cell Wall Components

The depolymerization of cell wall substances plays a decisive role in fruit softening, and this process is often accompanied by complex physiological and biochemical mechanisms. Some genes encoding cell wall enzymes and proteins synergistically promote the solubilization of cell wall substances [47]. In this study, *SlEXP1*, *SlXTH5*, *SlTBG4*, and *SlPL* were selected to be analyzed at the transcriptional level.

Analysis results showed that tomato plants overexpressing the RiPG2 gene were highly consistent with control plants in the expression trends of four genes (WT and UL strains). The expression levels of *SlTBG4* and *SlXTH5* showed a continuous upward trend (Figure 7A,B), while *SlEXP1* and *SlPL* increased at first and then decreased (Figure 7C,D). However, compared to the WT and UL strains, the expression levels of these four genes were significantly upregulated. This indicated that overexpression of *RiPG2* could promote the expression of *SlEXP1*, *SlXTH5*, *SlTBG4*, and *SlPL*. Among them, the expression level of *SlTBG4* was much higher than that of the other three genes in the middle and late development stages. This may be the reason why *RiPG2* promotes fruit softening at the molecular level. However, the specific regulatory mechanism is unclear until now, and further investigations are required. In summary, *RiPG2* can increase the degree of fruit softening by up-regulating other genes. This provides a basis for studying the molecular mechanism of fruit softening.

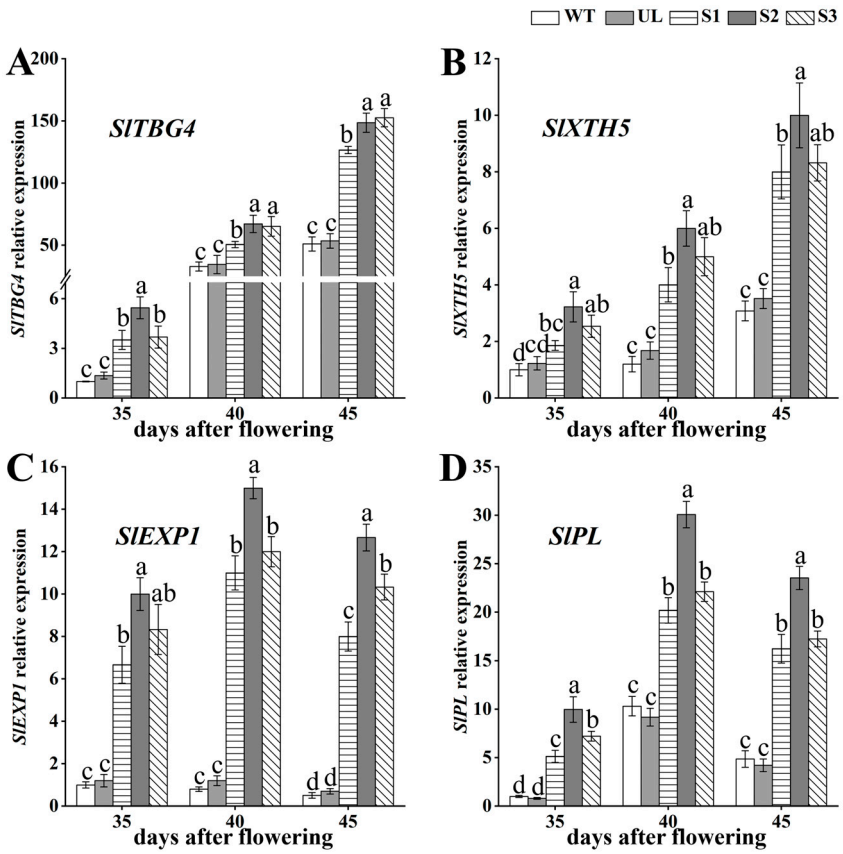

Figure 7. Expression levels of genes related to cell wall components in transgenic tomato fruits overexpressing *RiPG2*. (**A**) Relative expression levels of *SlTBG4*, (**B**) *SlXTH5*, (**C**) *SlEXP1*, and (**D**) *SlPL*. The data shown in the graph are the average of three replicates. Letters (a–d) indicate significant within-group variability ($p \leq 0.05$). The error bars in the bar graph indicate the standard deviation.

4. Discussion

Fruit softening is closely related to cell wall metabolism, and the softening of most fruits is accompanied by the degradation of cell wall polysaccharides [48]. As one of the major cell wall degrading enzymes, *PGs* have been isolated from many plants, such as wheat, maize, and *Arabidopsis thaliana* [49–51]. In the course of research, the function of *PGs* has been extensively characterized and analyzed. Studies have shown that *PGs* are involved in several physiological processes of fruit growth and development [52]. For example, *TAPG1*, *TAPG2*, and *TAPG4* were expressed only in abscission cells of leaves and flowers, suggesting that they are involved in the abscission process [53]. In *Musa nana* Lour., *MaPG3* and *MaPG4* were induced by ethylene and were involved in fruit softening. *MaPG2* was mainly related to aging, while *MaPG1* was hardly expressed in the fruit [54]. The tissue specificity of *PG* gene expression underlies their extensive involvement in fruit ripening regulation. The gene sequence of *RiPG2* was isolated for the first time from raspberry 'Polka' in this experiment. PG amino acid sequences with high amino acid homology to RiPG2 were also found in other species through the NCBI online website. Then, the amino acid sequences of these proteins were comparatively analyzed. The analysis revealed that the sequence of the RiPG2 protein was very similar to PG proteins from the other 11 species in four conserved domains (Figure 1A). These four conserved structural domains have also been reported in citrus CitPG proteins, and the *CitPG* gene could promote pectin lysis during fruit development [55]. This suggests that the RiPG2 gene may be functionally related to CitPG.

For some of the reported *PG* genes, due to their different functions, there were also certain differences in the localization of the genes in the cells. For example, *PG031* from soybean was located in the cell wall and had an effect on the seed coat cell wall [15]. In

this study, *RiPG2* was located in the chloroplast (Figure 2) and may be involved in some physiological pathways in chloroplasts, such as photosynthesis and glucose metabolism. Whether the *RiPG2* gene plays a key role in these processes needs to be further investigated.

The expression of the *RiPG2* gene increased significantly with fruit ripening and was highest at the red fruit stage (Figure 3). The expression pattern of *RiPG2* in raspberries suggests that this gene is closely related to fruit softening. Many reports on plants have investigated the important role of *PGs* for fruit ripening. For example, in tomato, fruit firmness was increased in *SlPG*-targeted mutant tomato lines obtained by gene editing technology, suggesting that knockdown of *SlPG* can delay tomato fruit softening [56]. Transient overexpression of *FcPG12* resulted in decreased fruit firmness and increased PG enzyme activity in fig tissues [18]. Similar results were observed in this study after overexpression of *RiPG2* in tomatoes (Figure 5C,D).

Tissue softening is usually caused by disruption of the cell wall structure, particularly the breakdown of polysaccharides (e.g., cellulose, pectin, and hemicellulose), the major components of the cell wall [57]. Protopectin can be degraded to WSP, and therefore WSP content increases with fruit maturity [58]. CSP content has been positively correlated with fruit firmness in many reports, such as *Annona squamosa*, pepper, and pear [17,59,60]. Therefore, changes in the content of these cell wall components are important indicators of fruit softening. In this study, the content of WSP increased significantly after overexpression of RiPG2, whereas the content of protopectin decreased significantly (Figure 6A,C). The content of CSP, cellulose, and hemicellulose decreased with fruit ripening (Figure 6D–F). These changes were consistent with the mechanism of cell wall degradation. It is noteworthy that differences in cellulose and hemicellulose contents were observed only at 40 and 45 days after anthesis, which was consistent with the trend of fruit firmness. This may be the reason why the fruit firmness of tomato plants overexpressing the *RiPG2* gene was similar to that of controls at the early stage of development. In addition, the ISP content showed an inconsistent trend in the four apple cultivars [61], while it decreased in pomelo [62]. In our study, the ISP content increased significantly with fruit softening (Figure 6B). This can be attributed to the accumulation of a large number of pectin molecules after the cleavage of the covalent bonds in CSP. The free pectin molecules bind to ions, resulting in an increase in ISP content.

The expression of *RiPG2* was significantly different between the transgenic lines, but this difference did not appear in physiological indicators. This may be related to the synergistic regulation of *RiPG2* and other cell wall-related genes. Cell wall degradation is the result of the synergistic action of several cell wall enzymes and proteins. Previous studies have shown that increased activity of these enzymes and proteins leads to fruit softening [63]. The low activity of pectinase synergistically inhibited the degradation of cell walls and increased their resistance to cleavage after the color change stage in grapes [64]. The genes encoding these enzymes and proteins may also have synergistic effects with each other [35]. Silencing the *PG* gene in strawberries reduced the expression of other genes encoding cell wall enzymes [24]. The results of the present study strengthened this hypothesis, as overexpression of *RiPG2* in tomatoes significantly up-regulated the expression of *SlEXP1*, *SlXTH5*, *SlTBG4*, and *SlPL* genes (Figure 7). The specific co-regulatory mechanism is not investigated in the present study, but it has been reported that it may be related to the release of oligogalactosides from PGs [23]. The role of OGAs will be the focus of our next study.

The mechanism of raspberry fruit softening through gene cloning and molecular biotechnology analysis has not been reported. In this study, the *RiPG* gene was cloned from raspberry fruit for the first time, and its function was verified by stable genetic transformation. This provides a new idea for improving the firmness and prolonging the shelf life of berry fruits using gene editing technology. However, since the genetic transformation system of raspberry has not been established, the *RiPG* gene was heterologously transformed into tomato in this study. Although research reports on heterologous transformation of model plants to verify gene function are common, the regulatory mechanism of *RiPG* in

model plants may be different from that in raspberry. Therefore, the stable genetic transformation system of raspberry will be further explored to verify the function of the *RiPG* gene in raspberry. And RNAi technology will be used to interfere with the expression of the *RiPG* gene to observe whether this technology has practical significance in improving berry quality.

5. Conclusions

Overexpression of the *RiPG2* gene affected the content of fruit cell wall components by significantly increasing the enzymatic activity of PG and upregulating the expression levels of genes related to cell wall metabolism. This resulted in the fruit softening. However, overexpression of *RiPG2* in tomatoes had no significant effect on fruit phenotype during ripening. In the future, our group will continue to explore the genetic transformation system of raspberries and further investigate the mechanism of fruit ripening and softening through gene editing technology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010160/s1, Table S1. List of primers used in this study. Figure S1. Nucleotide and deduced amino acid sequences of *RiPG2* gene. Figure S2. Hydrophilicity prediction of RiPG2 protein.

Author Contributions: G.Y. designed the experiments. T.L. and X.G. wrote and edited the manuscript. T.L. and X.G. analyzed the data and performed the genetic transformation and functional validation. Y.C., J.L., C.Y. and Z.G. performed the gene clone. All authors have read and agreed to the published version of the manuscript.

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References

1. He, P.; Zhang, J.; Lv, Z.; Cui, P.; Xu, X.; George, M.S.; Lu, G. Genome-wide identification and expression analysis of the polygalacturonase gene family in sweetpotato. *BMC Plant Biol.* **2023**, *23*, 300. [CrossRef] [PubMed]
2. Vaahtera, L.; Schulz, J.; Hamann, T. Cell wall integrity maintenance during plant development and interaction with the environment. *Nat. Plants* **2019**, *5*, 924–932. [CrossRef] [PubMed]
3. Kreitschitz, A.; Gorb, S.N. How does the cell wall 'stick' in the mucilage? A detailed microstructural analysis of the seed coat mucilaginous cell wall. *Flora* **2017**, *229*, 9–22. [CrossRef]
4. Liu, J.; Bi, J.; McClements, D.J.; Liu, X.; Yi, J.; Lyu, J.; Zhou, M.; Verkerk, R.; Dekker, M.; Wu, X.; et al. Impacts of thermal and non-thermal processing on structure and functionality of pectin in fruit-and vegetable-based products: A review. *Carbohydr. Polym.* **2020**, *250*, 116890. [CrossRef] [PubMed]
5. Wang, D.; Yeats, T.H.; Uluisik, S.; Rose, J.K.C.; Seymour, G.B. Fruit Softening: Revisiting the Role of Pectin. *Trends Plant Sci.* **2018**, *23*, 302–310. [CrossRef] [PubMed]
6. Xu, M.; Zhou, W.; Geng, W.; Zhao, S.; Pan, Y.; Fan, G.; Zhang, S.; Wang, Y.; Liao, K. Transcriptome analysis insight into ethylene metabolism and pectinase activity of apricot (*Prunus armeniaca* L.) development and ripening. *Sci. Rep.* **2021**, *11*, 13569. [CrossRef]
7. Gacura, M.D.; Sprockett, D.D.; Heidenreich, B.; Blackwood, C.B. Comparison of pectin-degrading fungal communities in temperate forests using glycosyl hydrolase family 28 pectinase primers targeting Ascomycete fungi. *J. Microbiol. Methods* **2016**, *123*, 108–113. [CrossRef]
8. Liu, S.; Tian, L.; Cong, Y.; Shi, Q.; Wang, L.; Lu, Y.; Wang, L.; Yang, G. Recent advances in polygalacturonase: Industrial applications and challenges. *Carbohydr. Res.* **2023**, *528*, 108816. [CrossRef]
9. Liu, J.; Li, C.; Jin, Y.; Zhang, S.; Wang, M.; Ge, Y. Repression of cell wall metabolism by calcium lactate enhances the postharvest quality maintenance of Jinfeng pear fruit. *Sci. Hortic.* **2023**, *322*, 112460. [CrossRef]
10. Guo, X.; Li, Q.; Luo, T.; Han, D.; Zhu, D.; Wu, Z. Postharvest Calcium Chloride Treatment Strengthens Cell Wall Structure to Maintain Litchi Fruit Quality. *Foods* **2023**, *12*, 2478. [CrossRef]

11. Cervone, F.; Castoria, R.; Spanu, P.; Bonfante-Fasolo, P. Pectinolytic activity in some ericoid mycorrhizal fungi. *Trans. Br. Mycol. Soc.* **1988**, *91*, 537–539. [CrossRef]
12. Zhai, Z.; Feng, C.; Wang, Y.; Sun, Y.; Peng, X.; Xiao, Y.; Zhang, X.; Zhou, X.; Jiao, J.; Wang, W.; et al. Genome-Wide Identification of the *Xyloglucan endotransglucosylase/Hydrolase* (*XTH*) and *Polygalacturonase* (*PG*) Genes and Characterization of Their Role in Fruit Softening of Sweet Cherry. *Int. J. Mol. Sci.* **2021**, *22*, 12331. [CrossRef] [PubMed]
13. Brummell, D.A. Cell wall disassembly in ripening fruit. *Funct. Plant Biol.* **2006**, *33*, 103–119. [CrossRef]
14. Verhage, L. Get in shape–how a polygalacturonase affects plant morphology. *Plant J.* **2021**, *106*, 1491–1492. [CrossRef] [PubMed]
15. Wang, F.; Sun, X.; Liu, B.; Kong, F.; Pan, X.; Zhang, H. A polygalacturonase gene *PG031* regulates seed coat permeability with a pleiotropic effect on seed weight in soybean. *Theor. Appl. Genet.* **2022**, *135*, 1603–1618. [CrossRef]
16. Ye, J.; Yang, X.; Yang, Z.; Niu, F.; Chen, Y.; Zhang, L.; Song, X. Comprehensive analysis of polygalacturonase gene family highlights candidate genes related to pollen development and male fertility in wheat (*Triticum aestivum* L.). *Planta* **2020**, *252*, 31. [CrossRef]
17. Wang, S.; Liu, C.; Su, X.; Chen, L.; Zhu, Z. Transcriptome analysis reveals key metabolic pathways and gene expression involving in cell wall polysaccharides-disassembling and postharvest fruit softening in custard apple (*Annona squamosa* L.). *Int. J. Biol. Macromol.* **2023**, *240*, 124356. [CrossRef]
18. Wang, Y.; Fan, Z.; Zhai, Y.; Huang, H.; Vainstein, A.; Ma, H. Polygalacturonase gene family analysis identifies *FcPG12* as a key player in fig (*Ficus carica* L.) fruit softening. *BMC Plant Biol.* **2023**, *23*, 320. [CrossRef]
19. Fabi, J.P.; Broetto, S.G.; Silva, S.L.; Zhong, S.; Lajolo, F.M.; do Nascimento, J.R.O. Analysis of Papaya Cell Wall-Related Genes during Fruit Ripening Indicates a Central Role of Polygalacturonases during Pulp Softening. *PLoS ONE* **2014**, *9*, e105685. [CrossRef]
20. Zhang, S.; Ma, M.; Zhang, H.; Zhang, S.; Qian, M.; Zhang, Z.; Luo, W.; Fan, J.; Liu, Z.; Wang, L. Genome-wide analysis of polygalacturonase gene family from pear genome and identification of the member involved in pear softening. *BMC Plant Biol.* **2019**, *19*, 587. [CrossRef]
21. Huang, W.; Chen, M.; Zhao, T.; Han, F.; Zhang, Q.; Liu, X.; Jiang, C.; Zhong, C. Genome-Wide Identification and Expression Analysis of Polygalacturonase Gene Family in Kiwifruit (*Actinidia chinensis*) during Fruit Softening. *Plants* **2020**, *9*, 327. [CrossRef] [PubMed]
22. Qian, M.; Zhang, Y.; Yan, X.; Han, M.; Li, J.; Li, F.; Li, F.; Zhang, D.; Zhao, C. Identification and Expression Analysis of Polygalacturonase Family Members during Peach Fruit Softening. *Int. J. Mol. Sci.* **2016**, *17*, 1933. [CrossRef] [PubMed]
23. López-Casado, G.; Sánchez-Raya, C.; Ric-Varas, P.D.; Paniagua, C.; Blanco-Portales, R.; Muñoz-Blanco, J.; Pose, S.; Matas, A.J.; Mercado, J.A. CRISPR/Cas9 editing of the polygalacturonase *FaPG1* gene improves strawberry fruit firmness. *Hortic. Res.* **2023**, *10*, uhad11. [CrossRef] [PubMed]
24. Paniagua, C.; Ric-Varas, P.; García-Gago, J.A.; López-Casado, G.; Blanco-Portales, R.; Muñoz-Blanco, J.; Schückel, J.; Knox, J.P.; Matas, A.J.; Quesada, M.A.; et al. Elucidating the role of polygalacturonase genes in strawberry fruit softening. *J. Exp. Bot.* **2020**, *71*, 7103–7117. [CrossRef] [PubMed]
25. Qian, M.; Xu, Z.; Zhang, Z.; Li, Q.; Yan, X.; Liu, H.; Han, M.; Li, F.; Zheng, J.; Zhang, D.; et al. The downregulation of *PpPG21* and *PpPG22* influences peach fruit texture and softening. *Planta* **2021**, *254*, 22. [CrossRef] [PubMed]
26. Bowen-Forbes, C.S.; Zhang, Y.; Nair, M.G. Anthocyanin content, antioxidant, anti-inflammatory and anticancer properties of blackberry and raspberry fruits. *J. Food Compos. Anal.* **2010**, *23*, 554–560. [CrossRef]
27. Elias, M.I.; Madureira, J.; Santos, P.M.P.; Carolino, M.M.; Margaça, F.M.A.; Cabo Verde, S. Preservation treatment of fresh raspberries by e-beam irradiation. *Innov. Food Sci. Emerg. Technol.* **2020**, *66*, 102487. [CrossRef]
28. De Ancos, B.; González, E.M.; Cano, M.P. Ellagic Acid, Vitamin C, and Total Phenolic Contents and Radical Scavenging Capacity Affected by Freezing and Frozen Storage in Raspberry Fruit. *J. Agric. Food. Chem.* **2000**, *48*, 4565–4570. [CrossRef] [PubMed]
29. Simpson, C.G.; Cullen, D.W.; Hackett, C.A.; Smith, K.; Hallett, P.D.; McNicol, J.; Woodhead, M.; Graham, J. Mapping and expression of genes associated with raspberry fruit ripening and softening. *Theor. Appl. Genet.* **2017**, *130*, 557–572. [CrossRef]
30. Monsalve, L.; Ayala-Raso, A.; Bernales, M.; Valdenegro, M.; Defilippi, B.; González-Agüero, M.; Cherian, S.; Fuentes, L. Dataset on quality and physiological changes of raspberry fruit during their development and under auxin in-vitro assay. *Data Brief.* **2018**, *21*, 1521–1525. [CrossRef]
31. Morales-Quintana, L.; Monsalve, L.; Bernales, M.; Figueroa, C.R.; Valdenegro, M.; Olivares, A.; Álvarez, F.; Cherian, S.; Fuentes, L. Molecular dynamics simulation of the interaction of a raspberry polygalacturonase (RiPG) with a PG inhibiting protein (RiPGIP) isolated from ripening raspberry (*Rubus idaeus* cv. Heritage) fruit as a model to understand proteins interaction during fruit softening. *J. Mol. Graph. Modell.* **2023**, *122*, 108502.
32. Gimeno, D.; Gonzalez-Buesa, J.; Oria, R.; Venturini, M.E.; Arias, E. Effect of Modified Atmosphere Packaging (MAP) and UV-C Irradiation on Postharvest Quality of Red Raspberries. *Agriculture* **2022**, *12*, 29. [CrossRef]
33. Yan, R.; Han, C.; Fu, M.; Jiao, W.; Wang, W. Inhibitory Effects of $CaCl_2$ and Pectin Methylesterase on Fruit Softening of Raspberry during Cold Storage. *Horticulturae* **2022**, *8*, 1. [CrossRef]
34. Ren, Y.; Sun, P.; Wang, X.; Zhu, Z. Degradation of cell wall polysaccharides and change of related enzyme activities with fruit softening in *Annona squamosa* during storage. *Postharvest Biol. Technol.* **2020**, *166*, 111203. [CrossRef]
35. Zheng, Y.; Duan, L.; Ran, Y.; Zhang, P.; Jiang, Y.; Zhao, Z.; Li, Z.; Chen, L.; Tang, Y.; Li, X.; et al. Transcriptomics analysis reveals molecular mechanism of softening and cell wall polysaccharides-disassembling in peaches treated by flow microcirculation of sodium nitroprusside medium. *Postharvest Biol. Technol.* **2023**, *196*, 112190. [CrossRef]

36. Yang, G.; Chen, Y.; Yu, H.; Zhang, H.; Han, D.; Guo, X.; Yan, E.; Quan, H.; Li, T. Raspberry (*Rubus idaeus* L.) *NCED1* gene enhances high salinity and cold tolerance in *Arabidopsis*. *In Vitro Cell. Dev. Biol. Plant* **2021**, *57*, 811–819. [CrossRef]
37. Yao, C.; Li, X.; Li, Y.; Yang, G.; Liu, W.; Shao, B.; Zhong, J.; Huang, P.; Han, D. Overexpression of a *Malus baccata* MYB transcription factor gene MbMYB4 increases cold and drought tolerance in *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2022**, *23*, 1794. [CrossRef]
38. Han, D.; Du, M.; Zhou, Z.; Wang, S.; Li, T.; Han, J.; Xu, T.; Yang, G. Overexpression of a *Malus baccata* NAC transcription factor gene MbNAC25 increases cold and salinity tolerance in *Arabidopsis*. *Int. J. Mol. Sci.* **2020**, *21*, 1198. [CrossRef]
39. Sparkes, I.A.; Runions, J.; Kearns, A.; Hawes, C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* **2006**, *1*, 2019–2025. [CrossRef]
40. Tan, B.; Yan, L.; Li, H.; Lian, X.; Cheng, J.; Wang, W.; Zheng, X.; Wang, X.; Li, J.; Ye, X.; et al. Genome-wide identification of HSF family in peach and functional analysis of *PpHSF5* involvement in root and aerial organ development. *PeerJ* **2021**, *9*, e10961. [CrossRef]
41. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ Method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
42. Fillatti, J.J.; Kiser, J.; Rose, R.; Comai, L. Efficient transfer of a glyphosate tolerance gene into tomato using a binary agrobacterium tumefaciens vector. *Nat. Biotechnol.* **1987**, *5*, 726–730. [CrossRef]
43. Debbarma, J.; Saikia, B.; Singha, D.L.; Das, D.; Keot, A.K.; Maharana, J.; Velmurugan, N.; Arunkumar, K.P.; Reddy, P.S.; Chikkaputtaiah, C. CRISPR/Cas9-mediated mutation in XSP10 and SlSAMT genes impart genetic tolerance to fusarium wilt disease of tomato (*Solanum lycopersicum* L.). *Genes* **2023**, *14*, 488. [CrossRef] [PubMed]
44. He, Y.; Karre, S.; Johal, G.S.; Christensen, S.A.; Balint-Kurti, P. A maize polygalacturonase functions as a suppressor of programmed cell death in plants. *BMC Plant Biol.* **2019**, *19*, 310. [CrossRef] [PubMed]
45. Wang, F.; Du, C.; Chen, J.; Shi, L.; Li, H. A new method for determination of pectin content using spectrophotometry. *Polymers* **2021**, *13*, 2847. [CrossRef] [PubMed]
46. Shi, L.; Liu, Q.; Qiao, Q.; Zhu, Y.; Huang, W.; Wang, X.; Ren, Z. Exploring the effects of pectate and pectate lyase on the fruit softening and transcription profiling of *Solanum lycopersicum*. *Food Control* **2022**, *133*, 108636. [CrossRef]
47. Wang, D.; Samsulrizal, N.H.; Yan, C.; Allcock, N.S.; Craigon, J.; Blanco-Ulate, B.; Ortega-Salazar, I.; Marcus, S.E.; Bagheri, H.M.; Perez Fons, L.; et al. Characterization of CRISPR mutants targeting genes modulating pectin degradation in ripening tomato. *Plant Physiol.* **2019**, *179*, 544–557.
48. Liu, R.; Shang, F.; Niu, B.; Wu, W.; Han, Y.; Chen, H.; Gao, H. Melatonin treatment delays the softening of blueberry fruit by modulating cuticular wax metabolism and reducing cell wall degradation. *Food Res. Int.* **2023**, *173*, 113357. [CrossRef]
49. Bu, Y.; Niu, F.; He, M.; Ye, J.; Yang, X.; Du, Z.; Zhang, L.; Song, X. The gene *TaPG* encoding a polygalacturonase is critical for pollen development and male fertility in thermo-sensitive cytoplasmic male-sterility wheat. *Gene* **2022**, *833*, 146596. [CrossRef]
50. Lu, L.; Hou, Q.; Wang, L.; Zhang, T.; Zhao, W.; Yan, T.; Zhao, L.; Li, J.; Wan, X. Genome-Wide Identification and Characterization of Polygalacturonase Gene Family in Maize (*Zea mays* L.). *Int. J. Mol. Sci.* **2021**, *22*, 10722. [CrossRef]
51. Xiao, C.; Somerville, C.; Anderson, C.T. POLYGALACTURONASE INVOLVED IN EXPANSION1 functions in cell elongation and flower development in *Arabidopsis*. *Plant Cell* **2014**, *26*, 1018–1035. [CrossRef] [PubMed]
52. Bai, M.; Tong, P.; Luo, Q.; Shang, N.; Huang, H.; Huai, B.; Wu, H. *CgPG21* is involved in the degradation of the cell wall during the secretory cavity formation in *Citrus grandis* 'Tomentosa' fruits. *Plant Cell Rep.* **2023**, *42*, 1311–1331. [CrossRef] [PubMed]
53. Kalaitzis, P.; Solomos, T.; Tucker, M.L. Three different polygalacturonases are expressed in tomato leaf and flower abscission, each with a different temporal expression pattern. *Plant Physiol.* **1997**, *113*, 1303–1308. [CrossRef] [PubMed]
54. Asif, M.H.; Nath, P. Expression of multiple forms of polygalacturonase gene during ripening in banana fruit. *Plant Physiol. Biochem.* **2005**, *43*, 177–184. [CrossRef] [PubMed]
55. Liu, Y.; Dong, T.; Lei, Y.; Deng, X.; Gu, Q. Isolation of a polygalacturonase gene from citrus sinensis fruit and its expression relative to fruit mastication trait, fruit development, and calcium or boron treatments. *Plant Mol. Biol. Rep.* **2011**, *29*, 51–59. [CrossRef]
56. Nie, H.; Shi, Y.; Geng, X.; Xing, G. CRISRP/Cas9-mediated targeted mutagenesis of tomato polygalacturonase gene (*SlPG*) delays fruit softening. *Front. Plant Sci.* **2022**, *13*, 729128. [CrossRef]
57. Lin, Y.; Lin, H.; Wang, H.; Lin, M.; Chen, Y.; Fan, Z.; Hung, Y.C.; Lin, Y. Effects of hydrogen peroxide treatment on pulp breakdown, softening, and cell wall polysaccharide metabolism in fresh longan fruit. *Carbohydr. Polym.* **2020**, *242*, 116427. [CrossRef]
58. Deng, L.; Pan, Z.; Zhang, Q.; Liu, Z.; Zhang, Y.; Meng, J.; Gao, Z.; Xiao, H. Effects of ripening stage on physicochemical properties, drying kinetics, pectin polysaccharides contents and nanostructure of apricots. *Carbohydr. Polym.* **2019**, *222*, 114980. [CrossRef]
59. Mi, S.; Li, T.; Shi, Q.; Zhu, W.; Wang, X. Cold shock precooling improves the firmness of chili pepper during postharvest storage and the molecular mechanisms related to pectin. *Food Chem.* **2023**, *419*, 136052. [CrossRef]
60. Li, J.; Dai, X.; Li, Q.; Jiang, F.; Xu, X.; Guo, T.; Zhang, H. Low temperatures inhibit the pectin degradation of 'Docteur Jules Guyot' pear (*Pyrus communis* L.). *Int. J. Biol. Macromol.* **2023**, *242*, 124719. [CrossRef]
61. Li, X.; Su, Q.; Jia, R.; Wang, Z.; Fu, J.; Guo, J.; Yang, H.; Zhao, Z. Comparison of cell wall changes of two different types of apple cultivars during fruit development and ripening. *J. Integr. Agric.* **2023**, *22*, 2705–2718. [CrossRef]
62. Liu, J.; Liang, L.; Jiang, Y.; Chen, J. Changes in metabolisms of antioxidant and cell wall in three pummelo cultivars during postharvest storage. *Biomolecules* **2019**, *9*, 319. [CrossRef] [PubMed]

63. Minic, Z.; Jouanin, L. Plant glycoside hydrolases involved in cell wall polysaccharide degradation. *Plant Physiol. Biochem.* **2006**, *44*, 435–449. [CrossRef] [PubMed]
64. Yu, J.; Wang, R.; Ma, W.; Lei, S.; Zhu, M.; Yang, G. Pectate yase gene *VvPL1* plays a role in fruit cracking of table grapes. *J. Agric. Food. Chem.* **2023**, *71*, 1643–1654. [CrossRef]