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Original Paper

Characterization of the expression of the stress-responsive *PpERS1* gene from peach and analysis of its promoter using transgenic tomato

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Abstract The *PpERS1* gene, which encodes an ethylene receptor and responds to abiotic and biotic stresses, was cloned from peach (*Prunus persica* L. Batsch cv Okubao). The genomic DNA sequence of *PpERS1* comprises seven exons which are separated by six introns, interestingly alternative splicing of the first intron produced three different *PpERS1* transcripts. In addition, a 2.8-kb sequence including the promoter of *PpERS1* was isolated and analyzed by placing expressing of the GUS reporter gene under its control. Several putative *cis*-elements were identified in the promoter of *PpERS1*, including two ethylene-responsive elements (EREs), five W boxes, and four putative binding sites for MYB-type transcription factors. Deletion analysis indicated the presence of an enhancer element in the *PpERS1* promoter. Temporal and spatial expression analysis of the *PpERS1* promoter using histochemical GUS staining showed GUS activity in all tissues examined throughout the development of transgenic tomato plants. Exposure to various stresses caused similar changes in expression patterns in peach and transgenic tomato plants. Overall, our results suggested that *PpERS1* gene might play important roles in response to multiple stresses via signal transduction mediated by ethylene receptors. The characterization of the *PpERS1* promoter contributes to our understanding of the transcriptional regulation of this ethylene receptor in peach.

Key words: ethylene receptor, *PpERS1*, promoter, stress responses, transcriptional expression.

The phytohormone ethylene regulates multiple aspects of plant growth, development and stress responses following its perception by ethylene receptors and subsequent activation of receptor-mediated signal transduction (Lin et al. 2009; Keunen et al. 2016; Kumar 2013). Great progress in understanding the ethylene signaling pathway by which plants respond to ethylene was achieved through binding to its receptors (Gallie 2015; Merchante et al. 2013). Ethylene receptors are a family of integral membrane members similar to bacterial two-component histidine kinases, which act as negative regulators of the ethylene response pathway (Hua and Meyerowitz 1998; Liu et al. 2010). To date, five ethylene receptors-*ETR1*, *ERS1*, *ETR2*, *EIN4*, and *ERS2*-have been identified in *Arabidopsis thaliana*, and components involved in the signaling pathways downstream of their activation have been characterized (Chang et al. 1993; Yang et al. 2015).

Since the cloning of the first ethylene receptor ETR1 from Arabidopsis, a variety of studies have steadily improved our knowledge of the receptors in ethylene signal transduction pathway. The ethylene receptor family has been divided into two subfamilies (type I and II) on the basis of structural similarities of Arabidopsis ethylene receptors (Gamble et al. 1998; Moussatche and Klee 2004). Several studies have demonstrated that ethylene receptors display spatial and temporal regulation at the transcriptional level following perception of internal (developmental) and external (environmental) stimuli, such as ripening, senescence, wounding, dehydration, and pathogen infection (Ciardi and Klee 2001; Wang et al. 2013). Ethylene receptors have been extensively studied in model plant, interestingly, analyses of their expression patterns have indicated that their regulation is complex and varies in relation to tissue, organ type and stress (Grefen et al. 2008; O'Malley et al. 2005). It is well known that expression of type-II receptor genes, such as *NTHK1* and *NTHK2*, either increases or decreases upon exposure to various environmental stresses, such as wounding, salt, and drought treatments (Cao et al. 2006; Cao et al. 2007). However, little information is available about how ethylene receptor response to adversity stress, although several ethylene receptors have been cloned in rosaceous plant.

Peach is becoming a very promising climacteric drupe model to enhance our insight into ethylene biology and ripening, and for genomic analysis of fruit tree

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species (Li et al. 2013; Verde et al. 2013), even though transformation events in peach have been rarely reported (Pérez-Clemente et al. 2004). Like other fruit species, stress restricts the yield and geographical area over which peach trees can be planted, and this continues to drive worldwide efforts to generate stress-tolerant cultivars using traditional and molecular genetic approaches (Liu et al. 2009). Insight into the transcriptional regulation of ethylene receptor genes will likely be essential to complement these efforts, given that elucidation of the function of ethylene in stress responses should be invaluable in guiding breeding strategies.

In this study, the ethylene receptor gene *PpERS1* was cloned from peach, and the effects of biotic and abiotic stresses on its expression were investigated. The promoter of *PpERS1* was isolated and characterized in order to investigate the regulatory mechanism(s) responsible for stress responsiveness. Our data showed that both levels of *PpERS1* transcripts and activation of the *PpERS1* promoter either increased or decreased in response to the abiotic stresses tested. These results suggest that *PpERS1* might play a crucial role in responding to multiple stresses in peach.

Materials and methods

Plant materials and treatment

In May 2010, green twigs of approximately 30 cm in length were collected from 7-year-old peach (*Prunus persica* L. Batsch cv Okubao) trees, which were grown in peach orchards of Huazhong Agricultural University, Wuhan, China. To investigate expressions of *PpERS1* gene upon exposure to environmental stresses, the twigs were treated with ethephon, wounding, pathogen, salt, dehydration and low temperature as described previously (Liu et al. 2009; Wang et al. 2011b). For ethephon treatments, the twigs were sprayed with 150 mg/L ethephon solution, containing 0.05% Triton X-100. For the wounding treatment, leaves were cut into discs with 1-cm diameters using a puncher, and incubated on wet filter paper. Infection with *Botryosphaeria rhodina* (anamorph *Lasiodiplodia theobromae*) was used for pathogen treatment. *L.theobromae* cells were grown on PDA at 25°C for 5 days prior to inoculation. 6-mm-diameter mycelial plugs were placed on the wound which were generated by a sterilized on the twigs. All inoculated twigs were placed in a plastic container which was covered with plastic film, with three inoculation sites for each twig. Some twigs were put in flasks containing salt (200 mM NaCl) or empty for salt and dehydration treatments, respectively. In addition, the twigs were kept at 4°C for low temperature treatment. Leaves were sampled 0, 1, 5, 12, 24, and 48 h after treatment with NaCl or low temperature; 0, 0.5, 1, 3, 6, and 12 h after dehydration or ethephon treatment; 0, 0.5, 1, 6, and 12 h after wounding treatment; and 0, 5, 12, 24, 48, and 72 h after treatment with *L.theobromae*. Samples were immediately frozen in liquid nitrogen, and stored at −80°C until use.

Amplification of genomic sequence and cDNA of PpERS1

Genomic DNA was isolated from fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) method described previously (Poresbski et al. 1997). Total RNA was extracted from the leaves as described previously (Meisel et al. 2005). 5 μg of DNA-free RNA that had been digested with 10 units of DNase I (RNase Free) (TaKaRa, Dalian, Liaoning, P. R. China) was used to synthesize the first strand cDNA with M-MLV First Strand kit according to the manufacturer's instructions (Invitrogen, Beijing, P. R. China).

Based on the sequence (Accession number AF316534) of *Prunus persica* deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/), the gene-specific primers (PpERS1-Forward Primer and PpERS1-Reverse Primer; Table 1) were designed to amplify the *ERS1* coding sequence in peach. The PCR reaction was carried out in a total volume of 50 μl, including 35.5 μl of H_2O, 5 μl of 10×TransTaq-T buffer, 5 μl of dNTP Mix (2.5 mM each), 2 μl of first-strand cDNA or genomic DNA as template, 1 μl primer (10 μM), and 0.5 μl TransTaq-T DNA Polymerase (5U, TransGen Biotech, Beijing, China). The temperature cycling program involved an initial 5-min denaturation at 94°C, followed by 35 cycles that each comprised 94°C for 30 s, 56°C for 35 s, and 72°C for 2-min (for cDNA) or 3-min (for genomic DNA), and a final 10-min extension at 72°C. The transcription start site of the *PpERS1* gene was identified by 5′ RACE (rapid amplification of cDNA ends) according to the instructions provided with the SMARTer™ RACE cDNA amplification kit user manual (Clontech Laboratories, Palo Alto, CA) (Wu et al. 2009). 5′ RACE was performed by using the gene specific reverse primers of PpERS1 (PpERS1-Race outer primer and PpERS1-Race inner primer; Table 1) and 5′-CDS primers A provided by SMARTerTM RACE cDNA amplification kit. The thermal cycling conditions used for RACE-PCR protocols involved 25 cycles that each comprised heating to 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min.

Isolation of the 5′ flanking region of the *PpERS1* gene was carried out using a Universal Genome Walker Kit (Clontech Laboratories, Palo Alto, CA). The primary PCR amplification involved the use of a *PpERS1* gene-specific reverse primer (GSP1) and universal walker primer (Adapor Primer) (Table 1), using genomic DNA as template. The diluted primary PCR product was used as a template for the secondary PCR with nested gene-specific reverse primer (GSP2) and adaptor walker primer (Nested Adapor Primer) (Table 1). These gene-specific primers were designed based on *PpERS1* gene sequence. The PCR products were gel purified, cloned into pMD 18-T vector (TaKaRa, Dalian, Liaoning, P. R. China), and then sequenced.

Quantitative RT-PCR

Quantitative RT-PCR was carried out using the SYBR Green RT-PCR master mix kit (TOYOBO, Japan). Specific primers (PpERS1-quantitative Forward Primer and PpERS1-quantitative Reverse Primer; Table 1) located in the seventh

Table 1. Primers used in this study (restriction site sequences are boxed).

Primer	Sequence (5′-3′)
GSP1	GGTAATGGTAGGTCCTTCCCTGTCCTCAAT
GSP2	CATGTTGCACTTGCAGATAATGAAGGGTTG
Adapor Primer	GTAATACGACTCACTATAGGGC
Nested Adapor Primer	ACTATAGGGCACGCGTGGT
PpERS1-Race inner primer	GAATGGAGAAATAGGCGAGGGCAATAAAG
PpERS1-Race outer primer	CTCCCGAGTTTTGACGCTTAGTAAATCCG
D0-Forward Primer	ACGCGTCGACGTTTGGAGGCTCTTGTTCTGCTA
D1-Forward Primer	ACGCGTCGACGAGCATAGGCGGAATCAGTAG
D2-Forward Primer	ACGCGTCGACTTAACCGTCCACAACGAACCA
D3-Forward Primer	ACGCGTCGACAACGACTCTTTCGGACTGACG
D4-Forward Primer	ACGCGTCGACCTACAGTTGCTCTGTCACCCT
D0-Reverse Primer	TCCCCCGGGCAAGGGAATGGAGAAATAGGCGA
PpERS1-Forward Primer	GATAATGGATTCCTGTGATTG
PpERS1-Reverse Primer	CAACGTGCTAAAGTGTCAAA
PpERS1-quantitative Forward Primer	GATTGAGAGTGAGGGCATTG
PpERS1-quantitative Reverse Primer	GCTGCTGTTGTATCACAAGG
18S-Forward Primer	GTTACTTTTAGGACTCCGCC
18S-Reverse Primer	TTCCTTTAAGTTTCAGCCTTG
ERS1-Forward Primer	CACCCTCACTTTGTCTTTGTCGTC
GUS-Reverse Primer	CGAGTCGTCGGTTCTGTAACTATCA

exon of *PpERS1*, and peach 18S rRNA (18S-Forward Primer and 18S-Reverse Primer; Table 1) were designed according to a previously described proceduret (Rasori et al. 2002). For each sample, three replicates were performed in a final volume of 10 μl, with each containing 4.2 μl H_2O, 0.4 μl cDNA, 0.2 μl of *PpERS1*-specific primers (10 μM), and 5 μl 2×SYBR Green PCR Master Mix according to the manufacturer's instructions. All PCR were performed using the LightCycler 480 Real Time System (Roche Diagnostics). Reactions were performed by an initial incubation at 95°C for 30 s, and then 40 cycles that each involved heating to 95°C for 5 s, then 56°C for 10 s, and 72°C for 15 s. finally terminate at 95°C for 60 s, then 50°C for 60 s, and 40°C for 30 s. Output data were generated by LightCycler® 480 genotyping software (Roche, Meylan, France). The quantitative RT-PCR reactions for the targeted gene (*PpERS1*) and internal reference gene (18S rRNA) were repeated three times for each sample.

Bioinformatic analysis

Sequence alignment was carried out using the BLAST programs provided by the NCBI. Molecular weight, isoelectric point and structure of PpERS1 protein were predicted using ScanProsite (http://www.expasy.ch/resources). Multiple alignments were performed using GeneDoc v.2.6.002 (Nicholas et al. 1997). A phylogenetic tree was constructed by the neighbor-joining method using MEGA 4 (Tamura et al. 2007). *Cis*-elements located in the 5′ flanking region of *PpERS1* gene were analyzed using two online programs: PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PlantCare database (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/) at the Advanced Biosciences computing Center.

Construction of promoter::GUS vectors

To construct plasmids containing the *PpERS1* promoter or its derivatives fused to the GUS reporter gene, the binary vector pCAMBIA1391Z (Cambia, Australia) was used to construct plasmids carrying the different parts of the *PpERS1* promoter. A 2,798-bp fragment of 5′ untranslated DNA upstream of the start (ATG) codon of the *PpERS1* gene was regarded as the putative full-length promoter (D0-2798). The putative full-length promoter and fragments generated by serial deletion from the 5′ end of D0-2798 were amplified using five pairs primers (D1-Forward Primer, D2-Forward Primer, D3-Forward Primer, D4-Forward Primer and D0-Reverse Primer; Table 1) containing a *Sal*I site, and one reverse primer containing a *Sma*I site. The resulting fragments were named D1-2158, D2-1536, D3-842, and D4-528, respectively. All fragments were digested sequentially with *Sal*I and *Sma*I (TaKaRa, China) and then inserted into pCAMBIA1391Z. The pCAMBIA1391Z plasmid harboring CaMV35S promoter was used as a positive control, whereas the original vector pCAMBIA1391Z was used as a negative control.

Generation of transgenic tomato plants

Recombinant constructs were introduced into tomato (*Solanum lycopersicum* cv. Micro-Tom) using *Agrobacterium tumefaciens*-mediated transformation. Leaf disc transformation and regeneration of transgenic plants were performed as described previously (Wang et al. 2011a). Transgenic shoots were selected and rooted on media containing 10 mg/L hygromycin B. After rooting, plants were transferred to a greenhouse with a 16-h photoperiod and a temperature maintained at 25°C. The transgenic nature of plantlets resistant to hygromycin B was confirmed by PCR of genomic DNA using the promoter-specific forward primer and the GUS-specific reverse primer

(ERS1-Forward Primer, GUS-Reverse Primer; Table 1). At least five lines carrying each construct were used for further experiments.

Assay of GUS activity

Histochemical assay and fluorometric assays of *GUS* reporter gene expression were performed essentially as described previously (Jefferson et al. 1987), but with some modification. For the histochemical staining, all tomato tissues were vacuum-infiltrated with a staining solution containing 1 mg ml^{-1} 5-Bromo-4-chloro-3-indolyl-BD-glucuronide (X-gluc), 100 mM $NaHPO_4$, pH 7.0, 5 mM $K_3(Fe(CN)_6)$, 5 mM $K_4(Fe(CN)_6)$, 10 mM EDTA and 0.1% Triton X-100. After 16 h of incubation at 37°C, the materials were decolored with 75% ethanol, and photographed.

The fluorometric assay was carried out by measuring production of 4-methylumbelliferone (4-MU) from 4-methylumbelliferone glucuronide (MUG). GUS activity was normalized to protein concentration in each of the crude extracts, and expressed as the pmol of 4-MU produced per milligram of soluble protein per minute. Protein content was assessed according to the Bradford method (Bradford 1976), using bovine serum albumin (BSA) as a standard.

Treatment with ethylene and abiotic stresses

To investigate the response of the *PpERS1* promoter to ethylene and abiotic stresses, T1 seedlings (in soil for 30-day old) of transgenic tomato plants harboring *PpERS1* full-length promoter::GUS fusions were identified by PCR, and then subjected to different treatments. Mechanical wounding experiments were carried out by pricking tomato leaves with a needle. For low-temperature treatment, tomato plants were placed at 4°C. To induce high salinity stress, the tomato plants were removed from soil, their roots were gently washed with water, and then soaked in 200 mM NaCl. For ethylene treatments, the tomato plants were sprayed with 0.05% Triton X-100 and 150 mg/L ethephon. Well-watered plants were taken as controls. Tomato plants were immediately frozen in liquid nitrogen after 6 h treatment and ready for GUS activity assays.

Results and discussion

The PpERS1 gene

The 2,604-bp *PpERS1* gene comprises a 1.935-bp open reading frame (ORF), which encodes a protein of 644 amino acids with a molecular weight of 72.4 kDa and a theoretical isoelectric point of 6.33. The genomic sequence of *PpERS1* includes seven exons, which are separated by six typical plant introns, as verified by comparing a *PpERS1* cDNA sequence with its genomic sequence (Figure 1). The ORF structure of the *PpERS1* gene is consistent with those reported in previous studies (Rasori et al. 2002), the difference is that two introns, found unexpectedly by 5′ RACE, are located in the 5′ UTR of *PpERS1*, whereas no other *ERS1* genes are reported to have introns in their 5′ UTRs (Figure 2A). The first intron of the *PpERS1* gene was either 108 bp or 119 bp, with its 5′ end located either 695 or 706 nucleotides upstream of the translation start codon (ATG). The second intron was 444 bp, with its 5′ end located 476 nucleotides upstream of ATG. The use of 5′ RACE also identified the location of the transcription start site (TSS) 777 bp upstream of the translation start codon (ATG) of *PpERS1*. Interestingly, 5′ RACE identify a range of differently sized fragments, suggesting alternative splicing of the *PpERS1* primary transcript. To clarify this hypothesis, the amplification products were recovered and more than 100 clones were sequenced (Figure 2A). Results indicated that at least three different transcripts (*PpERS1A, B*, and *C*) originated from the *PpERS1* gene (Figure 2B). The only difference among them was the length of the first intron in the 5′ UTR (Figure 2A). Although 5′ UTR introns have been identified and characterized in Arabidopsis and rice (Sivamani and Qu 2006; Karthikeyan et al. 2009), limited information is about the incidence of introns in the 5′ UTRs of peach genes. Our results indicated that the translation initiation site was located within the third exon (Figure 2B). A similar gene structure had been discovered in an Arabidopsis phosphate transporter gene *AtPht1; 4* (Karthikeyan et al. 2009).

A previous study suggested that the Arabidopsis genome contains at least five different genes that encode ethylene receptors, and these can be divided into two subfamilies based on structural and sequence similarities (Hua and Meyerowitz 1998). A phylogenetic tree was constructed based on the deduced amino acid sequences of known homologs of PpERS to analyze the phylogenetic relationships of ERS1 proteins from peach and other plants. This showed that *PpERS1* clustered into

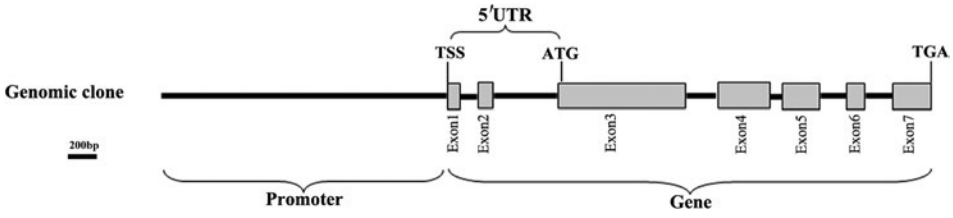

Figure 1. Detailed schematic representation of the *PpERS1* gene. TSS, transcriptional start site; ATG, translation initiation site; TGA, stop codon, 5′UTR, 5′untranslated region; exons are indicated by boxes; introns and non-coding regions are indicated by black lines. Scale bars=200 bp.

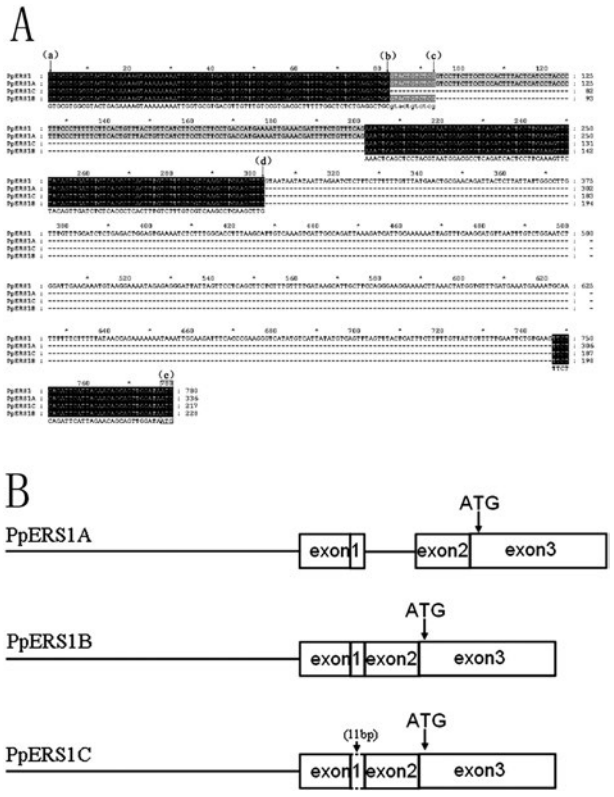

Figure 2. Alternative splicing of the first intron of the *PpERS1* gene, which is found in the 5′UTR of *PpERS1*. (A) The nucleotide sequences of the alternatively spliced transcripts *PpERS1A*, *PpERS1B*, *PpERS1C* were aligned using the CLUSTALX program with the default parameters selected. Whereas (a) denotes the transcriptional start site of *PpERS1*, the labels (b), (c), and (d) denote the alternative termination sites of the first exon in transcripts *PpERS1A*, *PpERS1B*, and *PpERS1C*, respectively. Label (e) indicates the translation initiation codon. (B) Schematic representation of the alternative processing of PpERS1 pre-mRNA. *Rectangular boxes* exons and *lines* introns, *dashed lines* deletion.

the ETR1 subfamily (Figure 3). Consistent with the fact that peach is a rosaceous plant, sequence analysis using the BLASTX algorithm provided by the NCBI revealed that *PpERS1* showed the highest homology to genes that encode ethylene receptors in rosaceous plants, such as *Prunus salicina* (ABU68267.1, 98%), *Pyrus communis* (AAL66197.1, 85%), *Pyrus pyrifolia* (BAD61002.1, 86%), *Malus domestica* (BAE97296.1, 87%), and *Fragaria*×*ananassa* (CAC48385.1, 83%). The result also demonstrated that ethylene receptor genes were relatively conserved in rosaceous species (Wang et al. 2011a).

Effects of biotic and abiotic stresses on the abundances of PpERS1 transcripts

Given that peach trees may be exposed to a range of different stresses during their life cycles, it is worthwhile to examine changes in the patterns of accumulation of *PpERS1* transcripts in response to different environmental stimuli. In the present work, expression of *PpERS1* gene was carried out with twigs that were proved validly as a approach (Liu et al. 2009),

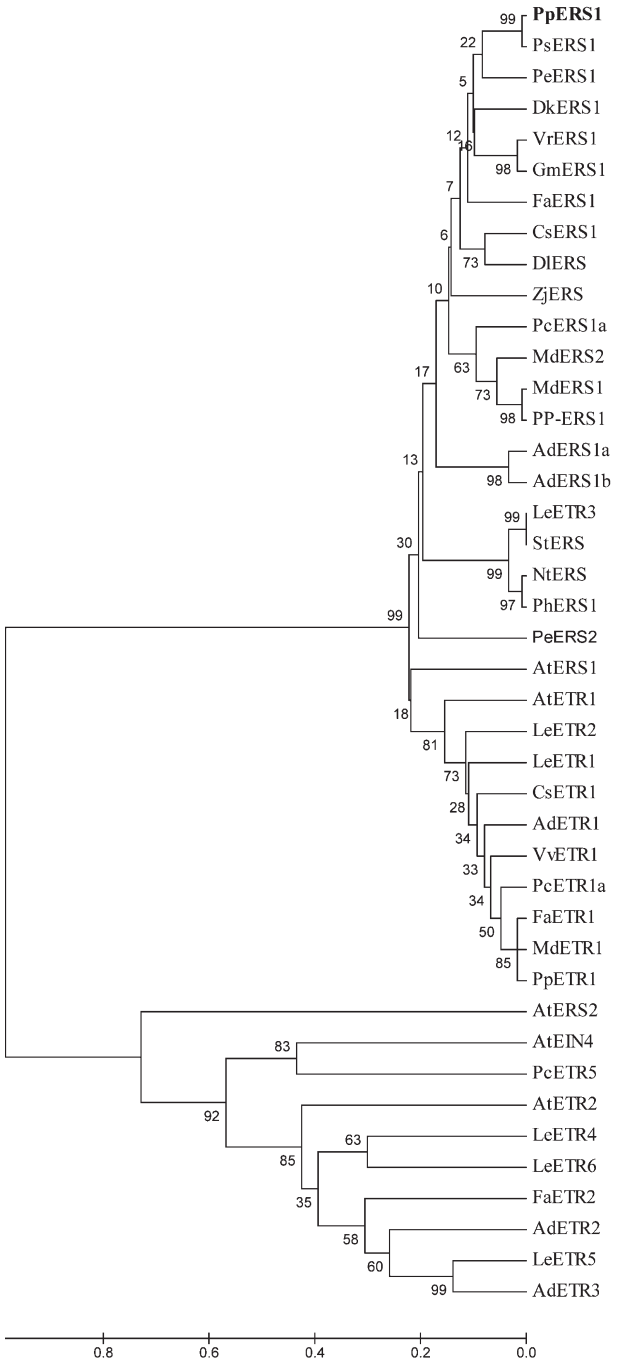

Figure 3. A phylogenetic tree constructed based on the amino acid sequences of PpERS1 (overstriking) and the closest known homologs of PpERS1. The amino acid sequences of other homologues ERS1 were obtained from the NCBI database, and accession numbers are as follows: *AtETR1* (AAA70047.1), *AtERS1* (NP_181626.1), *AtETR2* (NP_188956.1), *AtERS2* (AAC62209.1), *AtEIN4* (AAD02485.1), *LeETR1* (AAC02213.1), *LeETR2* (AAC02214.1), *LeETR3* (AAC49124.1), *LeETR4* (AAU34076.1), *LeETR5* (AAD31397.1), *LeETR6* (AAL86614.1), *MdETR1* (AAC31123.1), *MdERS2* (BAE97296.1), *MdERS1* (ABA03058.1), *PpETR1* (AAM73756.1), *PcERS1a* (AAL66197.1), *FaERS1* (CAC48385.1), *FaETR2* (CAC48386.1), *CsERS1* (AAC99435.1), *AdETR1* (ABY28264.1), *AdETR2* (ABY28265.1), *AdETR3* (ABY28266.1), *AdERS1a* (ABY28262.1), *AdERS1b* (ABY28263.1), *PsERS1* (ABU68267.1), *PP-ERS1* (BAD61002.1), *PeERS1* (BAA37137.1), *DkERS1* (BAD11810.1), *NtERS* (AAB96765.2), and *VvETR1* (AAF63755.1).

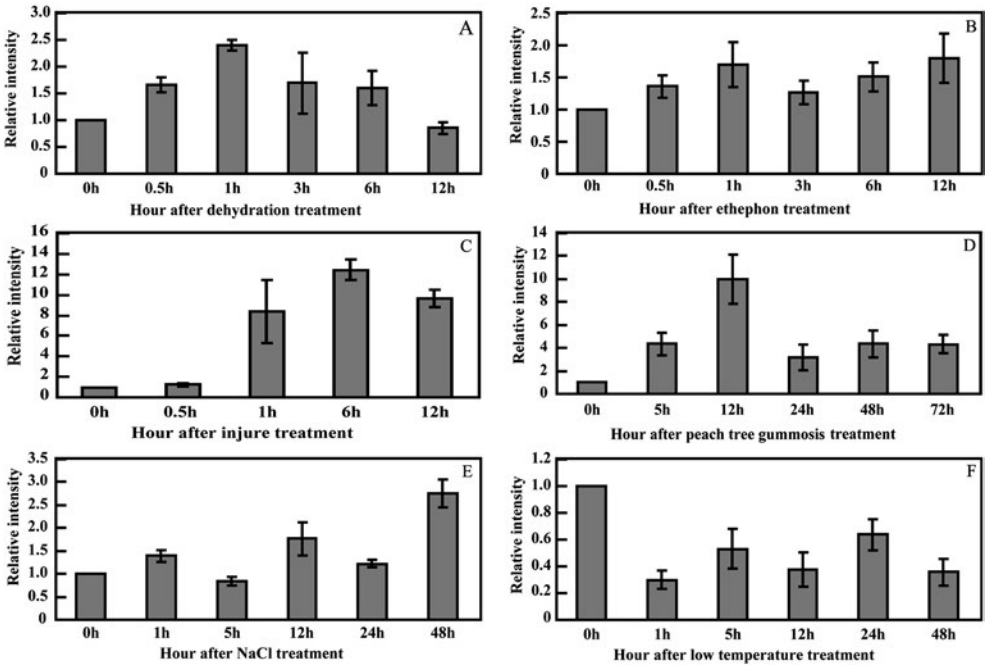

Figure 4. Changes in the abundances of *PpERS1* transcripts after exposure to several types of stresses. (A) Dehydration, (B) salt (200 mM NaCl), (C) ethephon (150 mg/L), (D) low temperature (4°C), (E) wounding, and (F) pathogen infection. Expression analysis was carried out by real time PCR. The values of each point represent mean values±SE of three replicates for the relative expression, which were standardized relative to the level of peach 18S rRNA.

various biotic or abiotic stresses, including wounding, dehydration, low temperature (4°C), salt (200 mM NaCl), ethephon (150 mg/L) and the causative agent of peach tree gummosis (*L.theobromae*) were imposed in twigs. The dehydration of a twigs can be different from a whole plants with a fully developed root system, twigs of similar length and seemingly uniform were selected, the twigs were put in containers added with distilled water and kept for 1h at 25°C in dark before they were used for dehydration treatment. For salt stress treatment, 200 mM NaCl that is not a physiological concentration was chosen just as an osmotic agent. As shown in Figure 4, changes in the abundances of *PpERS1* transcripts in response to stresses could be divided into three types: notable induction (wounding and *L.theobromae*), sustained up-regulation (dehydration, ethephon and salt), and repression (low temperature).

When the shoots experienced water loss for 0.5 h, the abundance of *PpERS1* transcripts increased appreciably and was maximal 1 h after imposition of the stress, whereafter levels decreased progressively until 12 h after the imposition of the stress, when levels of *PpERS1* transcript were comparable to those before dehydration (Figure 4A). The stimulatory effect of ethylene on *PpERS1* transcription was confirmed by monitoring changes in the abundance of *PpERS1* transcript after plants were sprayed with ethephon. Levels of *PpERS1* transcript started to increase 0.5 h after ethephon treatment, and were maintained at a stable level (approximately 50% higher than prior to ethephon treatment) throughout the entire 12-h treatment period (Figure 4B). Wounding and *L.theobromae* treatment exerted similar inductive effects on the accumulation of *PpERS1* transcripts. The level of *PpERS1* transcripts after wounding was notably increased until 6 h after the treatment, reaching levels approximately 12-fold higher than prior to wounding, which remained approximately 9-fold higher than initial levels 12 h after the treatment (Figure 4C). Exposure to the pathogen responsible for peach tree gummosis induced gradual accumulation of *PpERS1* transcript, with *PpERS1* transcripts maximal 12 h after inoculation, and then decreasing to a level approximately four-fold higher than before infection and remaining at this level until at least 72 h after infection (Figure 4D). Following exposure to NaCl and low temperature, levels of *PpERS1* transcripts fluctuated between the times when they were assayed, although overall, treatment with NaCl caused a slight increase in *PpERS1* transcript levels, whereas low temperature decreased the abundance of PpERS1 transcripts (Figure 4E, F).

Molecular genetic analyses have indicated that plants can regulate the activity of ethylene receptors at the transcriptional level (Ciardi and Klee 2001; Klee et al. 2002). As reported by Rasori (Rasori et al. 2002), ripening and senescence increased the levels of *PpERS1* transcripts. Similarly to *PpERS1*, the induction of ethylene receptor gene expression in response to ethylene or ethephon treatment was also observed in persimmon (Pang et al. 2007) and kiwifruit (Yin et al. 2008). Our

data obtained following exposure to the agent responsible for peach tree gummosis confirmed that ethylene plays a role in the defense response to certain plant-pathogen interactions (Ciardi et al. 2000).

Ethylene receptors are encoded by gene families in many plants. It is difficult to assign specific roles to individual ethylene receptors, because studies conducted in Arabidopsis and tomato suggest that they can compensate for each other (Klee and Tieman 2002; O'Malley et al. 2005), and that that they can, in all likelihood, physically interact with each other. Analysis of the structures and sequences of PpETR1 and PpERS1 indicates that they belong to the ETR1 subfamily, although the change in expression pattern of *PpETR1* in response to ethylene treatment differs from that for other members of the *ETR1* gene family (Rasori et al. 2002). A similar behavior has been observed for an *ETR*-type gene from kiwi fruit, whereas storage at low temperature increased the abundances of *AdERS1a* transcripts, the same treatment decreased the abundances of *AdERS1b* and *AdETR1* transcripts (Yin et al. 2009). The *NTHK1* and *NTHK2* genes, which encode ethylene receptors in tobacco, belong to ETR2 subfamily. However, similar to *PpERS1*, increases of the abundances of *NTHK1* and *NTHK2* transcripts have been observed in response to wounding and dehydration (Cao et al. 2007). The above-mentioned differences in expression patterns suggest that the *PpERS1* gene might play different roles in responses to different stresses.

Identification of the promoter of the PpERS1 gene

Gene function often depends substantially on the ability of gene promoters to respond to diverse signaling pathways, including those that regulate plant adaptation to biotic and abiotic stresses (Salazar et al. 2007). We used histochemical and fluorometric analysis of activity of the *GUS* reporter gene to investigate activity of the *PpERS1* promoter. To demonstrate the potential mechanism by which *PpERS1* was regulated in response to biotic and abiotic stresses, a 2,797-bp promoter sequence of *PpERS1* was isolated and designed as putative full-length promoter of *PpERS1*.

The putative *cis*-acting elements in the promoter region located upstream of the TSS were predicted by analysis of the PLACE and PlantCare databases. Several basic and potential regulatory elements associated with stress- and hormone-related responses in other plant promoters were located within the *PpERS1* promoter. A putative TAT A box sequence (TAT AAT) was found 29 bp upstream from the determined transcription start site (TSS), which resembles the position of the TAT A elements in other plant genes (Joshi 1987). A putative CAA T box sequence (CCA AT) was found 152 bp upstream from the TSS, which was twice the distance found in the promoters of most genes in other species. A highly conserved *cis*-element, the ethylene-responsive element (ERE), which is identical to the ERE of the carnation *GST1* gene (ATT TCA AA) was found in the *PpERS1* promoter (Itzhaki et al. 1994). Another ethylene responsive element, GCC box, which regulated the expression of defense-related genes by recruiting ERF (ethylene responsive factor) transcription factors, was observed downstream of the ERE site (Chakravarthy et al. 2003). The ERE and GCC boxes identified in the promoters of ethylene-inducible genes exhibit distinct roles in senescence-related and defense-related gene expression (Rawat et al. 2005). Meanwhile, we noted several W-boxes in the *PpERS1* promoter, which are pathogen-responsive *cis*-acting elements that are recognized by salicylic acid-induced WRKY DNA binding proteins (Xu et al. 2006) and may be involved in activation of *ERF3* gene by wounding (Nishiuchi et al. 2004). Some other putative *cis*-elements detected within the *PpERS1* promoter sequence include auxin and ABA response factor-binding sites, CBF (C-repeat binding factor) binding site, a circadian-response element, a cytokinin enhancer element, a binding site for the DRE/CRT transcription factor, a GA responsive element, a GT-1 element, a LTRE, bindings sites for MYB- and MYC-like transcription factors, a Q element, and a W-box.

Deletion analysis of the PpERS1 promoter in transgenic tomato

To identify the promoter region necessary for expression of the *PpERS1* gene, both full-length and truncated versions of the promoter were used to generate promoter::GUS fusion constructs (Figure 5A). Micro-Tom tomato was transformed to stably express five experimental constructs and two control constructs using Agrobacterium. Histochemical GUS staining and PCR identified at least twelve independent transgenic lines for each construct in screens of the T0 generations. A GUS histochemical assay showed that all of the *PpERS1* promoter constructs were functional in transgenic tomato and displayed distinct staining patterns on the leaves, except for the shortest one (D4-528) which showed no GUS staining (Figure 5B). Activity of the *PpERS1* promoter was quantified by fluorometric analysis of GUS activity on MUG (4-methylumbelliferone glucuronide). The results of quantitative analysis are consistent with those of histochemical analysis (Figure 5C). The observation that GUS activity was about 8-fold higher in transgenic plants that carry the full-length *PpERS1* promoter upstream of the *GUS* coding sequence, as compared with transgenic plants in which the D1-2158 promoter upstream of the *GUS* coding sequnce, indicated that the full-length promoter might contain enhancer elements that boost promoter activity. Notably, a Q element is found at

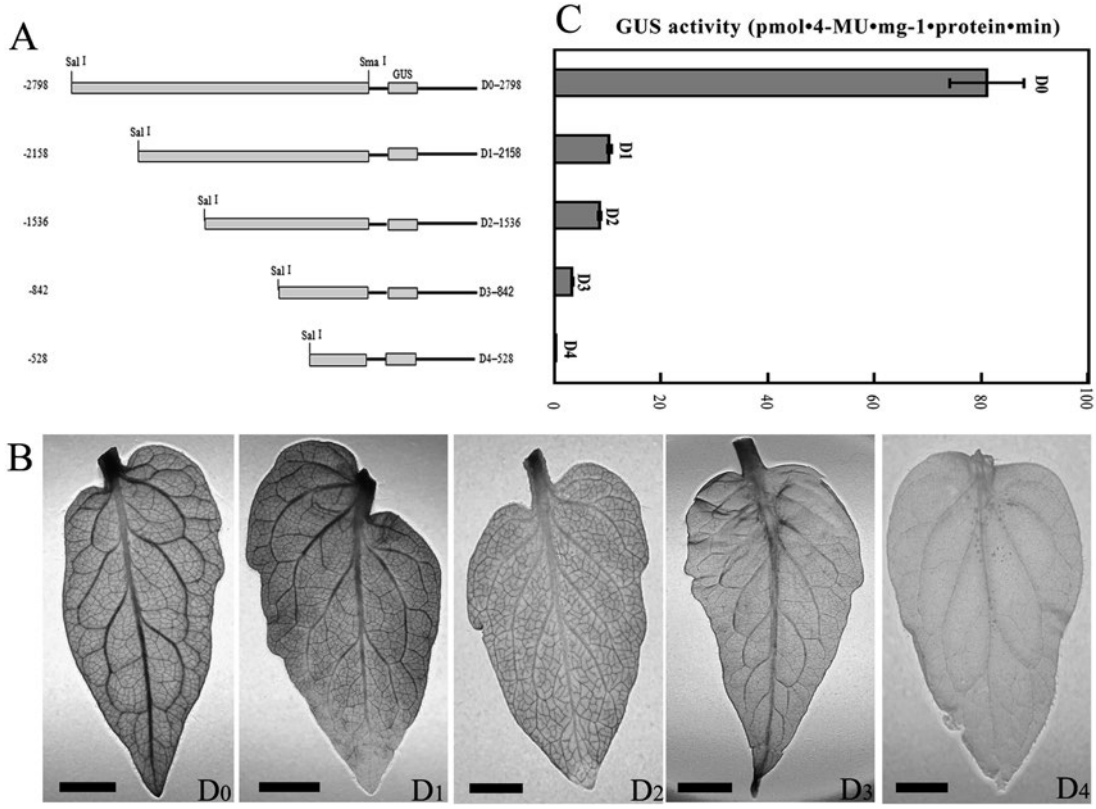

Figure 5. Assays for GUS activity driven by the full promoter of *PpERS1* and fragments left after serial deletions form its 5′ end. (A) Schematic diagram of *PpERS1* promoter constructs for assaying the activity of different parts of the *PpERS1* promoter. All promoter fragments were fused upstream of the *GUS* reporter gene in the vector pCAMBIA1391Z. (B) Fluorimetric GUS activity assays in transgenic tomato plants. The mean of each value were calculated by three independent replicates. (C) Histochemical staining for analysis function of *PpERS1* promoter and its deletions in transgenic tomato plants. D0, D0-2798; D1, D1-2158; D2, D2-1536; D3, D3-842; D4, D4-528. Scale bars=3 mm.

position −181 in the *PpESR1* promoter. Enhanced expression activity of the same Q element had been reported for the promoter of the *ZM13* gene from maize, which encodes the homolog of the product of the tomato *LAT52* gene (Hamilton et al. 1998) Transgenic plants that carry the D1-2158 and D2-1536 promoter promoter fragments cloned upstream of the *GUS* gene are characterized by GUS activities that are approximately 3-fold higher than those of transgenic plants carrying the D3-842 promoter. However, GUS activity was barely detectable in plants where GUS activity was placed under the control of the D4-528 promoter, which is consistent with the almost with histochemical signal was detected in the leaves of these plants (Figure 5C). The fact that the D3-842 promoter displayed a low level of GUS activity but that the D4-528 promoter barely displayed any evidence of GUS activity suggests that D3-842 should be regarded as a minimal sequence needed to initiate production of the *PpERS1* transcript. However, only the D1-2158 and D2-1536 promoters, which include all of the putative signal response elements identified by sequence analysis were able to direct the normal pattern of transcription of the *PpERS1* gene.

Activity of the PpERS1 promoter in transgenic tomato plants

Greater insight into the activity of the *PpERS1* promoter in different tissues and at different developmental stages should enhance our knowledge of the mechanisms that regulate expression of the *PpERS1* gene. GUS activities were determined in plants from the T0 and T1 generations of transgenic lines that carry the full-length *PpESR1* promoter and had been confirmed to be transgenic by growth on hygromycin B and PCR amplification of transgene sequences. GUS staining of T0 transgenic plants was used to analyze the tissue specificity of the *PpERS1* promoter. Our results showed that the *PpERS1* promoter was active in almost all plant organs, including roots, stems, leaves, flowers, and fruits, with the strongest activity observed in leaves and flower buds (Figure 6 A-1–A-5). GUS activity was intense in the mesophyll of CaMV35S::GUS transgenic plants, yet no GUS activity was observed in leaf veins (Figure 6 A-6), and there was no GUS expression in leaves and flower buds of transgenic plants that lack any promoter sequence upstream of the *GUS* gene (Figure 6 A-7, A-8). In fruit of the *PpERS1* promoter transgenic plants, both pericarp and sarcocarp tissues show little GUS activity,

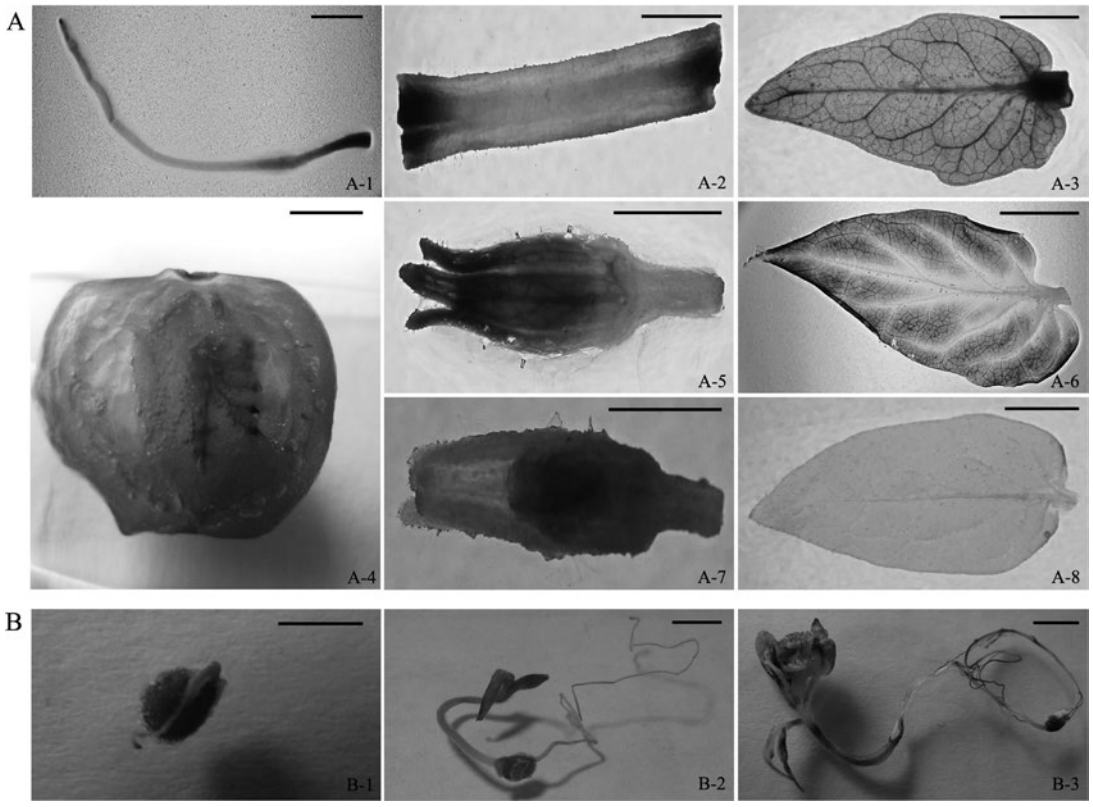

Figure 6. Tissue-specific expression and developmental regulation of the *PpERS1* promoter in transgenic tomato plants. (A) Histochemical localization of GUS activity in different organs of T0 transgenic tomato plants. **A-1** Root, **A-2** stem, **A-3** leaf, **A-4** fruit, **A-5** flower bud, **A-6** leaf of positive control (GUS gene driven by CaMV35S promoter), **A-7, 8** leaf and flower bud of negative control (no promoter upstream of *GUS* gene). (B) GUS histochemical staining at different developmental stages in T1 tomato plants expressing fusion of the full-length *PpESR1* promoter to *GUS*. **B-1** Three-day-old seeding, **B-2** Seven-day old seeding, **B-3** fifteen-day-old seeding. Scale bars = 5 mm

whereas the axile placenta and immature seed stained deep blue (Figure 6 A-4). Our findings resemble those obtained after analysis of the tissues specificity of the *Arabidopsis ERS1* promoter (Grefen et al. 2008).

Most noticeably, GUS activity was especially high at sites where roots, stems, and leaves were cut (Figure 6 A-1–A-3), whereas no such increase in GUS activity was apparent in transgenic plants that harbored the CaMV35S::GUS construct (Figure 6 A-6). This confirmed that the *PpERS1* promoter was activated by wounding in peach. A fluorometric assay based on the enzymatic conversion of MUG to 4-MU by GUS was conducted to quantify increases in activity of the *PpESR1* promoter upon wounding and other stresses. The result showed that whereas the promoter of *PpERS1* was positive regulated by wounding and ethephon, it was negatively regulated by low temperature (Figure 7). Patterns of activation or suppression of the *PpERS1* promoter in response to wounding, ethephon, and low temperature in the transgenic tomato plants resembled the patterns of accumulation of *PpERS1* transcripts in peach tissues exposed to these treatments. However, treatment with NaCl had different effects on the two systems. The discrepancy between the effects of NaCl on *PpERS1* promoter activity in tomato plants and *PpERS1*

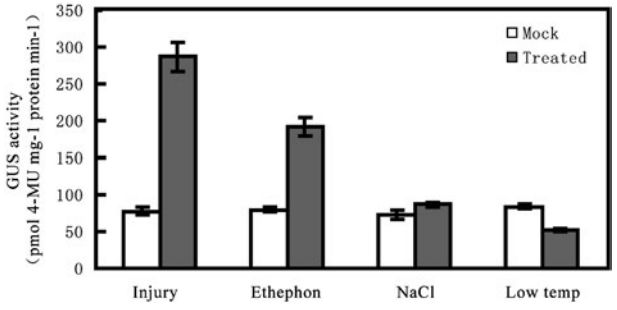

Figure 7. Effect of stresses on the expression of GUS conferred by the full-length promoter in transgenic tomato plants. GUS activity was examined 6 h after treatment with several stresses, including wounding, ethephon (150 mg/L), NaCl (200 mM) and low temperature (4°C). GUS activity was fluorometrically analyzed and expressed as pmol 4-MU mg^{-1} protein min^{-1}. Data are means ± standard deviations from three independent determinations.

transcript accumulation in peach might be attributed to GUS, which is a chimeric gene drive by *PpERS1* promoter in some transgenic plants. A similar result was obtained when the promoters of peach genes that encode ACC oxidases were characterized in transgenic tomato, which was selected as the heterologous host given that it bears fleshy fruits that resemble those of peach trees (Rasori et al. 2003). A previous study showed that activity

of the *AtERS1* promoter was developmentally regulated in Arabidopsis (Grefen et al. 2008). Here, in order to analyze the activity of the *PpERS1* promoter during peach development, we detected GUS activity of the full-length *PpERS1* promoter by histochemical staining throughout the life cycle of transgenic tomato plants. The results indicated that GUS activity was observed in the radicle tissue of seedling that germinated 3 day after imbibition (Figure 6 B-1). However, only cotyledons stained blue when the transgenic seedlings were 7 day old (Figure 6 B-2), although GUS activity was obvious in cotyledons, vascular tissues of the hypocotyls, parts of the first true leaves, taproots, and lateral roots of 15-d old seedlings (Figure 6 B-3).

Conclusion

The peach *PpERS1* gene, which encodes an ethylene receptor, shows a fairly high level of sequence conservation relative to *ESR1* sequences from other rosaceous species, and clustered with other members of the *ETR1* subfamily after classification based on their deduced amino acid sequences. The changes in patterns of *PpERS1* expression after exposure to different abiotic and biotic stresses suggest that these stresses affect the regulatory mechanisms responsible for transcriptional regulation of *PpERS1*. The length of the *PpESR1* promoter sequence upstream of the TSS affected the pattern of *PpESR1* expression. Bioinformatics analysis identified several *cis*-acting elements in the *PpERS1* promoter that are involved in hormone and stress responses. Similar expression patterns were revealed by assays of GUS activity in transgenic tomato plants in which expression of a GUS transgene was placed under the regulation of different fragments of the *PpESR1* promoter. Our study demonstrated that tomato might provide a much-needed model to study peach gene promoters, given that an efficient protocol to transform and regenerate peach plants remained to be reported.

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