Characterization of an S-locus receptor protein kinase-like gene from peach

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Summary A receptor-like protein kinase gene (Ppsrkl1) was isolated from a peach (Prunus persica (L.) Batsch.) bark cDNA library prepared with RNAs isolated from bark collected in December (cold acclimated). Sequence analysis indicated that this gene is related to the S-locus family of receptor protein kinases (SRKs) and that it shares greatest homology with ZMPK1 from maize and At4g32300 from Arabidopsis, both of which are intron-less genes. In bark tissues, Ppsrkl1 mRNA is induced by water deficit treatment, repressed by short-day photoperiods and showed no response to cold treatment. The Ppsrkl1 mRNA also increased in roots in response to water deficit. In fruit, Ppsrkl1 shows no response up to 6 h after wounding, but at 12 and 24 h after wounding, Ppsrkl1 mRNA shows an abrupt decline. This decline was prevented by the addition of salicylic acid to the wound site. The Ppsrkl1 mRNA rapidly decreased in fruit after 10-min exposure to UV-C radiation, followed by a return to normal levels within 1.5 h. Taken together, these experiments indicate that Ppsrkl1 is negatively regulated by light and positively influenced by salicylic acid treatment in fruit and water stress in bark and roots.

Keywords: photoperiod, salicylic acid, signal transduction, UV-C light.

Introduction Organisms have evolved complex pathways for extra- and intracellular communication. One of the major signaling routes is through a reversible phosphorylation system based on activation or deactivation of proteins by phosphorylation/dephosphorylation (reviewed in Graves and Krebs 1999, Bassett 2001, Klumpp and Krieglstein 2002). The first step in signal relay is perception of a chemical or physical signal, such as a change in temperature or light; one type of sensor commonly used to initiate a response to the signal is a receptor protein kinase (RPK). Plants utilize two types of RPKs: histidine-phosphorylating types, such as the ethylene receptor (Chang et al. 1993); and serine/threonine-phosphorylating types, such as the brassinosteroid receptor (Li and Chory 1997, Li et al. 2002). Although the list of histidine-phosphorylating RPKs is growing, serine/threonine-phosphorylating RPKs are by far the most abundant class in plants.

Serine/threonine RPKs can be classified into six groups based on differences in their extracellular ligand-binding domains (Kohorn et al. 1992, Nasrallah and Nasrallah 1993, reviewed in Walker 1994, Becraft et al. 1996, Hervé et al. 1996, Wang et al. 1996, Li et al. 2002). Of these groups, the S-locus receptor kinases (SRK) were the first to be identified with a specific trait, i.e., self-incompatibility in Brassica reproduction (Katchroo et al. 2002, Takayama and Isogai 2003). Incompatibility is controlled by the S-locus protein 11 (SP11)/S-locus cysteine-rich protein (SCR) genes expressed in the anther (Schopfer et al. 1999, Suzuki et al. 1999) in concert with the S-locus glycoprotein (SLG) and SRK genes expressed in the stigma (Nasrallah et al. 1988, Stein et al. 1991). At least 25 different SRK alleles have been identified in B. oleracea L. (Watanabe et al. 2001).

In Arabidopsis, several S-locus-like receptor-like kinases, e.g., ARK and RLK, have been identified (Walker 1993, Dwyer et al. 1994). Because Arabidopsis is self-fertilizing and because expression of these SRKs is not restricted to reproductive tissue, it is thought that they must function in some other capacity besides reproductive recognition. In maize, another self-compatible species, an RPK with 27% identity to the Brassica s13 allele was found to be mainly expressed in shoots and roots, but barely detectable in the silk (Walker and Zhang 1990). Although a gene from Ipomoea trifida (IRK1) with similarity to Brassica SLGs/SRKs was predominantly expressed in pistils and anthers, it did not segregate with the I. trifida S locus, providing additional evidence that not all SRKs are associated with self-incompatibility (Kowyma et al. 1996). An S-locus receptor-like kinase gene (SFR2) from Brassica was recently shown to be inducible by both wounding and pathogen infection (Pastuglia et al. 1997). When
leaves were wounded or infiltrated with *Xanthomonas campestris pv. campestris*, SFR2 responded similarly to authentic pathogenesis-related (PR) proteins, such as phenylalanine ammonia-lyase (PAL) and chitinase. Furthermore, salicylic acid (SA) also caused transient induction of SFR2 in leaves. These results are consistent with SFR2 having a role in defense in *B. oleracea*.

We are interested in identifying tree fruit genes that respond to environmental stresses, particularly cold and drought. In an effort to identify potential signaling components that may be associated with cold hardiness, we used a fragment containing a conserved portion of the protein kinase domain of ZMPK1 to screen a peach (*Prunus persica* (L.) Batsch.) cDNA library, prepared from bark collected in December, that had previously yielded genes associated with season-specific expression (Artlip et al. 1997). We succeeded in isolating Ppsrk11 (*P. persica* S-locus receptor kinase-like), a gene encoding a receptor-like kinase with strong similarity to the S-locus family of receptor protein kinases. We compared the derived amino acid sequence of Ppsrk11 with the complete SRK family in *Arabidopsis*, as well as with SRKs from other plants where these genes have not been associated with reproductive incompatibility. Because peach is a self-compatible species and the gene was originally identified in December bark tissue, our hypothesis was that expression of *Ppsrk11* might be responsive to environmental stresses related to winter, i.e., low temperatures, short-day (SD) photoperiod or water limitation. Here we discuss the results of these experiments and their implication for the evolution of this gene family and its expression in higher plants.

**Materials and methods**

**Plant materials and sampling**

For cDNA preparation, current-year branches from deciduous peach seedlings grown in an orchard in Kearneysville, WV, were collected each month for 1 year (Artlip et al. 1997, see below). For water deficit experiments, growth of trees, treatment and sampling are described in Artlip et al. (1997). The ultraviolet-C (UV-C) treatment of ‘Loring’ peach fruit and collection of samples is described in El Ghaouth et al. (2003). Wounding of ‘Loring’ peach fruit and treatment with salicylic acid (SA) was as follows. Fruit collected from the orchard was stored overnight at room temperature. Wounding was performed with a nail set in a wooden handle so that wound size (about 3 mm diameter) and depth (~5 mm) would be consistent among fruit. Fruit was surface-sterilized and punctured. Then either 20 µl of water or 25 mM SA was applied to the wound. Samples were taken with a 5-mm-diameter cork borer, trimmed to a uniform depth of 3–5 mm and immediately immersed in liquid nitrogen. Tissues were lyophillized and stored at –80 °C until RNA extraction. For the photoperiod and temperature stress experiments, actively growing 3-year-old trees (‘Canadian Harmony’ on ‘Tennessee Natural’) were moved from a greenhouse to an environmental chamber (Conviron, Winnipeg, MB, Canada). Trees were grown for 5 weeks at 25 °C in short days (SD, 8-h photoperiod). Each day, 8 h into the dark period, the tree groups were covered with light-impenetrable black plastic or black latex-covered fabric, and one group was exposed to 15 min of white light (night break, NB) beneath the plastic in order to simulate a long day (Zhu and Coleman 2001). Imposing this SD/NB regime ensured that the trees in both treatment groups had approximately equal exposure to light for photosynthesis and carbohydrate metabolism. Efficacy of the treatment was measured as a function of cessation of apical bud growth. Treatment at 5 °C was as described for the photoperiod treatment, except that the trees had been kept in the greenhouse for an additional 10 weeks before placement in the growth chamber.

**Isolation of Ppsrk11**

A cDNA library prepared from bark extracted from branches collected in December (Artlip et al. 1997) was screened with a 32P-labeled probe containing a restriction endonuclease fragment encoding a highly conserved region of the ZMPK1 kinase domain. Hybridization and wash conditions were the same as those described by Bassett et al. (2000).

A partial clone, ppkb5 (GenBank Accession No. AY645718), was obtained from the library, purified and sequenced from both strands (Sequetch, Mountain View, CA). Additional sequence upstream of the 5′ end of ppkb5 was obtained with a Genome Walker kit (Clontech, Palo Alto, CA) and a 5′ RACE (Rapid Amplification of cDNA Ends) kit (Invitrogen, Carlsbad, CA). The products obtained were sequenced with a rhodamine Terminator Cycle Sequencing Ready Reaction kit, a 9600 GeneAmp PCR System thermal cycler and an ABI Prism 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA). Sequences of the two products can be found under GenBank Accessions Nos. AY645719 and AY645720.

The derived amino acid sequence of the complete gene was analyzed for domains and motifs by ProSite MotifScan (Falquet et al. 2002) and the European Molecular Biology Laboratory’s European Bioinformatics Institute (EMBL-EBI; InterPro). For comparison of *Ppsrk11* with various SRKs, alignment was conducted with ClustalW Version 1.8. The neighbor joining tree was created in ClustalW at EMBL-EBI (http://www.ebi.ac.uk/services/index.html).

**RNA isolation and cDNA preparation from different peach tissues**

Total RNA was isolated from frozen peach bark tissues stored at –80 °C with a PureScript kit (Gentra Systems, Minneapolis, MN), as described in Wisniewski et al. (2003), or from frozen and lyophilized fruit samples according to the method of Callahan et al. (1993). Total RNAs were normalized to 1 mg ml⁻¹ and used as templates for the preparation of cDNAs with the Advantage RT for PCR kit (Clontech).

**Semiquantitative RNA analysis by PCR**

The cDNAs prepared from different tissues or at different times were separated in agarose gels, stained with SYBR-Gold (Molecular Probes, Eugene, OR) and quantified in a STORM 860 image analyzer (Molecular Dynamics, Sunnyvale, CA).
The cDNAs within each experiment were normalized, and 2 µl of each was used separately in each 50 µl PCR reaction. Touchdown PCR was conducted with the Advantage 2 PCR kit (Clontech). For Ppsrkl1, the primers were PKB950R (5′-CCC CTCTTCCCAAGTCTTCTCTTG-3′) and PKB613F (5′-GCTG CCTGAGCCTCTGAGTAGG-3′). For the peach proteasome subunit 7 (Bassett et al. 1998) controls, the primers were CH2313F (5′-GATCTTCCAAGGGTGTCCCTG-3′) and PAM-181R (5′-CGGTTGTTTGCATGAACTTCTTG-3′). All primers were synthesized by Invitrogen. Primers specific to the peach 26S ribosomal RNA were kindly supplied by Dr. Ann Callahan (USD, ARS, Kearneysville, WV). The touchdown thermocycling conditions were: 1 min at 94 °C, 20 cycles of 1 min at 94 °C, 1 min at 65 °C and 1 min at 72 °C, followed by 15 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The asterisk represents a decrease of 0.5 °C for each of the 20 programmed cycles.

Quantitative analysis of select RNAs
To obtain more accurate quantitative data, select RNA samples were subjected to competitive PCR with a PCR MIMIC Construction kit (Clontech). The MIMIC primers were MIMICPKB5′ (5′-GCTGCTTGGACCTGATGGGCGCAAGTC GAAATTCCTCCCG-3′) and MIMICPKB3′ (5′-CCCCTCTTTT CAGTTTCTTGTGATTGACCTGACCATTGC-3′). The Ppsrkl1 mRNA was quantified following the manufacturer’s protocol and as described in Bassett et al. (2000).

Results
A peach bark cDNA library prepared from samples collected in the orchard in December was screened with a probe derived from pZMK1 (Walker and Zhang 1990, Bassett et al. 2000). Sequence analysis of the initial clone obtained indicated that it was a partial cDNA encoding an S-locus receptor kinase-like (SRKL). We were able to identify the full-length sequence of the peach SRKL gene, Ppsrkl1, by genome walking and 5′ RACE. Comparison of the derived amino acid sequence with other members of the SRK family indicated a high degree of homology between Ppsrkl1 and ZMPK1 (Figure 1).

Several structural motifs and domains associated with members of the SRK family are also present in Ppsrkl1. For example, a lectin-like motif is found toward the amino-terminal end of the predicted polypeptide, and a PAN, or apple, motif encompasses the last six cysteine residues in the extra-cytoplasmic region. Like all serine/threonine plant receptor kinases identified to date, Ppsrkl1 has a predicted signal peptide, a membrane-spanning transmembrane region and a serine/threonine protein kinase domain. In addition to the standard motifs and domains associated with this class of receptor kinase, Ppsrkl1 and ZMPK1 both possess an epidermal-growth-factor-like (EGF-like) domain, a structure lacking in other SRK family members (Figure 2). The motif analysis program failed to recognize the S-domain in both Ppsrkl1 and ZMPK1, despite the presence of most of the conserved elements (e.g., WQSFDXPTDITLXXXL) found in the lectin-like domain of all S-locus-related proteins (Figure 2). Other conserved amino acids found in S-locus proteins are also found in Ppsrkl1 and ZMPK1, including the 12 conserved cysteines noted by Walker (1994).

Analysis of peach leaf genomic DNA with primers designed from the partial cDNA clone ppkb5, representing most of the Ppsrkl1 sequence, suggested that no introns were present in this gene (data not shown). Likewise, no introns were observed in the sequence obtained from the genome walk 5′-extension of ppkb5 with genomic DNA as the template. Consistent with this observation are the results of a detailed comparison of the derived polypeptide encoded by Ppsrkl1 with the complete SRK and SRK-related family of genes from Arabidopsis, indicating greatest similarity between Ppsrkl1 and the two Arabidopsis SRKs whose genes lack introns (Figure 3). Southern blot analysis under stringent wash and hybridization conditions indicated that Ppsrkl1 is a single-copy gene in the peach genome (data not shown).

Analysis of Ppsrkl1 mRNA abundance indicated that it was expressed at relatively low levels in the various peach tissues examined. Analysis of Ppsrkl1 expression by competitive PCR indicated small changes in gene expression in bark tissues in fall and winter (Table 1). Under water deficit conditions, there was a fivefold decrease in Ppsrkl1 mRNA in xylem tissues, but a 50-fold increase in concentration in roots (Table 1). Likewise, RT-PCR analysis of bark tissue indicated an increase in Ppsrkl1 expression in response to severe water limitation (Figure 4).

Bark tissues from trees subjected to SD or NB treatments at 5 or 25 °C were analyzed for Ppsrkl1 expression. Abundance of mRNA encoding Ppsrkl1 decreased dramatically under SD treatment at 25 °C (Figure 5); however, the effect was not as pronounced at 5 °C. Little or no difference was observed in response to NB treatment. As an internal control, the pattern of expression of subunit 7 of the 26S proteasome was relatively unchanged in response to either day length or temperature (Figure 5).

Levels of Ppsrkl1 decreased dramatically when samples were taken immediately after fruit had been exposed to UV-C light for 10 min (Figure 6). Levels returned to control values 1.5 h later. A second decrease of similar magnitude was observed by 12 h after UV-C exposure, but levels returned to control values at 48 and 96 h (not shown) after exposure. A similar cyclic decrease at T = 0 and at 12 h was seen with both subunit 7 and 1-aminocyclopropane-1-carboxylate (ACC) oxidase mRNAs (not shown). No such decrease was observed in the 26S RNA.

Peach fruit that had been wounded and immediately treated with water or with SA was sampled and assayed for Ppsrkl1 expression. In the wounded + water-treated samples, the expression of Ppsrkl1 began to decline about 12 h after wounding and was undetectable by 24 h (Figure 7). There was no decline in the 26S RNA population during this time; however, subunit 7 mRNA followed a pattern similar to that of Ppsrkl1 (not shown). Addition of SA prevented the decline in Ppsrkl1 (and subunit 7) 12 h after wounding, but did not further induce the mRNA in samples taken earlier.
Figure 1. Alignment of *Ppsrkl1* with other S-locus receptor kinases (SRKs) or SRK-related protein kinases. Derived polypeptides from SRKs or SRK-related protein kinases were aligned with ClustalW Version 1.8. The conserved amino acid residues associated with the extracellular domains of all SRKs (Walker 1994) are indicated by diamonds (♦) above the column. The 12 conserved cysteine residues are indicated by squares (□) above the column. Derived polypeptides were analyzed individually by InterProScan and ProSite MotifScan to identify similar motifs. The domains and motifs are delineated as follows: bent arrows = the lectin-like domain; arrowheads = the S-domain; horizontally expanded box = the EGF-like domain; and dash-dotted lines = the PAN motif. Putative transmembrane regions are underlined. Regions conserved in protein kinases are enclosed in vertically expanded boxes. Abbreviations: Ark1 = *Arabidopsis* receptor kinase 1 (Accession No. M80238); SFR2 = wound- or bacteria-induced SRK from *Brassica oleracea* (Accession No. X98520); SRK3 = S-locus receptor kinase 3 from *B. oleracea* (Accession No. X79432); Pch = *Ppsrkl1* (this study); ZMPK1 = *Zea mays* protein kinase 1 (Accession No. X52384); and RLK14 = receptor-like kinase 14 from *Oryza sativa* (Accession No. AF403126).
Discussion

We have described the isolation and characterization of a peach gene with significant similarity to the *Brassica* SRKs and related genes. It has been shown that the extracellular region of a receptor kinase is the region of the protein that interacts with the ligand and sometimes with other kinases to form a ligand–receptor complex (Trotochaud et al. 2000, Gómez et al. 2001, Kachroo et al. 2001, 2002, Takayama et al. 2001, Wang et al. 2001, Li et al. 2002). The *Ppsrkl1* has all the requisite features of a receptor protein kinase. It has all the conserved regions associated with serine/threonine activity (Hanks et al. 1988), as well as an amphiphilic helical region that may be associated with calmodulin binding (Vanoosthuyse et al. 2003), suggesting that its activity may be modulated through Ca\(^{2+}\) signaling. The *Ppsrkl1* also has many of the...
features associated with the extracellular regions of SRKs. For example, it has a lectin-like domain containing several highly conserved amino acids found in SLGs and SRKs. In addition, like other members of this class, it has a PAN motif consisting of seven cysteines located just upstream of the transmembrane region. This motif is associated with protein–protein and protein–carbohydrate interactions (Davie et al. 1991). Interestingly, neither Ppsrkl1, ZMPK1 nor At4g32300 is recognized as having an S-domain by the ProSite MotifScan program, which uses alignment analysis of polymorphic S-locus genes to define identities (Isogai et al. 1995, Sakamoto et al. 1998). Instead, Ppsrkl1 and ZMPK1, but not At4g32300, have EGF-like regions encompassing the first six of the 12 conserved cysteines. The EGF domains are generally associated with protein–ligand interactions (Downing et al. 1996).

There is considerable diversity in the extracellular regions of the polypeptides compared in Figure 1. For example, there is 12% amino acid identity in the lectin-like domain, 8% in the PAN motif, and only 4% in the S domain, making it the most diverse of the three regions. However, only the SRK3 polypeptide is associated with self-incompatibility, and the diversity among the other five polypeptides most likely reflects differences in function that may relate to differences in ligand recognition. If this is true, Ppsrkl1 and ZMPK1 may interact with similar ligands, because they retain closely related features and amino acid similarity.

Our hypothesis that Ppsrkl1 plays a role in stress responses necessitated monitoring expression in diverse tissues subject to various stresses. Because the gene was originally isolated from a cDNA library prepared from peach bark collected in December, we surmised that it might be associated with plant responses to cold stress, SD photoperiod or water deficit, as these environmental factors prevail in the mid-Atlantic region.

Figure 4. Response of Ppsrkl1 expression in bark tissues to water deficit. Three-year-old trees were potted in 4-l containers and acclimated in a greenhouse under well-watered conditions (Kearneysville, WV). Samples were taken from trees before potting and at time 0 (T0). At T0, water was withheld from the trees until pots were at 45% of saturated (field) capacity, and the trees were maintained under these drought conditions for 1 week before sampling, at which time water potentials were –2.0 MPa. The trees were then watered for 1 week, and samples were collected at the end of the week, at which time water potentials were –0.2 MPa. Total RNA was isolated, cDNA synthesized and PCR conducted. The PCR products were separated in 1% agarose gels, stained with SYBR-gold and visualized in the STORM image analyzer. Abbreviations: Pre-pot = sample taken before trees were placed in the gallon containers; T0 = sample taken after acclimation in the greenhouse under well-watered conditions; H2O deficit = sample collected after 1 week of water deficit; recovery = sample collected after re-watering trees for 1 week; M = molecular size markers; Ppsrkl1 = PCR product from Ppsrkl1 primers; and sub7 = PCR product from subunit 7 of the 26S proteasome primers.

Figure 5. Responses of Ppsrkl1 expression to photoperiod and low temperature. Three-year-old trees were acclimated in a greenhouse before being placed in a growth chamber under different photoperiod and temperature conditions. Samples were taken and RNA isolated. The cDNAs were normalized and used as templates in PCR reactions. The products were separated in agarose gels and visualized. Bands were quantified using ImageQuant software and computed as a percent of the control value. Abbreviations: Control = sample taken before trees were placed in the growth chamber; SD = short-day (8 h) photoperiod; NB = night break (16-h dark period interrupted after 8 h with 15 min white light). Results were obtained with primers specific for Ppsrkl1 (A) and for subunit 7 of the 26S proteasome (B).
at that time of the year. We quantified the abundance of Ppsrkl1 mRNA from bark tissues in October as trees were becoming cold acclimated, in December when tissues were optimally cold acclimated and full endodormancy was achieved and in April as trees became de-acclimated and were released from dormancy. We also analyzed expression of Ppsrkl1 in bark from trees placed in growth chambers in controlled light and temperature regimes. A small decrease (twofold) in mRNA abundance observed during October–December paralleled the modest decrease seen in response to SDs in the growth chamber experiments relative to the long day (LD) and NB controls. However, little change was noted in expression at 5 or 25 °C in NB-treated samples. Taken together these results indicate that Ppsrkl1 suppression in response to SD treatment, but not to cold exposure, could therefore be associated with early steps in cold acclimation.

In stem xylem tissues, we observed a marked decrease in mRNA abundance under severe water deficit (–2.0 mPa) conditions, whereas we detected a substantial (50-fold) increase in mRNA abundance in roots exposed to the same treatment. Based on a semiquantitative RT-PCR approach, we determined that expression of Ppsrkl1 in bark was also up-regulated in response to water deficit treatment. The Ppsrkl1 mRNA was barely detectable in leaves from seedling trees and did not change significantly in response to water stress treatment (data not shown).

To further characterize Ppsrkl1, we analyzed its expression in peach fruit under different stress conditions. Low-dose, hormic UV-C treatment of fruits results in temporary induction of disease resistance with a concomitant reduction in ripening and senescence (Stevens et al. 1998a, 1998b). A dramatic decrease in Ppsrkl1 mRNA was observed immediately after exposure to UV-C, but levels returned to normal within 1.5 h. A circadian-like rhythm was noted around 12 h after treatment; however, by 48 h after treatment, Ppsrkl1 expression had returned to normal. These results contrast with observations on the response of three pathogenesis-related (PR) genes in these same RNA samples. In those experiments, all three genes were induced between 6 and 24 h after exposure, reaching a maximum at 96 h (El Ghaouth et al. 2003). The rapid and opposite response of Ppsrkl1 mRNA to UV-C compared with PR genes indicates that it is regulated by a different mechanism (i.e., suppression versus induction) or associated with a completely different signaling pathway.

Expression of Ppsrkl1 in fruit after wounding is difficult to interpret. Wounding typically results in the transient expression of a subset of stress-responsive genes. However, Ppsrkl1 expression is suppressed by UV-C or wounding, albeit with different kinetics. On the other hand, like most PR genes, it responded positively to SA treatment, but only at those times when its concentration would have otherwise declined. This suggests that expression of Ppsrkl1 is typically maximal in ripe fruit.

The expression of Ppsrkl1 in response to different stress treatments is complex. A more direct approach to understanding the role of this gene would be to overexpress it in Arabidopsis or a similar model system having a Ppsrkl1 homologue to evaluate its effect on the host phenotype, particularly as it relates to water-use regulation. Another approach would be to
identify the ligand(s) and proteins that interact with Ppsrkl1 under different conditions. In this way we could begin to identify signaling components of the pathway controlled by this receptor kinase and understand its physiological function in greater detail.

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References


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