

Molecular characterization of peach PR genes and their induction kinetics in response to bacterial infection and signaling molecules

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Abstract ‘Venture’ and ‘BabyGold 5’ are two peach cultivars with a demonstrated resistance and susceptibility, respectively, to bacterial spot disease caused by *Xanthomonas campestris* pv. *pruni* (*Xcp*). To explore the differences between these cultivars at the molecular level, two PR1 (*Pp-PR1a*, *Pp-PR1b*) and three PR5 (*Pp-TLP1*, *Pp-TLP2* and *Pp-TLP3*) genes were isolated from peach (*Prunus persica* L.) and investigated by in silico and in situ approaches. The analysis of gene expression by qRT-PCR indicated that all PR genes, except *Pp-PR1a*, were induced to a significantly higher degree in the resistant cultivar. In response to signaling molecules, *Pp-PR1a* was induced chiefly by SA treatment, while other PR genes were induced mainly by ethephon or MeJA treatments. The induction of the same set of PR genes in response to bacterial infection, MeJA or ethephon suggests the involvement of jasmonic acid (JA)/ethylene (ET)-signaling pathways in mediating resistance against *Xcp*, which is consistent with the potential hemibiotrophic nature of this bacterium. The identification of binding sites for ERF and MYC2 transcription factors in the promoter of *Pp-TLP1* and *Pp-TLP2* genes further supported the role of JA/ET pathways in the transcription regulation of

these genes. The role of stomata in defense against *Xcp* was also investigated by measuring stomatal apertures in both ‘Venture’ and ‘BabyGold 5’ leaves after 1 and 3 HPI. While most stomata closed in both cultivars within 1 HPI, stomata reopened again at 3 HPI with a higher percentage recorded for ‘BabyGold 5’, suggesting a potential role of stomata in the susceptibility of this cultivar.

Keywords PR genes · *Xanthomonas campestris* pv. *pruni* · Plant hormones · Stomatal aperture · Thaumatin-like proteins

Abbreviations

aa	Amino acids
DPI	Days post-inoculation
ET	Ethylene
ETI	Effector-triggered immunity
DAMPs	Damage-associated molecular patterns
GFP	Green fluorescent protein
HPI	Hours post-inoculation
JA	Jasmonic acid
HPT	Hours post-treatment
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
PR	Pathogenesis-related
SA	Salicylic acid
SAR	Systemic acquired resistance
TLPs	Thaumatin-like proteins
<i>Xcp</i>	<i>Xanthomonas campestris</i> pv. <i>pruni</i>

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Introduction

Plants possess both constitutive and inducible mechanisms to resist pathogen attack. The constitutive physical and

chemical barriers on the plant surface represent the first line of defense against the establishment of pathogen infection structures. When these barriers are overcome by pathogens, another form of plant resistance, defined as inducible resistance, is expressed to counteract the invading organisms. Inducible defense mechanisms include the reinforcement of cell walls (Hahn et al. 1989), accumulation of phytoalexins (Darvill and Albersheim 1984), induction of lytic enzymes and proteinase inhibitors, and synthesis of several low-molecular-weight anti-microbial proteins referred to as pathogenesis-related (PR) proteins (Bowles 1990). PR proteins are induced in both mono- and dicotyledonous plants upon infection by fungi, oomycetes, viruses, bacteria, and by nematode or insect attack (Buchel and Linthorst 1999; Edreva 2004). PR proteins are also induced in response to defense signaling elicitors (e.g., salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Edreva 2005; van Loon et al. 2006). Based on their structure and serological relationships as well as their biological activities, PR proteins are classified into 17 families. These families include proteinase inhibitors (PR6), chitinases (PR3, 4), peroxidases (PR9), lipid-transfer proteins (PR14), endoproteases (PR7) and defensins (PR12) (van Loon et al. 2006).

PR1 proteins have frequently been used as markers for systemic-acquired resistance (SAR), but their biological activities have remained elusive (Mitsuhara et al. 2008). Twenty-four, 22 and 39 PR1 genes have been identified in tobacco, *Arabidopsis* and rice, respectively (van Loon et al. 2006). At the time of pathogen attack, PR1 genes were transcriptionally regulated by either SA, or ET/JA-signaling pathways that showed antagonistic relationships (Niki et al. 1998; Gupta et al. 2000). Tobacco *NtPRB1* and *Arabidopsis AtPRB1*, which encode basic proteins, were upregulated by JA and ET and downregulated by SA (Eyal et al. 1993; Niki et al. 1998; Santamaria et al. 2001), while tobacco *NtPR1a*, *b* and *c*, which encode for acidic proteins, were induced by SA (Ohashi and Matsuoka 1987). The induction kinetics of PR1 genes in response to pathogens varies among plant species. Among 22 PR1 genes identified in *Arabidopsis*, only one (PR1a) gene, which encodes for a basic protein, is known to be a pathogen-inducible gene (van Loon et al. 2006). By contrast, the 12 PR1 genes studied in rice were all upregulated in response to blast fungus infection, which predicts different evolution events for PR1 genes in *Arabidopsis* and rice genomes, presumably to provide resistance to a broad range of pathogens in the latter (Mitsuhara et al. 2008). In apple, three PR1 genes (*Md-PR1a*, *b* and *c*) showed no induction following inoculation of apple shoots with *Erwinia amylovora*, while other PR genes (e.g., *Md-PR5*) showed higher transcript levels upon inoculation (Bonasera et al. 2006).

PR5 proteins are another group of PR proteins with demonstrated antimicrobial activities (Hejgaard et al. 1991; Xu and Reddy 1997; Kuwabara et al. 2002). They are also referred to as thaumatin-like proteins (TLPs) because of their sequence and structural similarities to the sweet-tasting protein from *Thaumatococcus daniellii* Benth, thaumatin (Edens et al. 1982). The structure of most TLPs is characterized by the N-terminal signal peptide and the 16 cysteine residues which are conserved among most TLPs (Velazhahan et al. 1999). The overexpression of TLPs in transgenic plants conferred resistance mostly to fungi and oomycetes (Zhu et al. 1996; Datta et al. 1999; Velazhahan and Muthukrishnan 2003; El-Kereamy et al. 2011). Apparently TLPs exert their antifungal effects by permeabilizing fungal membranes (Batalia et al. 1996), binding and hydrolyzing β -1,3-glucans (Grenier et al. 1999; Menu-Bouaouiche et al. 2003), or by inhibiting fungal xylanases (Fierens et al. 2007). However, the role of TLPs in response to bacterial pathogens has been little investigated.

Bacterial spot, caused by *Xanthomonas campestris* pv. *pruni* (*Xcp*), is a severe disease of stone fruits, including peach. Symptoms on leaves include necrotic spots and shot holes that may coalesce, leading to tree defoliation. The bacteria invade peach leaves through the stomata (Miles et al. 1977; Aarrouf et al. 2008), which historically have been considered as passive barriers for the entry of pathogenic bacteria. Recent studies suggest an active role for stomata in restricting bacterial invasion (Underwood et al. 2007). While this role was confirmed during the interaction between *Pseudomonas syringae* and its host plants (Melotto et al. 2006), little is known about the regulation of stomatal aperture in response to other plant pathogenic bacteria (e.g., *Xcp*). In the present study, two peach cultivars, 'Venture' and 'BabyGold 5', which are known for their field resistance and susceptibility, respectively, to bacterial spot disease (Slingerland and Subramanian 2007) were used to elucidate the role of PR proteins during compatible and incompatible interactions of peach with *Xcp*. The signaling pathways, by which these proteins are regulated and the responses of stomata in resistant and susceptible peach cultivars following inoculation with *Xcp* were also studied.

Materials and methods

Plant materials

Twelve-year-old peach trees, 'Venture' and 'BabyGold 5', grown at the Vineland Research and Innovation Center farm, ON, Canada were used for this study. These two cultivars were chosen for their field resistance and susceptibility to bacterial spot disease, respectively

(Slingerland and Subramanian 2007). Trees of both cultivars were planted in two adjacent rows and maintained under similar growth conditions.

Bacterial inoculations

The *Xcp* was kindly provided by Dr. J. Tambong, (AAFC-Ottawa, ON, Canada). The bacteria were grown for 48 h at 28°C on plates of Luria–Bertani (LB) (Sigma) medium. A single colony was cultured at 28°C in LB broth supplemented with rifampicin (50 µg/µl) (Sigma, Oakville, ON, USA) until an OD₆₀₀ of 1:00 was reached. The bacteria were collected by centrifugation at 4,100 rpm for 10 min and suspended in 10 mM MgSO₄ solution containing 0.05% (v/v) Silwet (RoseCare.com, CA, USA). The density of the suspension was adjusted to an OD₆₀₀ = 0.2, which corresponded to a calculated number of 10⁸ cells/ml. For inoculation, three to five twigs, each containing the average of 6–8 leaves/twig, were collected randomly from each of ‘Venture’ and ‘BabyGold 5’ trees. The twigs were rinsed completely in 2% (v/v) sodium hypochlorite for 10 min, washed twice thoroughly with distilled water, and then kept under continuous light exposure on a lab bench for 3 h before inoculation. Inoculations were performed by dipping the twigs into a freshly prepared bacterial solution for 2 min. Inoculated twigs were transferred to a plastic bag partially sealed to maintain proper humidity level. Three leaves were randomly collected from independent twigs at 0, 1, 3 and 5 days post-inoculation (DPI) for analyzing bacterial growth (CFU/cm² leaf area). Mock inoculation (control) was carried out in a similar way but a 10 mM MgSO₄ solution was used instead of bacterial suspensions. The experiment was repeated three independent times, with similar number of replicate leaves each time. Leaf samples were collected randomly from each bag at 1, 4, 8, 24 and 48 h(hours) post-inoculation (HPI), quickly frozen in liquid nitrogen, and then stored at –80°C for RNA extraction. Mock-inoculated leaves were collected at 1 HPI for RNA analysis.

Treatment with signaling compounds

Twigs collected from separate ‘Venture’ trees were *disinfected* with *sodium hypochlorite* solution and washed with water as described above. Ten to fifteen fully expanded leaves were randomly collected from twigs and dipped for 1 min in signaling compound solutions, 50 µM MeJA (Sigma), 1 mM SA (Sigma), or 1 mM ethephon (Phyto-Technology, Shawnee Mission, KS, USA) supplemented with 0.1% (v/v) Triton X-100. For wounding treatment, leaves were punctured several times by a needle and then crushed with a hemostat. The experiment was repeated three independent times. Each time, a pool of leaf samples

were collected randomly after 1, 4, 8 and 24 h of treatment, frozen in liquid nitrogen and stored at –80°C for RNA extraction and further analysis of gene expression.

Isolation and in silico analysis of peach PR genes

Based on available sequence information in Genbank, degenerate primers were designed to isolate PR1 and TLP orthologs from *P. persica* (Online Resource 1). Total RNA was extracted from 100 mg of ‘Venture’ leaves inoculated with *Xcp* (1 HPI) using RNeasy Kit (Qiagen, Mississauga, ON, Canada). RNA samples were treated with DNase I (Invitrogen, Burlington, ON, Canada) prior to the synthesis of cDNA to remove any traces of genomic DNA. The first-strand cDNA was synthesized from 5 µg of total RNA using the RevertAidTM Premium First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). PCR reactions were carried out using 0.5 µl Platinum *Taq* DNA Polymerase (Invitrogen), 1× reaction buffer, 1.5 mM MgCl₂, 0.2 µM forward and reverse primers, and 1 µl of cDNA in a total of 50 µl. The amplified fragments were cloned into a pGEM-T Easy vector (Promega, Nepean, ON, Canada), sequenced, and compared with database sequences using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Extension of partial cDNA segments was carried out using the 3'- and 5'-RACE kit (Invitrogen) according to the manufacturer's instructions. Multiple comparisons of *P. persica* PR (Pp-PR) genes with orthologs from different plant species were conducted using ClustalX (Jeanmougin et al. 1998) and GeneDoc (Nicholas and Nicholas 1997). The phylogenetic tree was constructed according to the neighbor-joining method and visualized by Phyloheny.fr web tool (<http://www.phylogeny.fr/>). Names and accession numbers used for multiple-sequence alignments and phylogenetic analyses are listed in Online Resource 2. The prediction of a signal peptide was performed using SIGNALP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Deduced molecular weights and the theoretical isoelectric points of proteins were computed using the ProtParam tool (<http://www.expasy.org/tools/protparam.html>). The subcellular localization was predicted using PSORT (<http://psort.hgc.jp/form.html>).

Isolation of promoter regions from Pp-TLPs

Genomic DNA was extracted from ‘BabyGold 5’ and ‘Venture’ mature leaves using DNeasy Plant Maxi Kit (Qiagen). The 5'-upstream region of Pp-TLP1 and Pp-TLP2 genes was isolated using the Universal Genome Walker Kit (Clontech, Palo Arto, CA, USA). Nested PCR reactions were carried out using reverse primers from the coding region (Online Resources 3 and 4) and adaptor primers from the manufacturer. PCR-amplified fragments

were cloned and sequenced as explained above. Promoter sequences were analyzed using the Plant Promoter Analysis Navigator (PlantPAN), (<http://plantpan.mbc.nctu.edu.tw/index.php>).

RNA extraction and gene expression

Total RNA extraction and cDNA synthesis of each sample were done as described above. Quantitative real time-PCR (qRT-PCR) was performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada). The PCR reaction was performed in 25 µl of the reaction mixture containing 1× iQTM SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and about 10 ng of cDNA. The fold change in gene expression was calculated according to the comparative C_T method using *Pp-actin* (BU046508) as an internal control. The primers used to detect transcript levels of *Pp-PR* genes are listed in Online Resource 1.

Subcellular localization of TLP-GFP chimeric proteins

The coding sequences of Pp-TLP1 and Pp-TLP2 were PCR amplified using ‘Venture’ cDNA as a template and the primers listed in Online Resource 1. The amplified fragments were digested with *Bam*H1 restriction enzyme and cloned into a pGreen vector (Hellens et al. 2000) as C-terminal fusion with GFP protein and upstream of the CaMV dual 35S promoter. The chimeric constructs were verified by further sequencing and analysis. The subcellular localization of TLP-GFP chimeric proteins was studied in onion epidermal cells using PDS-1000/He Biolistic Particle Delivery System (BioRad), and following a method described by Weigel and Glazebrook (2002). The transfected onion tissues were observed by a Zeiss Axioplan fluorescence microscope equipped with Fluorescein isothiocyanate (FITC) and DAPI filters. The images were captured by Zeiss AxioCam MRc 5 digital camera. To confirm the position of the nucleus, onion strips were dipped in a 300-nM DAPI nucleic acid stain (Invitrogen) in phosphate-buffered saline (PBS) for 3 min and then washed with PBS thrice before microscopic observations.

Assessment of stomatal responses to *Xcp*

Three to five fully expanded young leaves from each cultivar were dipped in the bacterial suspension (prepared as described previously) for 2 min and then removed and placed on a glass surface with their adaxial surfaces facing up. After 1 and 3 HPI, square sections of leaves were mounted on microscope glass slides with their adaxial surface facing down and observed with an Olympus BX51 microscope equipped with an Olympus DP71digital

camera. Leaves dipped in MgSO₄ (10 mM) were used as controls and observed at the start (time 0). At various time points, pictures were taken from random leaf regions and both the width and height of the stomatal apertures were measured using the software Image-Pro version 6.2 for Windows (Cybernetics, Inc.). To exclude the effect of stomatal sizes on their width, the stomata were considered open when the ratio of aperture width/height was equal to or more than 0.25. The results of each experiment were an average of three replicates, each containing more than 18 measures of aperture width/height. Confocal Images for both cultivars were taken 3 HPI using a Leica DM IRBE microscope equipped with a laser confocal scanner TCS SP2 (Leica Microsystems Inc., Richmond Hill, ON, Canada).

Statistical analyses

Analysis of variance (ANOVA) was performed using the GLM or MIXED procedures of SAS statistical software (release 9; SAS Institute, Cary, NC, USA). The Tukey–Kramer HSD ($P < 0.05$) test was used to compare all means.

Results

Variation in bacterial spot resistance among peach cultivars

In vitro dip-inoculation of leaves with *Xcp* (10⁸ CFU/ml) resulted in wide necrotic areas in ‘BabyGold 5’ leaves at 3–4 DPI; these areas were scattered on the lower leaf surface and were similar to those seen in field conditions when the disease occurs (Fig. 1a, b). Only tiny purple spots could be seen on ‘Venture’ leaves around the midribs (Fig. 1b). The viable bacterial population (CFU/cm²) on the each leaves was counted after mock (10 mM MgSO₄ only) or after 1, 3 and 5 DPI (Fig. 1c). The results showed significant differences ($P = 0.0013$) between cultivars, with ‘BabyGold 5’ exhibiting more bacterial growth at all recorded times. The maximum number of viable bacteria (>10⁷ CFU/cm²) was recorded at 3 DPI in ‘BabyGold 5’, while only 10⁵ CFU/cm² was recorded for ‘Venture’ at 5 DPI.

Stomatal responses following inoculation with *Xcp*

Studies on the role of stomata during bacterial invasion often use the diameter of the stomatal aperture (µm) as a measure for stomatal opening or closure (Guimaraes and Stotz 2004; Melotto et al. 2006; Liu et al. 2009). In the present study we used the stomatal aperture width/height

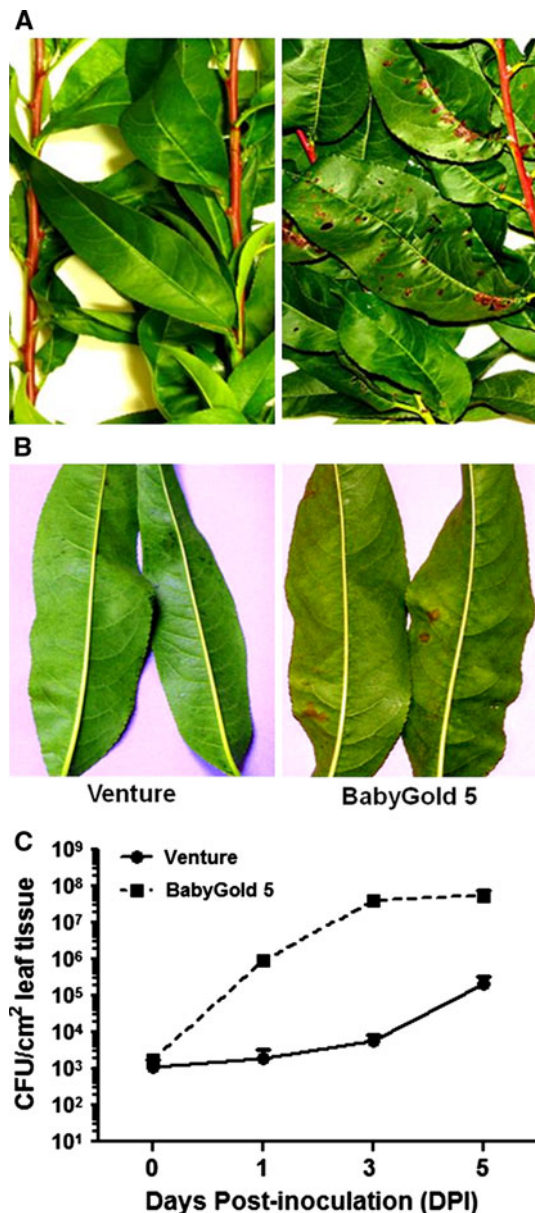


Fig. 1 Evaluation of ‘Venture’ and ‘BabyGold 5’ peach cultivars for resistance to *Xcp*. **a** Branches were collected from two adjacent trees in the field showing the susceptibility of ‘BabyGold 5’ and the resistance of ‘Venture’ to bacterial spot disease. **b** Leaves of ‘Venture’ and ‘BabyGold 5’ were collected after 7 days of dip-inoculation with *Xcp* (10^8 CFU/ml). **c** Values of CFU/cm² obtained after 0, 1, 3 and 5 DPI with *Xcp*. Values on the Y-axis represent means $\pm$ SE of three replicates

ratio in order to account for differences in stomatal sizes. With a ratio of ≥ 0.25 , the stomata were considered opened. Stomatal apertures were measured in ‘Venture’ and ‘BabyGold 5’ after mock inoculation (MgSO_4 alone) or following dip-inoculation of leaves with *Xcp* (10^8 CFU/ml) for 1 and 3 HPI. Most stomata ($\sim 90\%$) opened following mock-inoculation, while nearly 35% were opened at 1 HPI, without significant differences between cultivars (Fig. 2b).

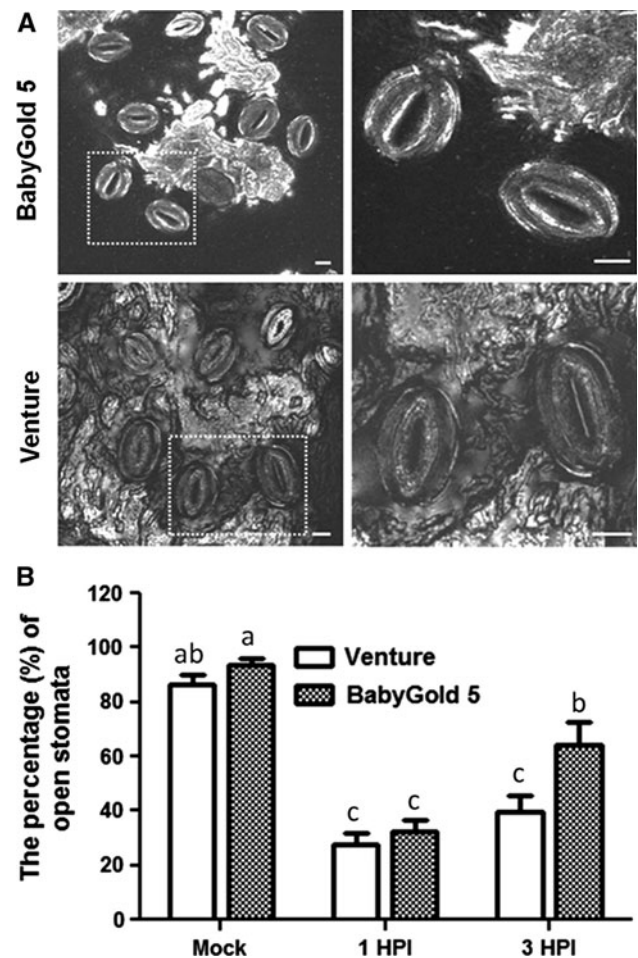


Fig. 2 Stomatal apertures in peach leaves after inoculation with *Xcp*. **a** Confocal images showing the width of stomatal apertures in ‘Venture’ and ‘BabyGold 5’ leaves at 3 HPI with *Xcp* (10^8 CFU/ml). **b** Stomatal responses were measured as the percentage of opened stomata ($W/H \geq 0.25$) in peach cultivars after mock (10 mM MgSO_4 alone) or after 1 and 3 h of dip-inoculation in a bacterial solution (10^8 CFU/ml) for 2 min. Values represent the mean $\pm$ SE of three replicates. Scale bar 20 μm . Columns connected with the same letter are not significantly different

At 3 HPI, the stomata resumed opening in both cultivars with a significantly higher percentage of opened stomata observed in ‘BabyGold 5’ (60%) than in ‘Venture’ ($\sim 40\%$). Microscopic images of leaves at 3 HPI further confirmed this observation (Fig. 2a).

Characterization of peach PR1 and TLP proteins

Full-length sequences of two PR1 (*Pp-PR1a* and *Pp-PR1b*) and three TLP (*Pp-TLP1*, *Pp-TLP2* and *Pp-TLP3*) genes were cloned, sequenced and deposited in the Genbank (accession numbers, JF694923, JF694924, JF694925, JF694926 and JF694927, respectively). These genes encode proteins with similar characteristics to their respective type members in tobacco (Table 1) as described

by van Loon et al. (1994). For the PR1 group, the proteins exhibited different lengths (161 and 190 aa), molecular masses (17.21 and 21.02 kDa), and isoelectric points (8.70 and 5.41) between Pp-PR1a and Pp-PR1b, respectively. Both proteins have an N-terminal signal peptide 24 and 23 aa in length, respectively (Fig. 3a). Based on the amino acid composition of proteins, Pp-PR1b is considered to be an acidic PR1, while Pp-PR1a is a basic PR1. The phylogenetic analysis of peach PR1 proteins with other orthologs showed that Pp-PR1a is located in the same clad of MdPR1a from apple and AtPRb1 and AtPR1a from *Arabidopsis* where it shares sequence similarities of 81, 75 and 72%, respectively, with these proteins. Pp-PR1b is quite divergent from Pp-PR1a, and is located in a separate branch (Fig. 3b). Peach PR1 proteins share six conserved cysteine residues with their respective orthologs, which is a characteristic of this group of proteins (Fig. 3a).

The three TLP genes identified in peach encode for proteins similar to the PR-5-type member in tobacco (Nt-PR5, CAA27548) (Table 1). Peach TLPs (Pp-TLP1, Pp-TLP2 and Pp-TLP3) have different molecular masses (25.70, 26.04 and 34.23 kDa), lengths (249, 248 and 330 aa), and isoelectric points (4.46, 4.83 and 4.73), respectively, but all of them are acidic proteins and share 16

conserved cysteine residues with other TLP orthologs (Table 1; Fig. 4a). Pp-TLPs have an N-terminal signal peptide with varied lengths (27, 26 and 24 aa for Pp-TLP1, Pp-TLP2 and Pp-TLP3, respectively), as predicted by the SIGNAL P 3.0 program. The three Pp-TLPs and nine TLP sequences available in the GenBank were used to establish a neighbor-joining phylogenetic tree (Fig. 4b). The results indicated that Pp-TLPs were located within different clusters. Pp-TLP1 shares high sequence similarity (85%) with cotton Gb-TLP1, while Pp-TLP2 was clustered together with the peach paralog PpAZ44 (96% sequence similarity), which has been studied before in response to abscission, organ senescence and wounding (Ruperti et al. 2002). Other sequences within the same cluster include apple Md-PR5 and pear Ppy-TLP with sequence similarities of 91 and 92%, respectively. Pp-TLP3 is probably a homolog of Pru p 2.04 (99% similarity), the putative allergen from (*P. dulcis* × *P. persica*) (Chen et al. 2008). Pp-TLP3 is located within the same cluster of *Brassica rapa* (Br-TLP). Based on the PSORT online tool, Pp-TLP1 and Pp-TLP2 were predicted (with certainty <70%) to be secreted into the extracellular matrix, while Pp-TLP3 was predicted (with certainty >90%) to be anchored in the plasma membrane.

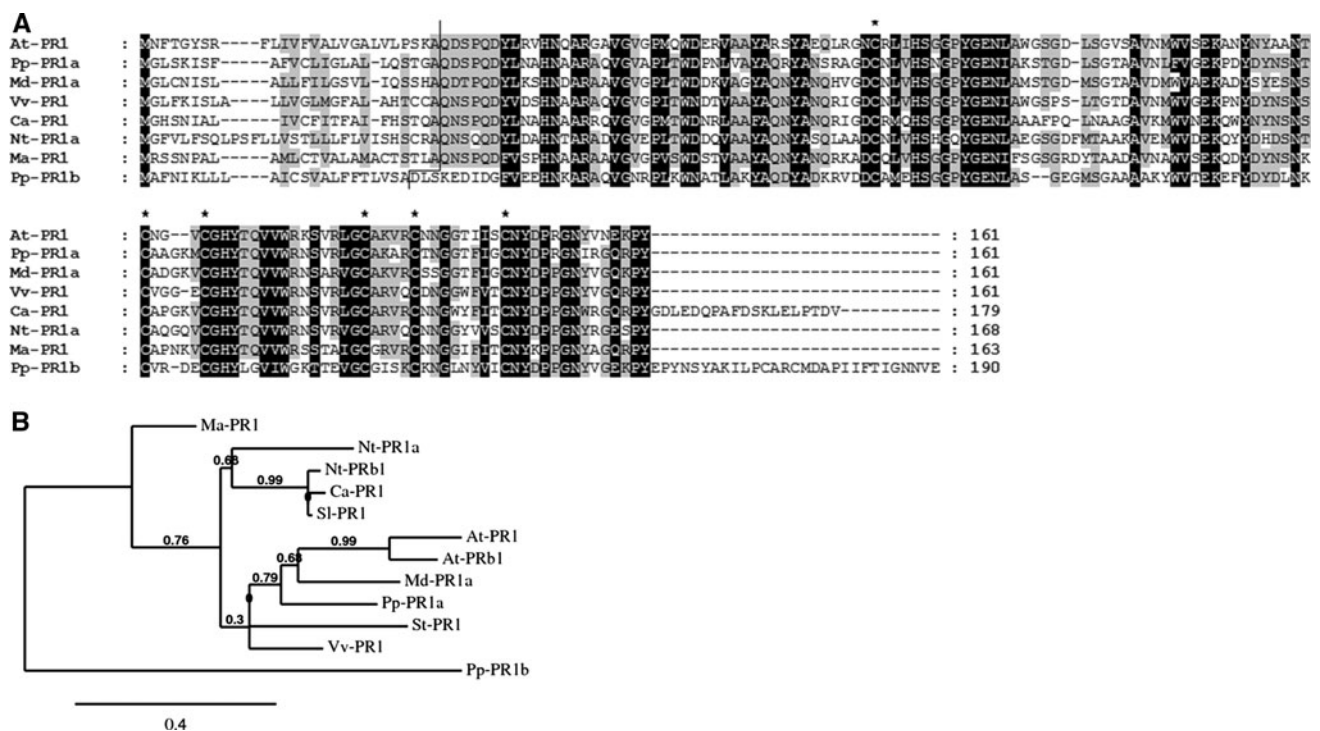


Fig. 3 Alignment and phylogenetic tree of PR1 sequences from various plant species. **a** Alignment of the deduced amino acid sequences of two peach PR1 genes (Pp-PR1a and Pp-PR1b) and their respective orthologs from apple, banana, *Arabidopsis*, pepper, grape and tobacco. The shading indicates sequence similarities of 100%

(black), or 80% (gray) among PR1 proteins. The stars indicate the six conserved cysteine residues among PR1 proteins. The vertical line shows the predicted signal peptide cleavage site. **b** Neighbor-joining phylogenetic tree of PR1 sequences from peach as well as other plant species

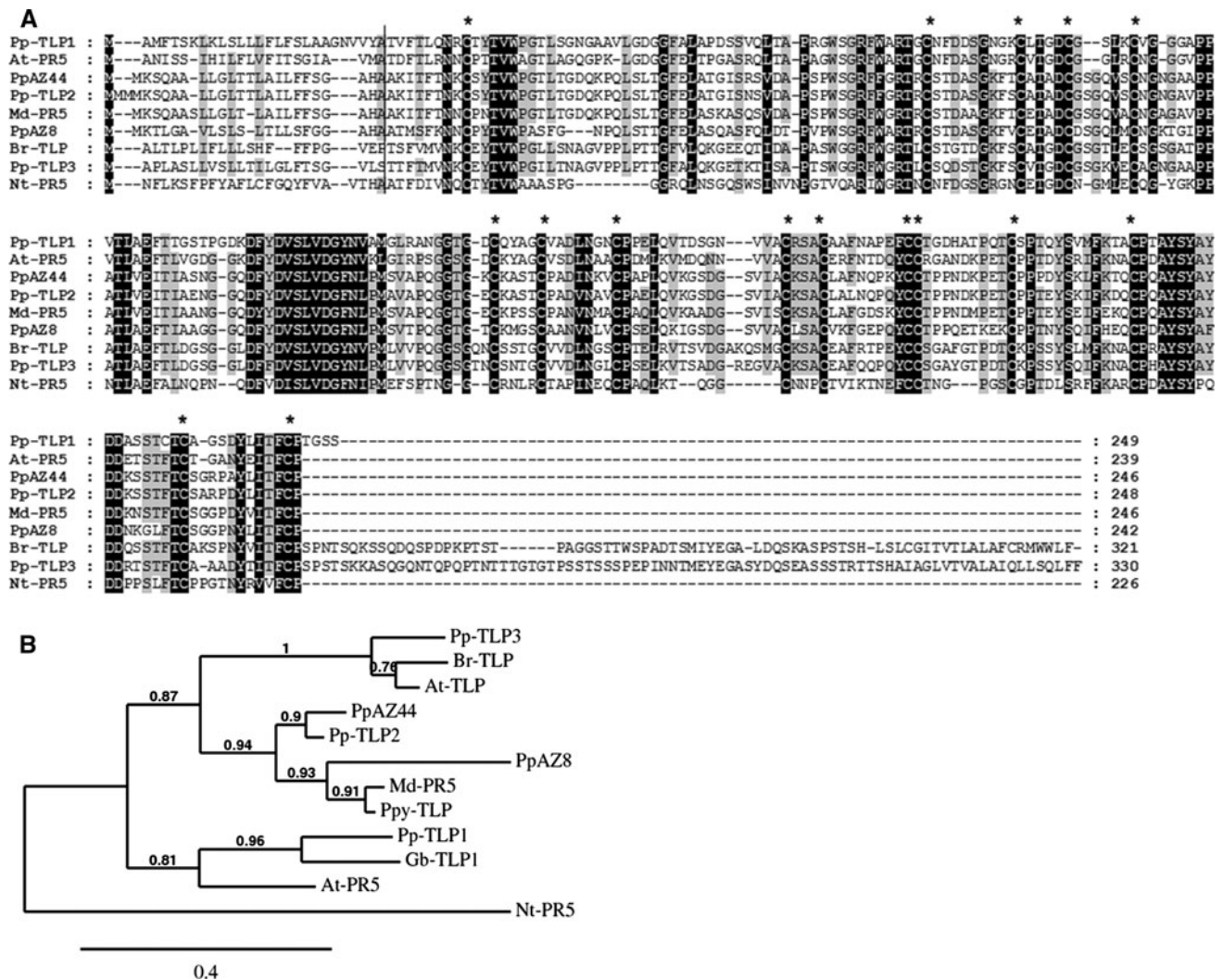


Fig. 4 Alignment and phylogenetic tree of TLP sequences from various plant species. **a** Alignment of the deduced amino acid sequences of three peach TLP genes (Pp-TLP1, Pp-TLP2 and Pp-TLP3) and their respective homologs from peach, apple, *Arabidopsis*, canola and tobacco. The shading indicates sequence similarities of

100% (black), or 80% (gray) among TLP proteins. The stars indicate the 16 conserved cysteine residues among the TLP proteins. The vertical line shows the predicted signal peptide cleavage site. **b** Neighbor-joining phylogenetic tree of TLP sequences from peach as well as other plant species

The subcellular localization of Pp-TLP1 and Pp-TLP2

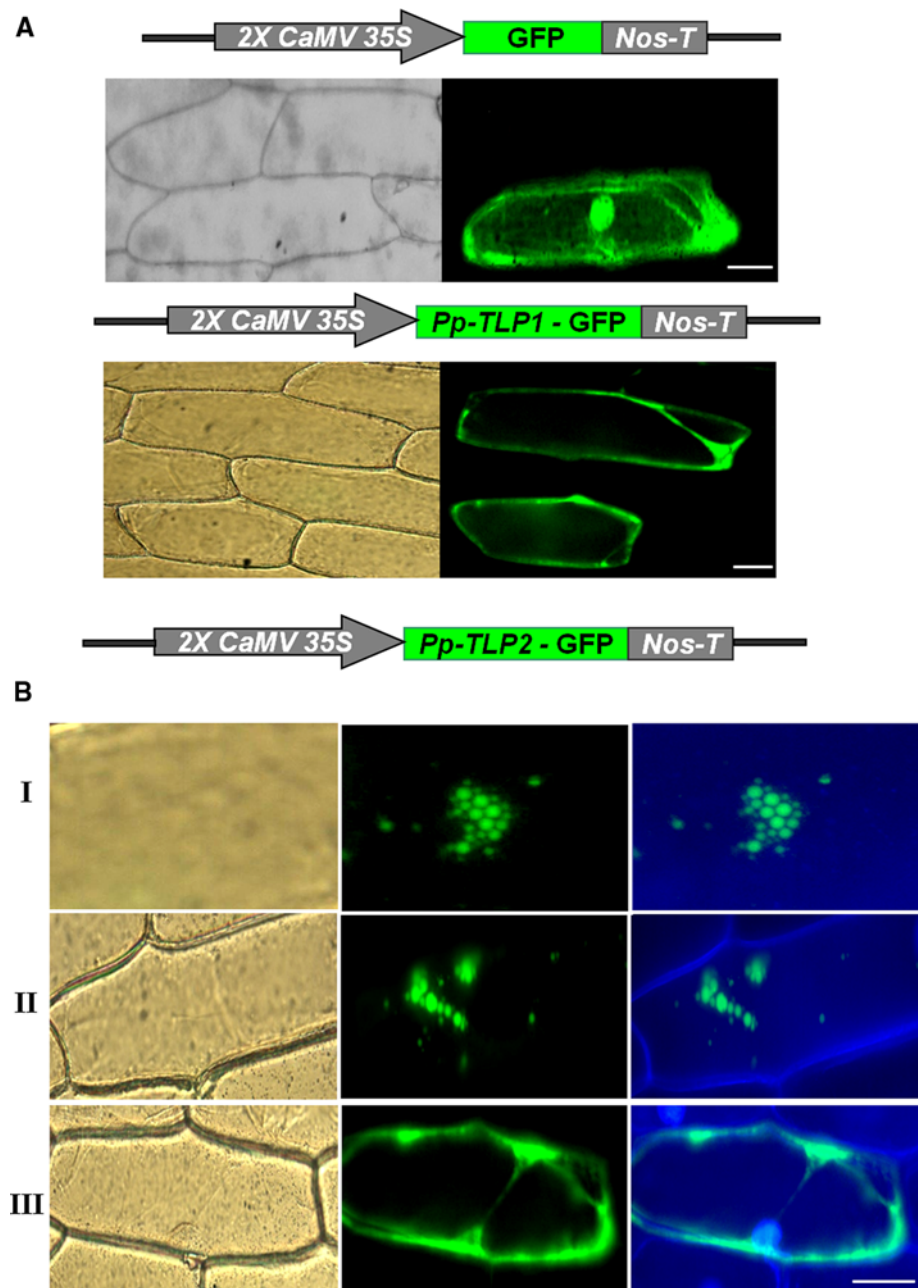
To confirm the results of in silico analysis for the subcellular localization of Pp-TLPs, the ORF of Pp-TLP1 and Pp-TLP2 was fused in-frame with the C-terminus of GFP protein and transiently expressed in onion-epidermal cells under the control of the CaMV 35S promoter. In contrast to the control (GFP alone), which was uniformly distributed throughout the onion cell, the chimeric Pp-TLP1-GFP protein was localized in the apoplast, comprising the intercellular space and the cell wall (Fig. 5a). The existence of the N-terminal signal peptide suggests the secretion of Pp-TLPs through the endomembrane system. Consistent with that, the observation of Pp-TLP2-GFP at different stages showed the translocation of chimeric

proteins in Golgi-like vesicles. These vesicles were found either scattered in the cytoplasm or arranged together toward the intercellular spaces (Fig. 5b).

Accumulation of peach PR transcripts in response to Xcp

Transcript profiles of peach PR1 and TLP genes were investigated by qRT-PCR after inoculation with *Xcp*. Generally, these genes can be classified into three classes based on their induction kinetics after inoculation with *Xcp* (Fig. 6). The first class includes *Pp-PR1a* whose expression was downregulated in the resistant ‘Venture’ and the susceptible ‘BabyGold 5’ cultivar after bacterial infection. The second class of genes includes *Pp-PR1b*, *Pp-TLP1* and

Fig. 5 Subcellular localization of peach TLP proteins. Onion epidermal cells were bombarded with plasmids expressing GFP alone (control), Pp-TLP1-GFP and Pp-TLP2-GFP chimeric proteins. **a** GFP protein is uniformly spread over all the cell (*upper panel*), while Pp-TLP1-chimeric protein localizes between the cell wall and plasma membrane (*lower panel*). **b** Different stages during Pp-TLP2 localization. The protein is packed in Golgi-like vesicles aggregating in cytoplasm (*I*), moving toward cell wall (*II*) and eventually localized to the apoplast (*III*). Images were taken under white light (*left column*) or FITC filter (*middle column*). The *right column* shows the merged images from DAPI and FITC filters. Scale bar 50 μ m



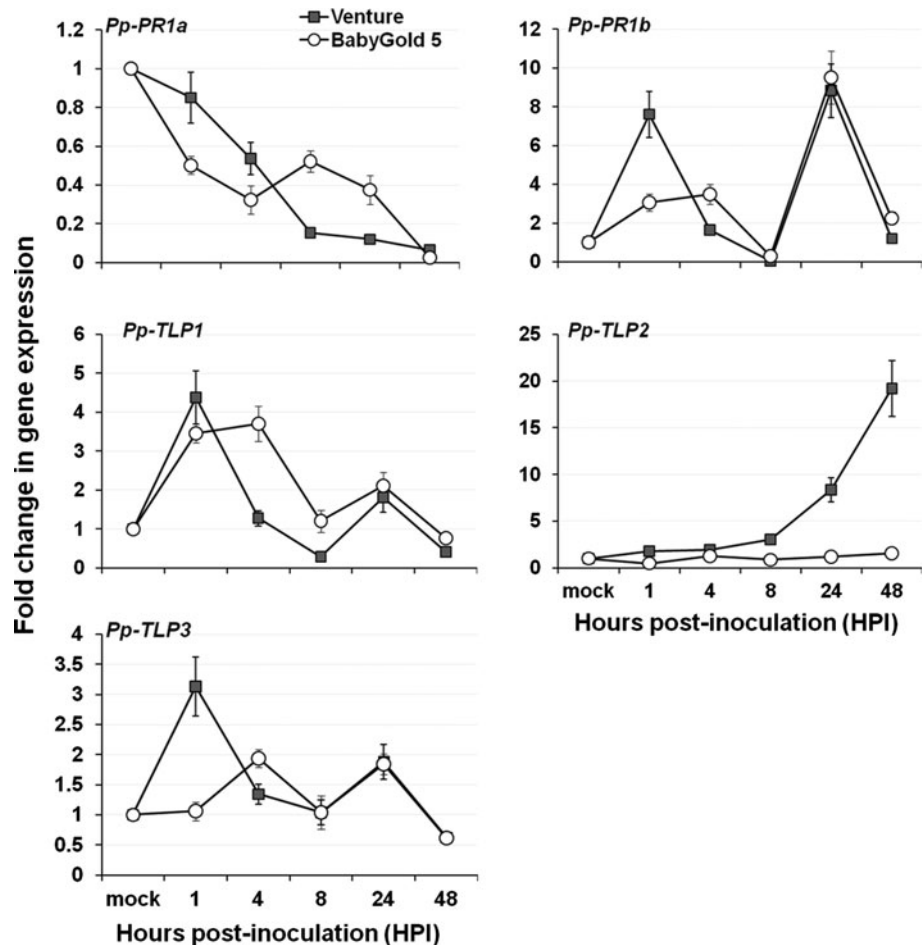
Pp-TLP3. The expression of these genes was induced in both cultivars following *Xcp* infection; however, there were differences in kinetics and magnitude of gene induction between the resistant and the susceptible cultivar. At 1 HPI, the expression of these genes was induced to higher levels in ‘Venture’ than in ‘BabyGold 5’, with a maximum induction of $\sim$ sevenfold over mock treatment for *Pp-PR1b*. At 4 HPI, transcripts of these genes were accumulated to higher levels in ‘BabyGold 5’ than in ‘Venture’, with a maximum transcript abundance of $\sim$ threefold over mock treatment for *Pp-TLP1*. The expression of these genes decreased in both cultivars at 8 HPI, peaked again at 24 HPI and then diminished at 48 HPI. The third class

includes *Pp-TLP2* whose expression increased to high levels in the resistant cultivar only after *Xcp* infection. *Pp-TLP2* transcripts accumulated gradually in the resistant cultivar and reached a peak ($\sim$ 19-fold over mock treatment) at 48 HPI, whereas in the susceptible cultivar, *Pp-TLP2* transcript levels were similar to those observed in mock treatment (Fig. 6).

Expression PR genes after treatments with signaling compounds

In the PR1 group, *Pp-PR1a* was upregulated by all signaling molecules within 1 h post-treatment (HPT), with SA

Fig. 6 Gene expression analyses of peach PR1 and TLP genes in peach cultivars, ‘Venture’ and ‘BabyGold 5’, after 1, 4, 8, 24 and 48 HPI with *Xcp*. The fold change in gene expression is shown as the ratio of the relative gene expression of each sample to that in mock-inoculated leaves of the same cultivar, and values are the mean and standard error of three biological replicates



exerting more induction (>fourfold over untreated controls) than ethephon (~threefold) or MeJA (~twofold). *Pp-PR1a* transcripts declined at 4 HPT, then accumulated again in response to SA only at 8 HPT. At 24 HPT, the gene expression reached its lowest levels after SA and ethephon treatments. For *Pp-PR1b*, transcripts accumulated to higher levels after ethephon or MeJA than after SA treatment. The increase of *Pp-PR1b* after ethephon treatment occurred as early as 1 HPT and peaked at 8 HPT with approximately 12-fold over untreated controls whereas, after MeJA treatment, transcripts accumulated slowly and reached their maximum levels (>55-fold) at 24 HPT (Fig. 7).

Among the TLPs, *Pp-TLP1* was induced at high levels by ethephon and MeJA, *Pp-TLP2* was upregulated mainly by MeJA; and *Pp-TLP3* was induced by all signaling compounds. Like *Pp-PR1b*, the gene expression of *Pp-TLP1* was induced rapidly by ethephon and reached its maximum levels (~150-fold over control) at 8 HPT, then declined steeply at 24 HPT. On the other hand, the induction of *Pp-TLP1* by MeJA treatment occurred late (at 4 HPT), but continued to increase gradually to ~130-fold over control at 24 HPT. Similarly, *Pp-TLP2*

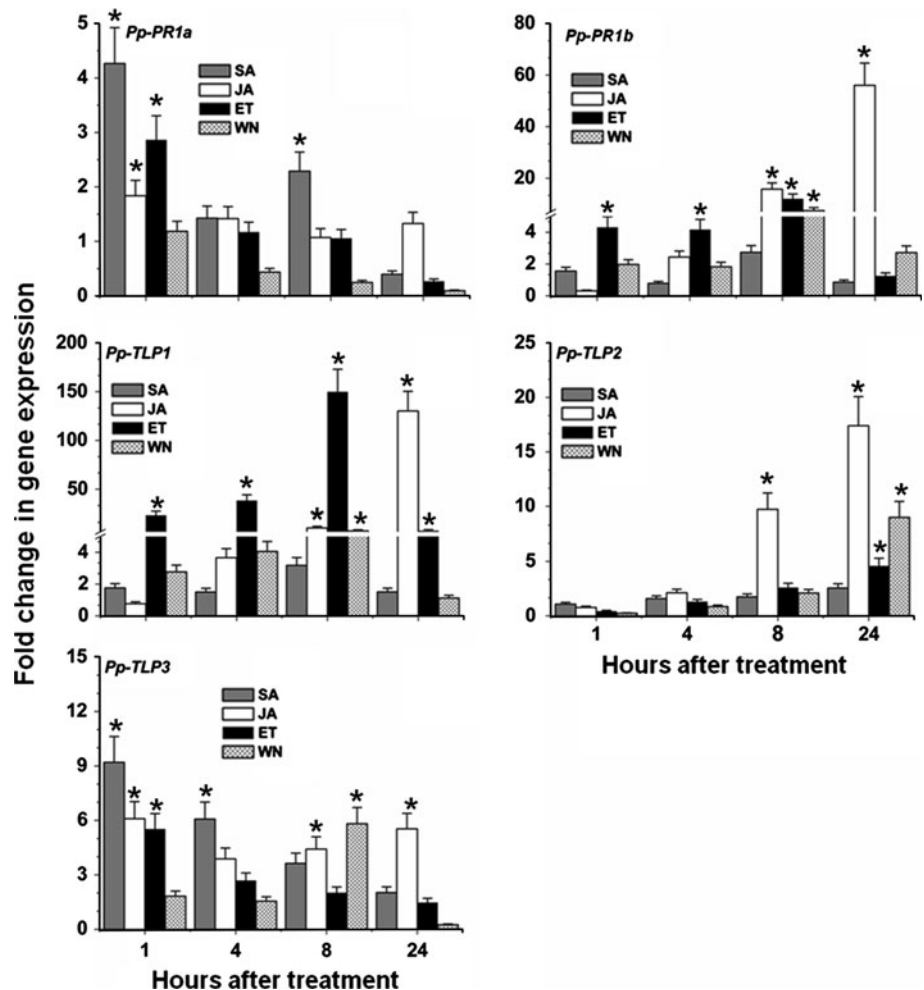
transcripts were accumulated in response to MeJA treatment and reached their maximum levels (>15-fold) at 24 HPI. The expression of *Pp-TLP3* was induced rapidly (1 HPT) in response to all signaling molecules but SA showed the highest induction (~ninefold over control) (Fig. 7).

Since it has previously been reported that PR genes are induced after herbivore attack and wounding (Edreva 2005), the gene expression of peach PR genes was monitored after different times of mechanical wounding. Although different expression patterns were observed, it can be concluded that, except for *Pp-PR1a*, the maximum induction of PR transcripts by wounding occurred at 8 or 24 HPT. Among the PR1 group, *Pp-PR1b* was induced by wounding with a maximum induction (>sevenfold) observed at 8 HPI. Among TPLs, *Pp-TLP2* had the highest induction (>ninefold) at 24 HPT, followed by *Pp-TLP1* (~eightfold) at 8 HPT and *Pp-TLP2* (~fivefold) at 8 HPT (Fig. 7).

The identification of *cis*-acting elements in TLP promoters

Promoter sequences with sizes of 733 and 644 bp were retrieved for *Pp-TLP1* and *Pp-TLP2* genes, respectively

Fig. 7 Gene expression analyses of PR1 and TLP genes in peach leaves after 1, 4, 8 and 24 h of treatments with MeJA (50 μ M), SA (1 mM), ethephon (1 mM) or wounding (WN). The fold change in gene expression is shown as the ratio of the relative gene expression of each sample to that in untreated leaves, and values are the mean and standard error of three biological replicates. Treatments marked by an asterisk are significantly greater than untreated controls ($P < 0.05$)

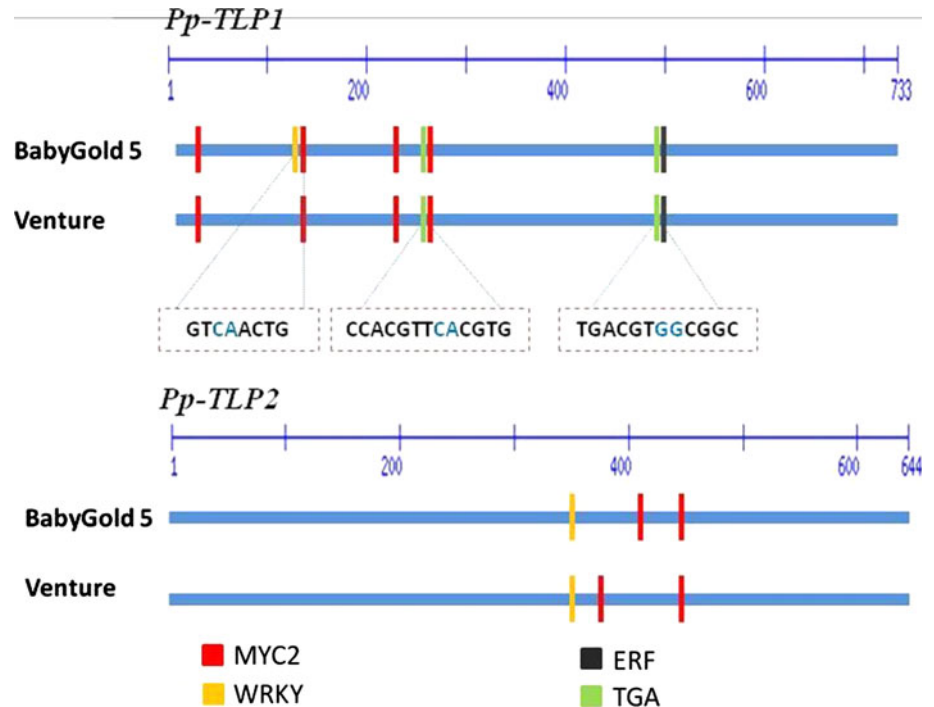


(Online Resources 3 and 4). The analysis of sequences from ‘Venture’ as well as ‘BabyGold 5’ revealed the existence of similar *cis*-acting elements in both cultivars (Fig. 8). Regardless of many putative elements identified in the PlantPAN database (Chen et al. 2008), only those with a previously demonstrated role in disease resistance or hormone signaling were considered. The binding sites for transcription factors MYC2 (JA signaling), ERF (JA/ET signalling), WRKY (SA signalling) and TGA (SA signalling) were recognized in both plus and minus DNA-strands. MYC2-binding sites were identified in four positions in *Pp-TLP1* and two positions in *Pp-TLP2*. Different locations for MYC2-binding sites in ‘Venture’ and ‘BabyGold 5’ were detected. The GCC-box, the binding site for ERFs, was found only in *Pp-TLP1* (one position). TGA binding sites were recognized only in *Pp-TLP1* (two positions), while WRKY-binding sites were found in both *Pp-TLP1* (‘BabyGold 5’ only), and *Pp-TLP2* (Fig. 8). There was an overlap between TGA and WRKY binding sites and those of MYC2 and the GCC-box (Fig. 8).

Discussion

Although entry of *Xcp* through peach leaf stomata has already been demonstrated (Miles et al. 1977; Aarrouf et al. 2008), the role of the stomata in opposing such entry has not yet been investigated. Two peach cultivars, ‘Venture’ and ‘BabyGold 5’, are known for their field resistance and susceptibility, respectively, to bacterial spot disease. In an attempt to demonstrate whether stomata might contribute to the different responses of these cultivars, stomatal apertures were measured in both cultivars following inoculation with *Xcp*. While most stomata closed within 1 HPI in both cultivars as a preinvasive defense mechanism (Melotto et al. 2006; Underwood et al. 2007), the stomata started reopening again at 3 HPI, with ‘BabyGold 5’ exhibiting a higher percentage of opened stomata than ‘Venture’ (Fig. 2). In a similar bacterial pathogen–plant interaction involving *Arabidopsis* and *P. syringae* pv. tomato DC3000 (*Pst* DC3000), the induction of stomatal reopening at 3 HPI has been attributed to the phytotoxin,

Fig. 8 Putative *cis*-acting elements in peach TLP promoters. The sizes of 733 and 644 bp were retrieved from the promoter region of *Pp-TLP1* and *Pp-TLP2* genes, respectively. The comparison of the same region in ‘Venture’ and ‘BabyGold 5’ identified a number of binding sequences for transcription factors with relevance to SA, JA and ET signalling pathways. These transcription factors include MYC2 (JA signalling), ERF (ET and JA), TGA (SA) and WRKY (SA). Sequences in *dashed boxes* indicate the overlapping binding sites for transcription factors



coronatine (COR), produced by *Pst* DC3000. Although it is unknown whether *Xcp* produces COR, it can be safely assumed that elicitors might be produced from *Xcp* to evade a plant's basal defense and to induce stomatal reopening at 3 HPI. The significant differences observed between the stomatal apertures of 'Venture' and 'BabyGold 5' after inoculation with *Xcp*, suggest that the stomata may play a significant role in resistance of peach to *Xcp*.

To better understand the molecular mechanisms underlying the resistance to *Xcp* in peach, 'Venture' and 'BabyGold 5' were used as candidates for incompatible and compatible interactions, respectively. It was hypothesized that the changes in their transcriptomes upon pathogen attack would reflect their phenotypic responses. Plant microarray data collected in the past few years indicated that resistance and susceptibility rely on the same set of genes that fluctuate in the timing and magnitude of their expression during interaction with pathogens (Tao et al. 2005; van Loon et al. 2006). One set of these genes encodes PR proteins that, in many cases, exert direct antimicrobial activities against invading pathogens (Edreva 2005).

In the present study, two genes (*Pp-PR1a* and *Pp-PR1b*) were isolated from the PR1 family and three (*Pp-TLP1*, *Pp-TLP2* and *Pp-TLP3*) from the TLP family. The induction kinetics of these genes in 'Venture' and 'BabyGold 5' indicated that four of the five genes, *Pp-PR1b*, *Pp-TLP1*, *Pp-TLP2* and *Pp-TLP3*, were upregulated in response to inoculation with *Xcp* (Fig. 6). The association between the stomatal closure at 1 HPI and the rapid induction of *PR*

genes at the same time probably illustrates the PAMP-triggered immunity (PTI) responses of epidermal and guard cells. PTI is triggered by pathogen-associated molecular patterns (PAMPs) (e.g., flagellins and peptidoglycans) and are associated with the induction of many defense-related genes (Asai et al. 2002; Nürnberger et al. 2004; Zipfel et al. 2004). PTI is different from effector-triggered immunity (ETI) that is triggered after the recognition of specific effectors from pathogens (e.g., *avr* proteins) by plant R proteins (Jones and Dangl 2006).

ETI is often characterized by the initiation of HR at the site of pathogen infection, and hence are effective against biotrophic pathogens, which derive their nutrients from living host tissues. However, for necrotrophic pathogens which feeds on dead cells, or hemibiotrophs that obtain nutrients from both living and dead tissues, ETI is likely beneficial for their pathogenicity (Ding et al. 2011; Pieterse et al. 2009). For *Xcp*, the bacterial growth in 'BabyGold 5' leaves increased from 10^3 to 10^7 CFU/cm² during the first and third day post-inoculation, while the development of necrotic lesions on leaves was only observed at 3 DPI. These observations indicate that *Xcp* might adopt a biotrophic phase before the destructive necrotrophic phase, during which the symptoms develop. The potential hemibiotrophic nature of *Xcp*, along with the absence of HR symptoms on the leaves of the resistant cultivar following inoculation with *Xcp*, indicate that ETI is unlikely to be involved in resistance of 'Venture' to *Xcp*.

For necrotrophic and hemibiotrophic pathogens, disease development might be associated with damage-associated

molecular patterns (DAMPs) (Schwessinger and Zipfel 2008; Nürnberger and Kemmerling 2009). The stomatal opening at 3 HPI and the spread of *Xcp* in the intercellular spaces of mesophyll cells is often followed by the degradation of cell wall components (Aarouf et al. 2008). Polysaccharides released from the degradation of cell walls might serve as DAMPs, leading eventually to the induction of defense responses in mesophyll cells. Accordingly, genes such as *Pp-TLP2*, which increased to higher levels in the resistant cultivar at 24 and 48 HPI, and *Pp-PR1b*, which showed another induction peak at 24 HPI (Fig. 6) might be a part of PTI responses which are also triggered by DAMPs (Jha et al. 2007; Göhre and Robatzek 2008).

On the other hand, the expression of *Pp-PR1a* showed no induction in response to inoculation with *Xcp* in either cultivar (Fig. 6). This expression pattern of *Pp-PR1a* did not differ from that of its nearest ortholog in apple (*Md-PR1a*) after inoculation with fire blight bacteria *Erwinia amylovora* (Bonasera et al. 2006) or the apple scab fungus, *Venturia inaequalis* (Gau et al. 2004). In the latter example, the authors analyzed the protein content of *Md-PR1* in the apoplastic fluid following inoculation of apple with *V. inaequalis*, but did not detect any PR1 protein even after 21 DPI (Gau et al. 2004), which predicts that *Pp-PR1a* expression would not be induced after 48 HPI with *Xcp*. On the other hand, *Pp-PR1b*, which lies on a separate phylogenetic branch, was induced in both resistant and susceptible cultivars following inoculation with *Xcp*. Among the 22 *Arabidopsis* PR1 genes, only one gene (NP_179068) is induced by pathogens, insects, or defense elicitors, while the others are constitutively expressed in roots or pollen (van Loon et al. 2006). These results taken together indicate that, despite their sequence similarities, only certain members of the PR1 proteins in each organism have a significant relevance to biotic stress.

SA-, JA- and ET-dependent defense signaling pathways regulate the expression of many PR genes upon pathogen attack. While ET/JA-signaling pathways confer resistance against necrotrophic pathogens, the SA-signaling pathway

is substantial for resistance against biotrophs. For hemi-biotrophic pathogens, both SA and ET/JA are proposed to contribute to defense responses; with SA signaling is activated during early hours after infection, while ET and JA pathways are activated at later hours and probably combined with the transition to the necrotrophic phase (Ding et al. 2011). Comparative analysis of gene expression after bacterial infection and after treatments with signaling compounds indicated that JA- and ET-inducible PR genes were more associated with resistance to *Xcp* than SA-inducible genes (Fig. 7). For instance, *Pp-PR1a*, which exhibited no induction of gene expression following bacterial infection, was rapidly induced by SA treatment whereas *Pp-PR1b*, which was induced to more than sevenfold by *Xcp* in the resistant cultivar, was expressed at low levels following SA treatment. The treatment by ethephon or MeJA induced the expression of *Pp-PR1b* to more than 15- and 50-fold over the control, respectively. In addition, *Pp-TLP2* transcripts, which accumulated to higher levels in the resistant than in the susceptible cultivar after inoculation with *Xcp*, were mainly triggered by MeJA treatment. SA treatment had almost no effect on the expression of *Pp-TLP2*.

The subcellular localization of PR proteins, as well as their induction by signaling molecules, cannot be revealed from protein sequence composition. Previous investigations have indicated that acidic PR proteins are localized in the apoplast and are induced by SA, while basic PR proteins are localized in vacuoles and are regulated by JA/ET-signaling pathways (Ohashi and Matsuoka 1987; Eyal et al. 1993; Niki et al. 1998; Santamaria et al. 2001; Gupta et al. 2000; van Loon et al. 2006). These findings are contrary to those reported in the present study where *Pp-PR1a*, which was induced rapidly and to high levels by SA treatment, encodes a basic protein, while *Pp-PR1b*, which was upregulated mainly by MeJA and ethephon treatments, encodes an acidic protein (Table 1). Moreover, *Pp-TLP1* and *Pp-TLP2* (basic PR proteins) showed apoplastic localization when expressed in onion epidermal cells

Table 1 Comparison of peach PR1 and TLP proteins with their respective type members

	Pp-PR1	Pp-PRb1	Nt-PR-1 ^a	Pp-TLP1	Pp-TLP2	Pp-TLP3	Nt-PR-5 ^a
Amino acid residues	161	190	168	249	248	330	226
Molecular weight (kDa)	17.21	21.02	18.57	25.70	26.04	34.23	24.55
Isoelectric point	8.70	5.41	4.83	4.46	4.83	4.73	5.33
Basic amino acids	14	19	8	12	16	16	14
Acidic amino acids	10	25	17	19	21	24	16
Signal peptide probability	0.999	0.997	0.998	1.00	1.00	0.995	0.957
Predicted subcellular localization	Extracellular	Extracellular	Extracellular	Extracellular	Extracellular	Plasma membrane	Extracellular

^a Tobacco PR1 (CAA29392) and Tobacco PR5 (CAA27548) are the type member proteins as described by van Loon et al. (1994)

(Fig. 5), and their coding genes were induced chiefly by ethephon and MeJA treatments.

The transcription induction of peach PR genes by JA/ET-signaling pathways was further demonstrated with the analysis of *Pp-TLP1* and *Pp-TLP2* promoter sequences that identified binding sites for transcription factors with central roles in these pathways (Fig. 8). A GCC-box was recognized in the promoter sequence of *Pp-TLP1*, which might explain the high expression of this gene after ethephon and MeJA treatments, respectively. The GCC-box is a *cis*-regulatory element found in the promoter of several PR genes, among them the plant defensin *PR12* (*PDF1.2*) (Penninckx et al. 1998; Lorenzo et al. 2003). The transcription regulation of *PDF1.2* by JA/ET pathways occurs via ethylene response factor 1 (ERF1), a transcription factor which binds to the GCC-box leading to the induction of *PDF1.2* upon pathogen attack or after treatments with jasmonic acid (50 μ M) or ethylene (100 ppm) (Lorenzo et al. 2003). In addition, MYC2-binding sites were found in four positions within *Pp-TLP1* and two positions in *Pp-TLP2*. The MYC2 transcription factor regulates the expression of many JA- and wounding-responsive genes (Boter et al. 2004; Lorenzo et al. 2004), which might explain the elevated levels of *Pp-TLP1* and *Pp-TLP2* transcripts after MeJA and wounding treatments. On the other hand, binding sites for WRKY and TGA transcription factors, which are involved in the SA-signaling pathway (Zhou et al. 2000; Yu et al. 2001), were found in *Pp-TLP1* promoter sequences. The sequences of WRKY- and TGA-binding sites overlapped with those of MYC2 and the GCC-box (Fig. 8), suggesting a competitive occupancy of these sites among different signaling pathways. This competition might serve as an underlying molecular mechanism by which the antagonistic interactions between SA and JA/ET pathways take place.

Altogether, it can be concluded that ‘Venture’ and ‘BabyGold 5’ were reliable candidates for a comparative molecular analysis of resistance and susceptibility, respectively, to *Xcp*. Stomatal responses to *Xcp* were different between ‘Venture’ and ‘BabyGold 5’ indicating a putative role for stomata in resistance. The temporal and quantitative analysis of peach PR genes after *Xcp* infection showed faster and stronger gene expression in ‘Venture’ than in ‘BabyGold 5’. The induction kinetics of PR genes after treatments with signaling compounds indicated that ET- and JA-inducible genes are more involved than SA-inducible genes in defense against *Xcp*, which is consistent with the potential hemibiotrophic nature of this bacterium. Additional investigations on ET/JA-signaling pathways may enhance our understanding of the upstream components that regulate the expression of PR genes and other defense-related genes during peach–*Xcp* interaction.

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