Plant Molecular Biology **50:** 667–685, 2002.

The proline-rich, extensin-like receptor kinase-1 (PERK1) gene is rapidly induced by wounding

Nancy F. Silva[1,2] and Daphne R. Goring[1,*]
[1]*Department of Botany, University of Toronto, 25 Willcocks Street, Toronto, Ontario, Canada M5S 3B2 (*author for correspondence; e-mail goring@botany.utoronto.ca);* [2]*Biology Department, York University, Toronto, Ontario, Canada M3J 1P3*

Received 11 July 2001; accepted in revised form 22 March 2002

Key words: extensins, fungal pathogen, receptor-like protein kinases, wounding

Abstract

We report the isolation and characterization of PERK1 (Proline Extensin-like Receptor Kinase 1), a novel plant RLK from *Brassica napus* that is predicted to consist of a proline-rich extracellular domain with sequence similarity to extensins, a transmembrane region, and a catalytic domain possessing serine/threonine kinase activity. Database searches with the predicted PERK1 amino acid sequence also led to the identification of a predicted family of related genes in the *Arabidopsis* genome. Using biolistic bombardment of onion epidermal cells, we have shown that a PERK1-GFP fusion is localized to the plasma membrane as predicted for a receptor kinase. Given the similarity of PERK1's extracellular domain to extensins, a possible role in plant defense responses was investigated by treating *B. napus* tissue with mechanical stresses and infection with the fungal pathogen, *Sclerotinia sclerotiorum*. Various wounding stimuli resulted in a dramatic and rapid accumulation of PERK1 mRNA. Levels of PERK1 mRNA also increased moderately in response to infection by the fungal pathogen *S. sclerotiorum*. Given the kinetics of PERK1 mRNA accumulation in response to these treatments, PERK1 may be involved early on in the general perception and response to a wound and/or pathogen stimulus.

Introduction

Plants are subjected to a variety of external stimuli and have evolved mechanisms in order to respond to environmental changes. In plants, as in other eukaryotes, a diverse group of cell surface receptor-like protein kinases (RLKs) play a fundamental role in signal transduction processes (Stone and Walker, 1995; Lease *et al.*, 1998). These membrane-spanning proteins perceive the initial stimulus and transmit the information intracellularly through a signaling cascade, which ultimately results in the appropriate cellular responses. Members of the plant RLK family share highly conserved catalytic domains known to possess serine/threonine substrate specificity, yet the extracellular domains of these receptors are quite divergent which enable these proteins to selectively respond to diverse extracellular signals (Walker, 1994; Lease *et al.*, 1998). There are several classes of plant RLKs distinguished according to characteristic amino acid sequence motifs of their extracellular domains (Shiu and Bleecker, 2001).

The *S* domain RLK class has a distinct extracellular domain sharing sequence identity to the *S* locus glycoprotein (SLG) (Stone and Walker, 1995). Among this class are the *Brassica S* receptor kinases (SRKs) expressed exclusively in reproductive tissues and implicated in controlling the sporophytic self-incompatibility response (Stein *et al.*, 1991; Goring and Rothstein, 1992; Takasaki *et al.*, 2000; Silva *et al.*, 2001). Other RLKs of this type are represented in *Arabidopsis* by ARK1, ARK2, ARK3 (Tobias *et al.*, 1992; Dwyer *et al.*, 1994; Tobias and Nasrallah, 1996), in maize by ZmPK1 (Walker and Zhang, 1990) and by OsPK10 in rice (Zhao *et al.*, 1994). The diverse expression patterns among members of this class suggest that these plant receptor kinases may be involved in

mediating a variety of cellular signalling processes (Walker, 1994).

The largest plant RLK class is characterized by the leucine-rich repeat (LRR) motif involved in peptide ligand recognition and implicated in mediating protein-protein interactions (Braun and Walker, 1996; McCarty and Chory, 2000; Shiu and Bleecker, 2001). Analyses of *Arabidopsis* mutants with altered morphological phenotypes have led to the identification of several LRR RLKs with genetically defined functions. Examples include CLV1 in the regulation of floral and meristem development in *Arabidopsis* (Clark *et al.*, 1997; Trotochaud *et al.*, 2000), BRI1 in the perception and signal transduction of brassinosteroids (Li and Chory, 1997), ER in the control of organ differentiation (Torii *et al.*, 1996), and HAESA in the control of floral organ abscission (Jinn *et al.*, 2000). In addition, the Xa21 disease resistance gene in rice has been shown to encode a receptor kinase belonging to the LRR class (Song *et al.*, 1995).

The lectin-like class of plant receptor kinases, represented in *Arabidopsis* by Ath.lecRK1 (Hervé *et al.*, 1996) and LRK1 (Swarup *et al.*, 1996) contain extracellular domains sharing sequence similarity with legume lectins which are known carbohydrate-binding proteins implicated in the transduction of oligosaccharide signals in plant cellular communication processes (Hervé *et al.*, 1996). The *Arabidopsis* wall-associated kinase (WAKs) class is composed of at least 22 members in the completed *Arabidopsis* genome and contains epidermal growth factor (EGF)-like repeats as well as limited amino acid identity to the tenascin superfamily, collagen or neurexins in their extracellular domains (Kohorn *et al.*, 1992; He *et al.*, 1996, 1999; Shiu and Bleecker, 2001). Recent studies have implicated WAKs in the developmental control of cell expansion and cell elongation (Lally *et al.*, 2001; Wagner and Kohorn, 2001), in addition to the involvement of one of its members (WAK1) in mediating pathogen defense responses in *Arabidopsis* (He *et al.*, 1998). WAKs are the only class of RLKs that have been demonstrated to provide transmembrane coupling between the plant cell wall and the cytosol (He *et al.*, 1999). Other classes of plant RLKs are represented by the *Arabidopsis* PR5K (Wang *et al.*, 1996), which contains an extracellular domain related to plant defense proteins, and the maize CRINKLY4 harboring a region showing similarity to the cysteine-rich repeat motif of the tumor necrosis factor receptor (TNFR) (Becraft *et al.*, 1996).

In this study, we report the isolation of a cDNA designated PERK1 (Proline Extensin-like Receptor Kinase 1) that encodes a putative novel receptor protein kinase in *Brassica napus* and defines a new class of plant RLKs with an extracellular domain rich in proline and sharing sequence similarity to the extensin family of cell wall proteins. We have found that there is a rapid and transient accumulation of PERK1 mRNA in response to mechanical stresses. Transcript levels were also moderately increased by treatment with the fungal pathogen *Sclerotinia sclerotiorum*. Taken together, this evidence suggests a role for PERK1 in mediating the early events in the defense response of a plant to mechanical injury, perhaps by sensing changes in the cell wall via its extensin-like extracellular domain and triggering a wound response signaling cascade through its catalytic domain.

Materials and methods

Construction of λ-Pistil cDNA library

Pistils were collected from floral buds of *Brassica napus* Westar and W1 cultivars 1–2 days before anthesis. Total RNA was isolated as described by Jones *et al.* (1985) and enriched for poly(A)$^+$ RNA with pre-packed oligo $(dT)_{25}$-cellulose beads (New England Biolabs). Pistil poly(A)$^+$ RNA (5 μg) was used for the construction of a cDNA library with the ZAP-cDNA synthesis kit as described by the manufacturer's procedures (Stratagene). Infection of *Escherichia coli* host strain XL1-Blue yielded a primary library with an average titer of 1.0×10^6 plaque-forming units per milliliter (pfu/ml). The primary library was subsequently amplified to obtain an average total of 6.6×10^{10} pfu/ml on 150 mm petri dishes. *In vivo* mass excision of the pBluescript phagemids from the library was carried out as outlined by the manufacturer (Stratagene).

Generation of putative novel receptor-like protein kinase clones

In order to isolate novel *B. napus* receptor-like protein kinases, a combination of degenerate oligonucleotide primers designed against conserved kinase subdomains I and VII (Hanks and Quinn, 1991) were used to amplify mass excised phagemid DNA from a newly constructed λ-pistil cDNA library. Two oligonucleotide combinations were used

in a polymerase chain reaction (PCR): RK1 (5′-GGiGGTTTCGGiAT$^{T}/_{c/A}$GTiTT$^{A}/_{T}^{T}/_{C}$ AA$^{A}/_{G}$GG-3′) and RK2 (5′-AAiATiC$^{T}/_{G}$iGCCATiCC$^{A}/_{G}$ AA$^{A}/_{G}^{T}/_{C}$-3′), or RK1 and RK3 (5′-AAiATiC$^{T}/_{G}$iGCCATiCC$^{A}/_{G}$ AA$^{A}/_{G}^{T}/_{C}$-3′). The RK1 primer was constructed based upon a conserved amino acid consensus sequence (GGFGIV$^{F}/_{Y}$KG) within subdomain I of the catalytic domain. The degeneracy of the RK2 primer reflects a conserved amino acid consensus sequence (DFGMARIF) of subdomain VII that closely resembles the *S* receptor kinase in *Brassica*. The RK3 primer was generated on the basis of conserved amino acids (DFGLAKLL) within subdomain VII prevalent among the RLKs isolated in *Arabidopsis thaliana*. Standard PCR reactions were performed using Tsg polymerase (Biobasics). Amplifications were carried out for 35 cycles of 1 min at 95 °C, 1.5 min at 50 °C, and 1 min at 72 °C. PCR products of the expected size (ca. 450 bp) were gel-purified, cloned into the pT7Blue plasmid (Novagen) and transformed into *E. coli* DH5α cells. Positive clones were sequenced using an ABI automated sequencer (Model 373 Stretch DNA; Perkin Elmer). Sequence analyses were performed with the DNAsis software (Hitachi Software). PCR products showing less than 85% and 70% sequence identity to protein kinases in the database at the nucleotide and amino acid levels, respectively, were further pursued, one of these being PERK1.

Screening of λ-pistil cDNA Library

The original 351 bp PERK1 PCR product was used to screen the λ-pistil cDNA library. About 2×10^6 plaques from the amplified library were screened and plated at a density of 1×10^5 pfu per 150 mm plate. Duplicate colony lifts were performed and prehybridized for 2 h at 42 °C in 50% v/v formamide, 5× Denhardt's solution, 5× SSC, 0.1% w/v SDS, 1 mM EDTA and 100 μg/ml salmon sperm DNA. Filters were subsequently hybridized overnight in the same solution containing the PERK1 PCR product labeled with [^{32}P]α-ATP by random priming as described by Feinberg and Vogelstein (1983). The filters were then washed twice with 2× SSC, 0.1% w/v SDS at room temperature for 15 min, followed by two 25 min washes with 0.5× SSC, 0.1% w/v SDS at 55 °C. Plaques containing putative positive clones were cored and subjected to several rounds of screening until single isolates representing the PERK1 clone were obtained. Single-clone excision to liberate the pBluescript phagemid was performed on each isolate according to the procedure recommended by the manufacturer (Stratagene). Phagemid DNA digested with *Eco*RI/*Xho*I to release the cloned cDNA was subjected to standard plasmid Southern blot analysis and probed with the [^{32}P]α-ATP-labeled PERK1 PCR product. The membrane was pre-hybridized at 42 °C in 5× SSPE, 10× Denhardt's solution and 0.5% w/v SDS for 2 h and hybridized overnight at the same temperature in a buffer containing 50% w/v formamide, 5× SSPE and 0.5% w/v SDS. Washes were performed twice at room temperature for 15 min in 2× SSC, 0.1% w/v SDS followed by 30 min washes at 60 °C in 0.1× SSC, 0.1% w/v SDS. Several positive clones were detected by autoradiography and were sequenced using both universal and sequence specific primers to generate the 1512 bp consensus sequence representing the PERK1 cDNA isolated from the *Brassica* λ-pistil cDNA library.

Rapid amplification of cDNA ends (5′-RACE)

Despite the presence of an open reading frame, the 1512 bp cDNA did not encode the full-length transcript; therefore, the remaining 5′ end of the cDNA was isolated using the 5′-RACE system, Version 2.0 kit (Invitrogen). First-strand cDNA was synthesized from ca. 300 μg of mixed Westar and W1 pistil total RNA using a gene-specific primer GSP1 (5′-TAACCAACAAGAGACA-3′) designed to anneal ca. 300 bp from the 5′ end of the partial 1512 bp PERK1 cDNA. A homopolymeric tail was added to the 5′ end of the cDNA with TdT (terminal deoxynucleotidyl transferase) and dCTP. This tailed cDNA was then amplified with a second gene-specific primer GSP2 (5′-CCACTCCCAACTTTCAAC-3′) designed to anneal 3′ to the GSP1 primer, and an abridged anchor primer which annealed to the homopolymeric tail (Invitrogen). Amplification was carried out using the following cycling protocol: 1 min initial denaturation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 30 s at 63 °C, and 2 min at 72 °C. A PCR product of the expected size corresponding to the 5′ end of PERK1 was gel-purified, cloned into the pT7Blue plasmid (Novagen) and transformed into *E. coli* DH5α. Confirmation of the 5′-RACE product was obtained by plasmid Southern blot analysis as described above and by sequential primer-based sequencing.

Cloning of full-length PERK1 cDNA

A PCR-based approach was used to generate a full-length PERK1 cDNA by combining the 5′-RACE product cloned into the *Eco*RV site of pT7Blue (Novagen) with the cDNA isolated from the library screen cloned into the *Eco*RI/*Xho*I sites of the pBluescript SK phagemid. A forward primer (5′-GGAAAGCTTGCATGCCTGAGGTCGAC-3′) containing an internal *Pst*I site was designed to anneal upstream to the *Eco*RV cloning site of pT7Blue. A reverse primer (5′-CGCCTGCAGGTAATACGACTCACTATAGGG-3′) also containing a *Pst*I site was designed on the basis of the pBluescript phagemid sequence immediately 3′ to the *Eco*RI/*Xho*I cloning site. The full-length PERK1 cDNA was generated from a 100:μl PCR reaction containing 1:μl (ca. 20 ng) of each template (cDNA in pT7Blue and pBluescript phagemid) according to the following cycling protocol: 5 min initial denaturation at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 53 °C and 3 min at 72 °C. The PCR product of the expected size (ca. 2.2 kb) was gel-purified and cloned into the *Pst*I restriction site of pBluescript KS ($^{+}$/$_{-}$) II. The full-length PERK1 cDNA sequence was confirmed by sequencing with both universal and sequence specific primers as previously described. All DNA and protein sequence analyses was performed with the DNAsis software (Hitachi Software).

Plant material

B. napus Westar seeds were surface-sterilized with 30% v/v commercial bleach solution and rinsed in sterile water. The seeds were placed on moistened Whatman paper in a petri dish and germinated in a growth chamber under continuous light at 22 °C. Westar seedlings were potted and soil grown in a growth chamber at 22 °C with a 16 h light period followed by an 8 h dark period at 16 °C. Genomic southern blot and multiple-tissue RNA blot analyses were conducted on tissue harvested from flowering plants. Treatments were performed on 2-month old *B. napus* plants grown under the conditions stated above. Root tissue was harvested from two-month old *B. napus* plants grown hydroponically under the same day/night cycle in a growth medium described by Sommerville and Ogren, (1982).

Genomic DNA isolation and Southern blot analysis

Genomic DNA was extracted from 1g of young *B. napus* leaf tissue as described by Goring and Rothstein (1992). Genomic DNA (5 μg) was digested with several restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Xba*I, *Xho*I), fractionated through a 0.8% w/v agarose gel and transferred overnight in 10× SSC onto Zetaprobe membrane (BioRad). This was performed in duplicate to test hybridization conditions under low- and high-stringency conditions. After drying, the membranes were pre-washed in 0.1× SSC, 0.5% w/v SDS for 25 min at 60 °C. The membranes were then pre-hybridized and hybridized as previously described for plasmid southern blots with the inclusion of 10% w/v dextran sulfate and 50 μg/ml salmon sperm DNA in the hybridization buffer. Washing conditions for genomic southern blots varied depending on the stringency tested. One membrane was washed under conditions of low stringency for 15 min at room temperature in 2× SSC, 0.1% w/v SDS followed by a second 15 min room temperature wash in 1× SSC, 0.1% w/v SDS and three final washes at 50 °C in 1× SSC, 0.1% w/v SDS. The second membrane was washed under conditions of high stringency by lowering the salt concentration to 0.1× SSC, 0.1% w/v SDS and increasing the temperature to 65 °C. Membranes were probed with the [^{32}P]α-ATP-labeled PERK1 cDNA generated by random priming as described by Sanger *et al.* (1977) and subjected to autoradiography (XAR-5 film, Kodak) overnight at −80 °C.

Isolation and RNA blot analysis of multiple-tissue RNA

Total RNA was extracted as described by Jones *et al.* (1985) from petal, anther and pistil tissues harvested from floral buds 1–2 days before anthesis. Total RNA was isolated from root, leaf and stem tissues using a protocol optimized for high-carbohydrate tissues described by Cock *et al.* (1997). Poly(A)$^{+}$ RNA was partially purified with the polyA Spin mRNA isolation kit (New England Biolabs) as outlined by the manufacturer's procedure. A 5 μg portion of the RNA was fractionated on a 1.2% w/v formaldehyde gel and transferred onto Zetaprobe membrane (BioRad) in 10× SSC. Hybridization and high-stringency wash conditions were conducted as previously described for genomic southern blot analysis. The membrane was subsequently probed with the cyclophilin EST clone (No. mBN086) used as an internal control for even loading (Lippuner *et al.*, 1994; Chuck *et al.*, 1996).

Production and purification of PERK1 wild-type and mutant recombinant proteins

For use in the kinase assay and phosphoamino acid analyses, a construct containing only the catalytic domain of the PERK1 cDNA was generated by PCR amplification with the forward gene-specific primer (5′-CGCGGATCCAGAAGAAACGACGGAGAGAC-3′) designed to anneal immediately following the transmembrane domain of PERK1 and the reverse gene-specific primer (5′-CGCGGATCCGCGCCCATCTGG TTTAAAGAGAAG-3′) designed to anneal at the end of the cDNA. Both primers were engineered with *Bam*HI restriction sites to facilitate subcloning of these PCR products. The mutant PERK1 catalytic domain was constructed by mutating the invariant lysine of subdomain II, involved in phosphotransfer, to a glutamic acid using the QuikChange site-directed mutagenesis kit (Stratagene). Both the wild-type and the mutant kinases were cloned in-frame into the pMAL-C expression plasmid which generates recombinant fusion proteins with a maltose-binding protein (MBP) domain used for purification (New England Biolabs). The MBP domain is ca. 40 kDa in size. *E. coli* (BL21pLysS) cells carrying either the wild-type or mutated pMAL-C/PERK1$_{CD}$ were grown in a 200 ml culture with shaking at 37 °C until an OD_{600} of 0.6. Isopropyl β-thiogalactoside was added to a final concentration of 0.1 mM to induce the production of the recombinant protein, and the culture was incubated overnight at 20 °C with shaking. The cells were harvested and the pellet was resuspended in 20 ml of M-lysis buffer (50 mM Hepes pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 200 μM PMSF). After sonication, the insoluble proteins were removed by centrifugation at 12 000 $\times$ *g* for 10 min and the solubilized protein extract was incubated with a 10% v/v amylose resin solution (New England Biolabs) for 30 min at 4 °C. The protein extract was washed three times in M-wash buffer (50 mM Hepes pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 200 μM PMSF, 10% glycerol). The extract was resuspended in 6 ml of M-wash buffer and loaded onto a 1 ml syringe for column purification. The protein was eluted by the addition of 500 μl of elution buffer (50 mM Hepes pH 7.4, 100 mM maltose, 10% glycerol) to the column and 5–7 fractions of eluate were collected. Purified recombinant fusion proteins were subjected to SDS-PAGE. Western blot analysis with an anti-MBP antibody (New England Biolabs) was then performed to confirm the identity of the wild-type and mutant recombinant PERK1 proteins.

Kinase assay and phosphoamino acid analysis

The purified PERK1 wild-type and mutant recombinant proteins (1 μg) were mixed with 10$\times$ kinase buffer (200 mM Pipes pH 7.0, 100 mM $MgCl_2$, 20 mM $MnCl_2$, 100 μg/ml aprotinin) and 10 μCi of [^{32}P]γ-ATP in a 12 μl final volume reaction and incubated at room temperature for 30 min. The kinase reaction was stopped by the addition of 6 μl of 3$\times$ SDS sample buffer (187.5 mM Tris-HCl, 6% w/v SDS, 0.03% w/v phenol red), boiled for 5 min, and electrophoresed through a 10% w/v SDS-PAGE gel. The SDS-PAGE gel was subsequently stained with Coomassie blue, dried down, and exposed for 10 min to X-ray film. The fusion protein on the Coomassie-stained gel corresponding to the phosphorylated wild-type protein detected on the autoradiogram was excised from the gel and subjected to phosphoamino acid analysis as described by Boyle *et al.* (1991) using the Hunter Thin Layer Peptide Mapping Electrophoresis System 700 (C.B.S. Scientific).

Transient transformations and subcellular localization of PERK1-GFP

Chimeric fusion constructs with the GFP described by Friedrichsen *et al.* (2000) were generated to investigate the subcellular localization of PERK1 in onion epidermal cells. Two GFP gene-specific primers, 5′-CGCGGATCCATGAGTAAAGGAGAAGAACTTT-3′ and 5′-CGCGGTACCCGCTTATTTGTATAGTT CATCC-3′ engineered to introduce *Bam*HI and *Kpn*I sites respectively were used to amplify GFP from the BRI1-GFP template (Friedrichsen *et al.*, 2000). The nopaline synthase terminator (Nost) present in pBI221 (Clontech) was re-amplified with gene-specific primers, 5′-CGCGGTACCGAATTTCCCCG ATCGTTCAAAC-3′ and 5′-CGGCCAGTGAATTC CCGATC-3′, containing convenient *Kpn*I and *Eco*RI sites. Each PCR product was digested with the corresponding restriction enzymes and both products were simultaneously ligated into pBluescript KS ($^+$/$_-$) and sequenced. This GFP-Nost cassette was then cloned as a *Bam*HI-*Eco*RI fragment into pBI221 (Clontech) downstream of the 35S cauliflower mosaic virus (CaMV) promoter. The 35S-GFP-Nost cassette was subsequently cloned into the pCAMBIA 2301 (Cambia) plant transformation vector as a *Hin*dIII-*Eco*RI fragment and would serve

A.

1 MSSAPSPGTGSPPSPPSNSTTTTPPPASAPPPTTPSSPPPPSTIPTSPPPSSRSTP

57 SAPPPSPPTPSTPGSPPPLPQPSPPAPTTPGSPPAPVTPPTRNPPPSVPGPPSNP

112 SREGGSPRPPSSPSPPSPSSDGLSTGVVVGIAIGGVALLVIVTLICLLCKKKRRRDE

169 EDAYYVPPPPPPGPKAGGPYGGQQQQWRQQNATPPSDHVVTSLPPPPKAPSPP

222 RQPPPPPPPPPFMSSSGGSDYSDRPVLPPPSPGLVLGFSKSTFTYEELARATN GFS
I

277 EANLLGQGGFGYVHKGVLPSGKEVAVKQLKVGSG QGEREFQAEVEIISRV HHRHL
II III IV

332 VSLVGYCIA GAKRLLVYEFVPNNNLELHLHGEGR PTMEWSTRLKIALGSAKGLSYL
V VI

388 HEDCNPKIIHRDIKASNLIDFKF EAKVADFGLAKIASDTNTHV STRVMGTFGYLAPE
VII VIII

446 Y AASGKLTEKSDVFSFGVVLLELITGRRPVDANN VYVDDSLVDWARPLLNRASEQ
IX X

501 GDFEGLADAKMNNGYDRE EMARMVACAAACVRHSARRRPRMSQIVRALEGNVS
XI

554 LSDLNEGMRPGQSNVYSSYGGSTDYDSSQYNEDMKKFRKMALGTQEYNATGEY

607 SNPTSDYGLYPSGSSSEGQTTREMEMGKIKRTGQGYSGPSL*

B.

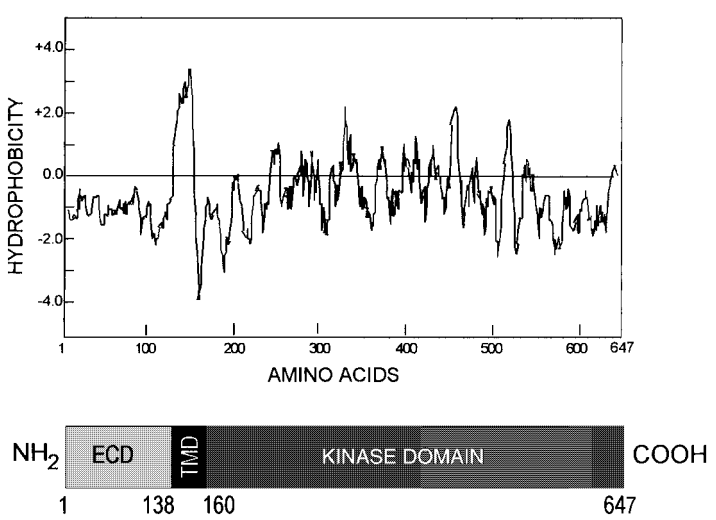

Figure 1. Predicted PERK1 amino acid sequence and protein structure. A. Predicted amino acid sequence of the PERK1 protein. The dashed underline indicates the extensin $(Ser\text{-}Pro)_4$ pentapeptide signature motif and the transmembrane region is denoted by the double underline. The roman numerals indicate the eleven characteristic subdomains of protein kinases as defined by Hanks and Quinn (1991). Amino acids in bold face type represent invariant residues and italicized amino acids indicate groups of conserved amino acids. The PERK1 sequence has been deposited in GenBank (accession number AY028699). B. Structural features of the PERK1 protein. A hydropathy plot deduced from the PERK1 amino acid sequence was generated by the Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) where increased hydrophobicity is denoted by positive values. A linear schematic representation of PERK1 denote the three domains comprising this polypeptide. ECD, extracellular domain; TMD, transmembrane domain.

as a control for all subcellular localization experiments. Two PERK1 gene-specific primers, 5′-CGCGGATCCAAGCAGACATGTCCTCGGC-3′ and 5′-CGCGGATCCAAGAGAAGGTCCACTATAACC-3′, containing *Bam*HI sites were used to amplify the full-length PERK1 cDNA such that its starting methionine was retained but the stop codon omitted, enabling PERK1 to be cloned in frame with GFP. The 35S-GFP-Nost cassette in pCAMBIA was digested with *Bam*HI and PERK1 was then cloned into this construct as a *Bam*HI fragment. Transient expression of GFP-fusion proteins in onion epidermal cells was performed with a Biolistic Particle Delivery System (BioRad) using 5 μg of each plasmid DNA precipitated onto M-17 tungsten particles (Dyer and Mullen, 2001; Rodríguez-Concepción *et al.*, 2001; Lee *et al.*,

2001). A GFP-AtROP6Q64L construct containing the AtROP6 gene encoding a small Rho GTPase localized exclusively to the plasma membrane was used as a positive control for our localization studies (Bischoff *et al.*, 2000). Onion peels were incubated at 25 °C on solid MS medium supplemented with sucrose and Amphotericin (Sigma) for 24 h after bombardment after which GFP was visualized with a Zeiss Axioskop2 MOT fluorescence microscope under the 10× objective (Carl Zeiss). Image capture was performed with Northern Eclipse 5.0 (Empix Imaging). Plasmolysis of the onion epidermal cells was induced upon addition of 0.8 M mannitol as described by Friedrichsen *et al.* (2000).

Plant treatments

Treatments were conducted on 2-month old plants and every experiment used one plant per time point. Leaf material was wounded by punching out leaf disks 1 cm around the perimeter of the leaf blade ensuring that the midvein remained intact. Stems and roots were wounded by slicing these tissues into 1–3 cm segments. The wounded tissues were placed in petri dishes containing filter paper moistened with 20 mM sodium phosphate buffer supplemented with 50 μg/ml chloramphenicol to prevent bacterial contamination of the wounded tissues as described by Shirsat *et al.* (1996). A control time point (0 h) for this experiment was performed by incubating unwounded tissue as mentioned above. Tissue was harvested at 0 h, 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, and 4 h after the wound stimulus. Several wounding treatments were applied to *B. napus* tissue to investigate whether changes in the steady-state levels of PERK1 mRNA were responsive to these different wound stimuli. The rubbing treatment involved gently rubbing the leaves with gloved hands for 2 min, whereas the abrasive wounding treatment involved rubbing stem tissue and the undersides of leaf tissue with abrasive sand paper (Pastuglia *et al.*, 1997). In both cases wounded tissue was harvested at the indicated time points.

The fungal pathogen treatment with *Sclerotinia sclerotiorum* (O-CEF-34) was performed according to the excised leaf protocol described by Kim *et al.* (2000). This procedure was slightly modified in that young fully expanded leaves were excised and inoculated with the fungal agar plugs 2 h after excision, to allow for PERK1 mRNA to return to basal levels. A control experiment was simultaneously performed, in which excised leaves were inoculated with agar plugs not colonized by the fungus. Total RNA was extracted from treated tissue according to the method described by Cock *et al.* (1997). Total RNA (40 μg) was electrophoresed on a 1.2% w/v formaldehyde gel and subjected to standard RNA blot analysis. Hybridization and washing conditions were performed as outlined for the multiple-tissue RNA blot. After autoradiography, the radioactive signals were quantified by Instant Imager Electronic Autoradiography (Packard). Membranes were subsequently reprobed with the cyclophilin EST clone (No. mBN086) as a control for even loading (Lippuner *et al.*, 1994; Chuck *et al.*, 1996), and the amounts of hybridized radiolabeled cyclophilin were quantified in the same manner. The relative amounts of RNA hybridized to the full-length PERK1 cDNA probe were determined after correction for differences in the amounts of cyclophilin RNA. Ethidium bromide-stained gel photographs were used to show the relative amounts of total RNA loaded for each time point.

Results

Isolation and sequence analyses of PERK1 cDNA

The PERK1 cDNA was isolated during a search for novel receptor kinases in *Brassica*. A region of the PERK1 kinase domain was initially isolated by PCR using degenerate oligonucleotide primers and a *Brassica* λ-pistil cDNA library as the template. The 351 bp PCR cDNA product was then used to screen the λ-pistil cDNA library to obtain longer cDNAs, followed by 5′-RACE to isolate the remaining 5′ end of the cDNA. The full-length PERK1 cDNA sequence isolated is 2189 bp and consists of one large open reading frame of 1944 bp, encoding a predicted protein of 647 amino acids (Figure 1A) with an estimated molecular mass of 69 kDa. The first methionine of this open reading frame is preceded by two in-frame stop codons indicating that the entire translated region was identified. In addition, a favorable translation initiation site is present directly adjacent to the AUG codon (Lutcke *et al.*, 1987). Based on the overall structure, PERK1 is predicted to encode a novel receptor-like kinase with three distinct domains: an extracellular domain, a single membrane-spanning domain, and a kinase domain (Figure 1B).

One notable absence is the N-terminus signal peptide. However, PERK1 was predicted by the PSORT database (http://psort.nibb.ac.jp/) to be a Type Ib integral membrane protein with its hydrophilic amino

terminal domain exposed on the exterior of the membrane despite the absence of a cleavable signal sequence preceding this domain. Singer (1990) proposed that, despite the lack of a signal peptide, Type Ib integral membrane proteins are inserted into the membrane via the usual ER-translocator protein machinery with slight modifications. Hydropathy analysis of the PERK1 protein predicts a single membrane-spanning region of 23 amino acids followed by a characteristic stop transfer sequence rich in charged amino acids (Figure 1B; Kyte and Doolittle, 1982; Weinstein *et al.,* 1982).

All known serine/threonine/tyrosine protein kinases display amino acid sequence similarities in their catalytic domains and consist of eleven conserved subdomains (Hanks and Quinn, 1991). The overall features of this organization are present in the catalytic domain (residues 274–548) of PERK1 including all the absolutely conserved amino acids and the highly conserved amino acid groups (Figure 1A). Furthermore, the amino acid sequences in subdomains VI (DIKASN) and VII (GTFGYLAPE) are consistent with the consensus sequences, DLKXXN and G(T/s)XX(Y/F)XAPE respectively, prevalent among serine/threonine kinases. This suggests that PERK1 may possess serine/threonine rather than tyrosine substrate specificity.

Based on the predicted extracellular domain, PERK1 is proposed to encode a novel class of plant RLKs. The extracellular domain of PERK1 consists of 137 amino acids and is rich in proline residues (41% prolines). FASTA3 amino acid searches (http://www.ebi.ac.uk/fasta33/) revealed similarities between the extracellular domain of PERK1 and hydroxyproline-rich glycoproteins (HRGPs), a family of plant cell wall-associated proteins (Cassab, 1998). The HRGP family includes several classes of proteins such as the arabinogalactan proteins, glycine-rich proteins, proline-rich proteins, extensins, and chimeric proteins with extensin-like domains (Cassab, 1998). A defining feature of extensin and extensin-like domains is the presence of the $Ser(Pro)_4$ pentapeptide signature motif and variations of this motif such as $Ser(Pro)_{3–7}$ (Memelink *et al.*, 1993; Showalter, 1993; Cassab, 1998). The predicted extracellular domain of PERK1 is predominantly composed of $Ser(Pro)_{2–3}$ motifs in addition to the presence of one conserved $Ser(Pro)_4$ signature motif (Figure 1A). The highest scores in the FASTA3 amino acid searches with PERK1's extracellular domain were with extensins and putative HRGPs containing $Ser(Pro)_x$ motifs. The relatedness between these proteins with the extracellular domain of PERK1 was primarily restricted to the Ser/Pro-rich regions. Thus, based on these criteria, we have defined PERK1's extracellular domain as being extensin-like.

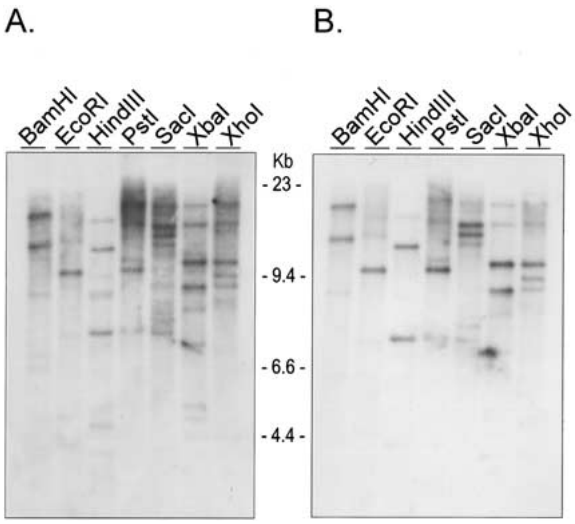

Figure 2. Genomic DNA southern blot analysis for PERK1. *Brassica napus* genomic DNA (5 μg) was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, *Xba*I and *Xho*I. Digested DNA was subjected to electrophoresis, blotted and hybridized with the full-length PERK1 cDNA under (A) low-stringency and (B) high-stringency conditions. The sizes (in kilobases) of the λ/*Hin*dIII molecular weight markers are given in the middle.

Southern blot analysis of the PERK1 gene

To study the complexity of the PERK1 gene in the *B. napus* genome, southern blot analysis was performed on genomic DNA under low-stringency (Figure 2A) and high-stringency (Figure 2B) conditions. The full-length PERK1 cDNA probe hybridized to restriction fragments consistent with known restrictions sites within the cDNA. In the case of *Hin*dIII- and *Sac*I-digested DNA, two fragments hybridized intensely to the PERK1 probe as expected given that both sites are present once in the cDNA (Figure 2). However, some additional hybridization bands detected in these lanes under both conditions of stringency suggests that there exists another gene with a high degree of sequence homology to PERK1. In addition, less prominent hybridization bands detected in the lanes corresponding to genomic DNA digested with *Sac*I, *Xba*I and *Xho*I under both low- and high-stringency conditions indicates the presence of possibly another three to four genes sharing sequence similarity to PERK1. This evidence suggests that PERK1

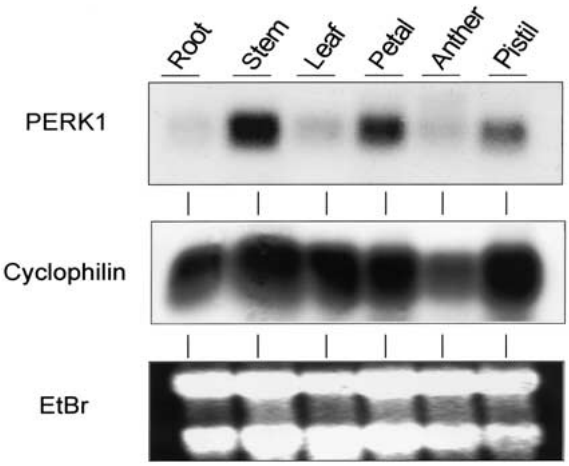

Figure 3. RNA blot analysis for the expression of PERK1 in various *Brassica napus* tissues. A 5 μg portion of partially enriched poly(A)$^+$ RNA isolated from root, stem, leaf, petal, anther and pistil tissues was electrophoresed, blotted and hybridized with the full-length PERK1 cDNA (top panel) . The blot was subsequently hybridized with the cyclophilin cDNA probe used a control for even loading (middle panel). The corresponding ethidium bromide gel image shows the relative levels of RNA loaded for each tissue sample. EtBr; ethidium bromide.

appears to exist as a member of a larger multi-gene family in the *B. napus* genome.

Analysis of PERK1 expression patterns in various tissues of B. napus

RNA blot analysis was performed with poly(A)$^+$ RNA extracted from a variety of *B. napus* tissues in order to examine the expression patterns of PERK1 throughout the plant (Figure 3). After hybridization with the full-length PERK1 cDNA probe, a single transcript ca. 2.2 kb in size was detected which is consistent with the size of the full-length PERK1 cDNA clone. The PERK1 transcript is ubiquitously expressed, with highest levels detected in stem, petal and pistil tissues and significantly lower levels detected in root, leaf and anther tissues (Figure 3, upper panel). The blot was subsequently probed with the cyclophilin cDNA as an internal control for even loading (Lippuner *et al.*, 1994; Chuck *et al.*, 1996) (Figure 3, middle panel). An ethidium bromide-stained gel photograph (Figure 3, lower panel) illustrates the relatively equal amounts of RNA used for each tissue. Therefore, the observed difference in the intensity of the cyclophilin signal in the anther sample is attributed to the lower abundance of cyclophilin mRNA present in the anther tissue as reported by Gasser *et al.* (1990).

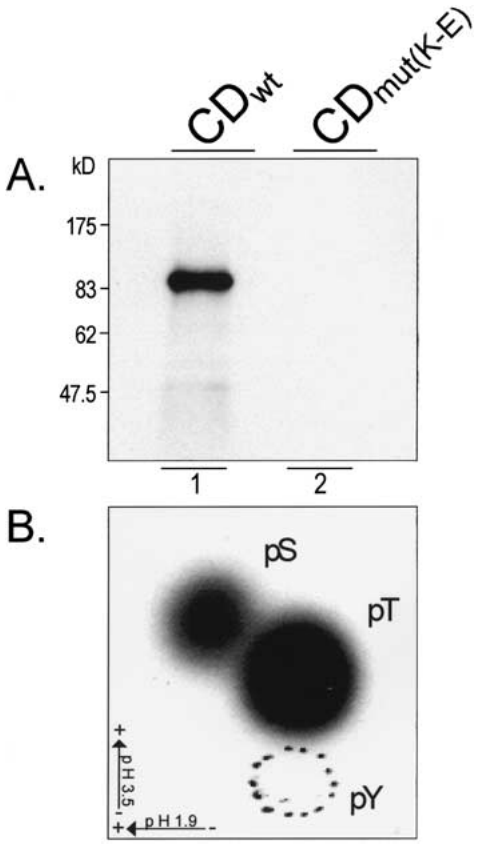

Figure 4. PERK1 kinase activity. A. Analysis of kinase activity of recombinant PERK1 protein purified from *E. coli.* The PERK1 kinase domain was produced in *E. coli* as a MBP-kinase fusion and tested for kinase activity in the presence of [^{32}P]γ-ATP, separated on a 10% SDS-PAGE gel, and subjected to autoradiography. CD_{wt}, wild-type catalytic domain form of PERK1; $CD_{mut(K-E)}$, kinase inactive form of PERK1. The sizes of the protein markers in kD are given on the left. B. Phosphoamino acid analysis of autophosphorylated PERK1. [^{32}P]γ-ATP-labeled PERK1 was hydrolyzed with HCl and subjected to two-dimensional thin-layer electrophoresis. The position of phosphotyrosine (pY) is indicated by the dashed circle, however only radiolabeled phosphoserine (pS) and phosphothreonine (pT) residues were detected.

Autophosphorylation and phosphoamino acid analyses of recombinant PERK1 protein

In order to ascertain whether the PERK1 protein encodes a functional kinase and to determine the specificity of the kinase activity, the catalytic domain of the PERK1 cDNA was expressed in *E. coli* and the kinase activity of the recombinant protein was examined (Figure 4). To ensure that the phosphorylation of the fusion protein was not a direct result of bacterial kinase contamination, a mutant PERK1 kinase was constructed in which the invariant lysine of subdomain II, involved in phosphotransfer, was mutated to

a glutamic acid. Substitution mutations involving this amino acid should render the kinase inactive (Hanks and Quinn, 1991). In Figure 4A, an autoradiograph of the protein gel shows that only the wild-type PERK1 fusion protein has autophosphorylation activity in the presence of [^{32}P]γ-ATP (Figure 4A, lane 1) when compared to the mutant kinase which did not show any detectable kinase activity (Figure 4A, lane 2). Therefore, this strongly suggests that the PERK1 protein encodes a functional kinase, and that mutation of the invariant lysine to a glutamic acid successfully abolished kinase activity.

In order to determine the amino acid specificity of this autophosphorylating activity, the phosphorlyated wild-type PERK1 fusion protein was extracted from the gel and subjected to phosphoamino acid analysis. As expected, only phosphorylated serine and threonine residues were detected (Figure 4B) indicating that PERK1 encodes a functional serine/threonine kinase.

Subcellular localization of PERK1

Despite the absence of a signal peptide, PERK1 is predicted to encode an integral membrane protein based on the presence of a predicted transmembrane domain. In order to determine the subcellular localization of PERK1 *in vivo*, a C-terminal GFP-PERK1 construct was engineered and transiently expressed under the control of the 35 CaMV promoter in onion epidermal cells (Figure 5). Onion epidermal cells transiently expressing the GFP alone control exhibit nuclear and cytoplasmic green fluorescence characteristic of the GFP localization (Figure 5A), whereas the PERK1-GFP fusion protein re-directed fluorescence throughout the periphery of transformed cells consistent with the proposed localization of PERK1 to the plasma membrane (Figure 5C). In order to differentiate between the plasma membrane and the cell wall, PERK1-GFP transformed cells were treated with 0.8 M mannitol which induces plasmolysis resulting in the internalization of the plasma membrane along with cellular organelles while the cell wall remains unchanged (Friedrichsen *et al.*, 2000). Figure 5E and F illustrate fluorescence and DIC images of a plasmolysed cell, in which the PERK1-GFP fluorescence at the plasma membrane was internalized or pulled away from the cell wall as indicated by the open-face arrows. As a positive control for plasma membrane localization, a GFP-RhoGTPase (AtRop6Q64L; Bischoff *et al.*, 2000) fusion was also bombarded into the onion epidermal cells. The GFP-AtRop6Q64L fluorescence is localized to the periphery of the cell in a similar pattern to that seen for PERK1-GFP (Figure 5G).

Identification of Arabidopsis thaliana *PERK1 family members*

A. thaliana and *B. napus* are both members of the Brassicaceae (Cruciferae) family, and exhibit a high degree of sequence similarity within their genomes. This prompted us to investigate the existence of related PERK1 genes in the *Arabidopsis* genome. To this end, the predicted PERK1 amino acid sequence was used to search databases containing the annotated *Arabidopsis* genome sequence. Results from the BLASTP search identified fourteen *A. thaliana* genes that were each predicted to encode a proline-rich, extensin-like extracellular domain, followed by a transmembrane domain and kinase domain. The members of this *Arabidopsis* PERK family exhibited varying degrees of amino acid similarity to the PERK1 sequence. The majority displayed less than 70% sequence similarity within the first 200 amino acids corresponding to the extracellular and transmembrane domains as well as part of the juxtamembrane region. However the amino acid similarities among the kinase domains of these genes and PERK1 were significantly higher. Strikingly, the AtPERK1 gene was determined to be over 80% similar to PERK1 throughout the entire region searched. A more detailed sequence comparison between the entire predicted coding sequence of AtPERK1 and the full-length PERK1 cDNA confirmed that these genes do indeed share a high degree of homology, corresponding to 84% and 83% sequence identity at the nucleotide and amino acid levels, respectively. Since *Brassica* genes typically show more than 85% nucleotide conservation in the coding region with their *Arabidopsis* orthologues (Arabidopsis Genome Initiative, 2000) we propose that AtPERK1 represents the PERK1 orthologue in *A. thaliana*. In addition, no other PERK-like sequences exhibit higher similarity to Bn-PERK1 than AtPERK1 in the completed *Arabidopsis* genome.

Within the *Arabidopsis* PERK family, we investigated sequence relationships by constructing a protein similarity tree (CLUSTAL W) based on the amino acid sequences corresponding to the juxtamembrane regions and the kinase domains of these genes (Figure 6A; Thompson *et al.*, 1994, 1997). Members of other receptor-like protein kinase classes were used in a rooted tree to illustrate that the predicted *Arabidopsis* PERK family members were found in a distinct cluster

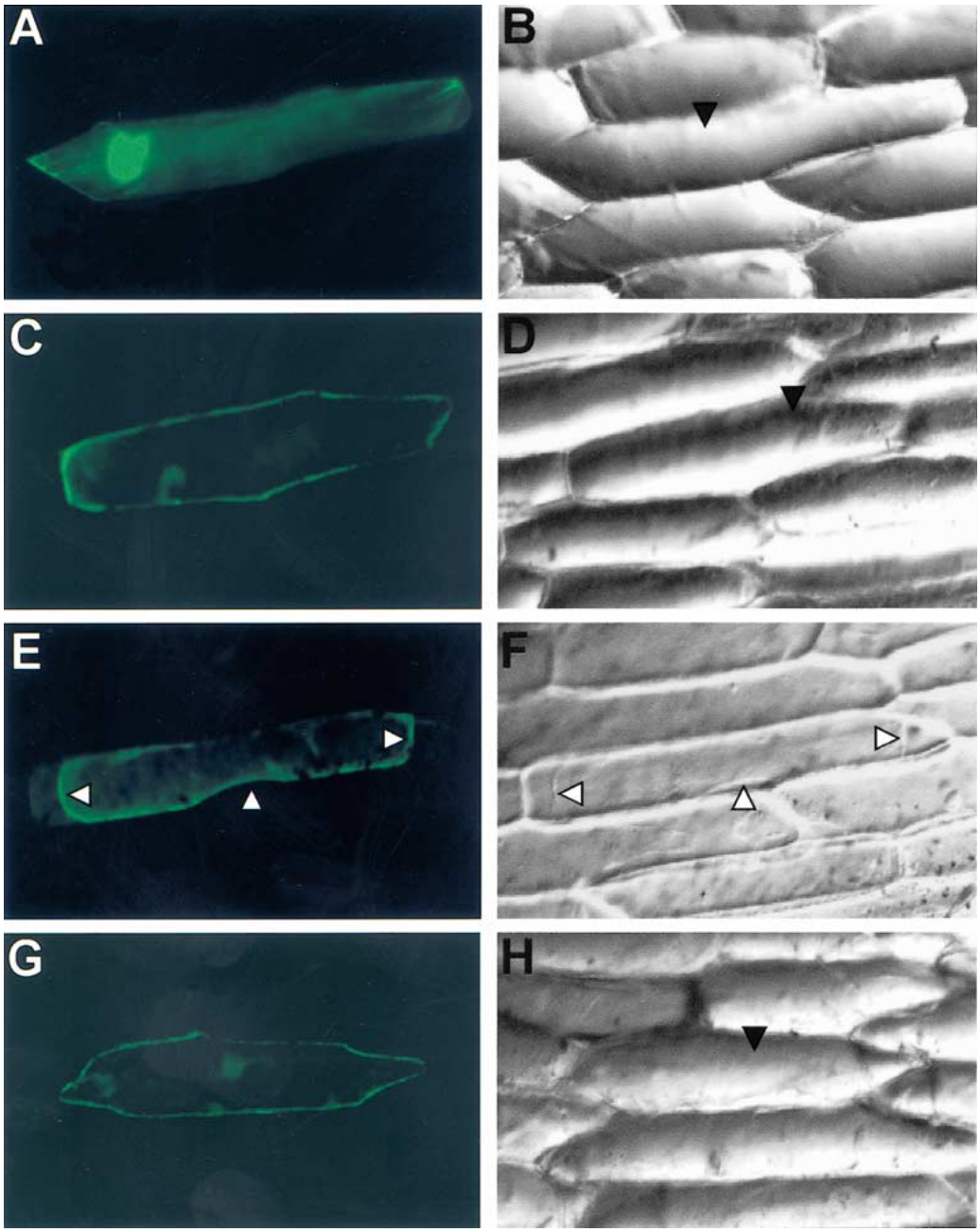

Figure 5. Plasma membrane localization of PERK1-GFP. Immunofluorescence images of GFP-tagged proteins are shown at 24 h afer biolistic bombardment of onion epidermal cells in A, C, E, and G. The corresponding DIC images delineating the transformed onion cell (marked by a bold face arrow) are shown in B, D and H. A, B. Transformed cell transiently expressing GFP. Green fluorescence is found in the nucleus and cytoplasm as expected for GFP localization. C, D. PERK1-GFP-transformed cell. Green fluorescence is found on the periphery of cell, consistent with plasma membrane localization. E, F. PERK1-GFP-transformed cell upon plasmolysis with 0.8 M mannitol. Bold face arrows indicate where the plasma membrane has detached from the cell wall as a result of plasmolysis and where the corresponding PERK1-GFP fluorescence is localized. G, H. GFP-AtRop6Q64L (Rho GTPase)-transformed cell. This positive control for plasma membrane localization (Bischoff *et al.*, 2000) also shows green fluorescence at the periphery of the cell.

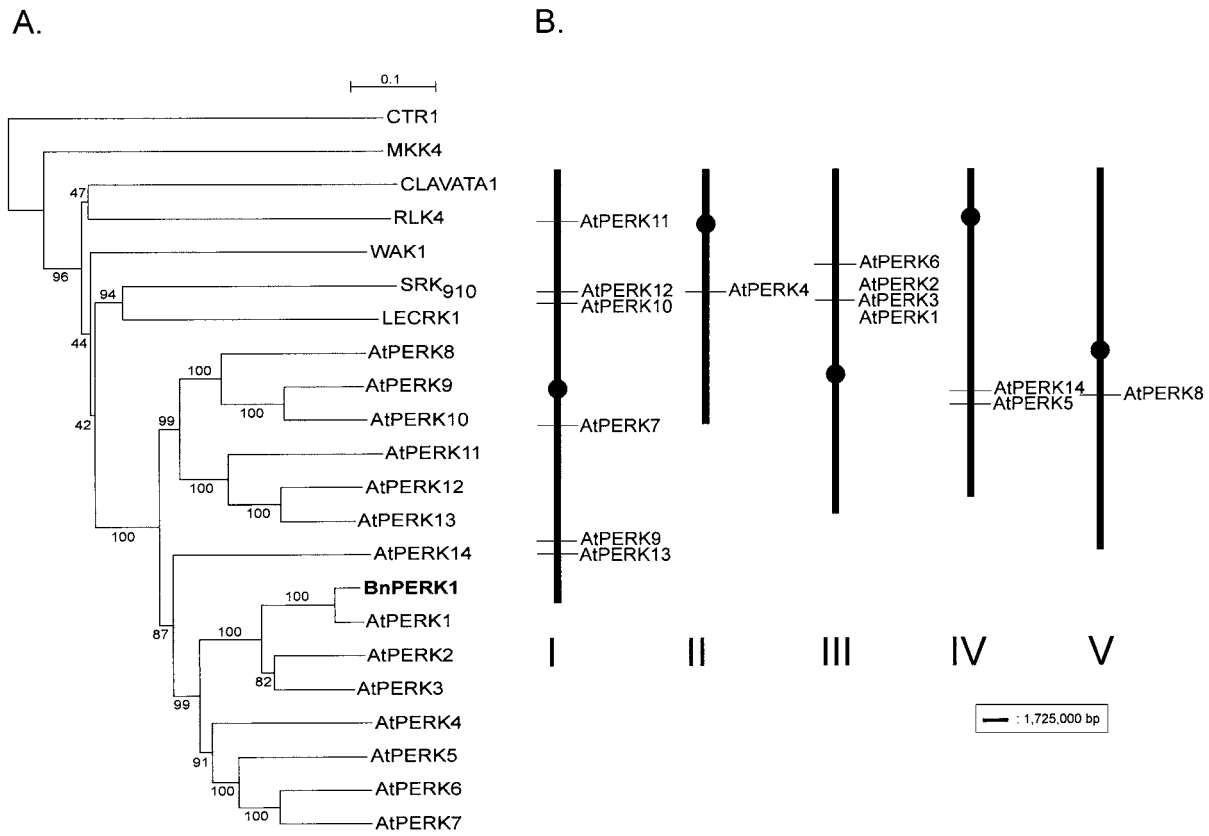

Figure 6. Identification and location of *Arabidopsis thaliana* PERK family members. A. Neighbor-joining tree representing the fourteen *A. thaliana* PERK family members, constructed based on the alignment of amino acid sequences corresponding to a region from the end of the transmembrane domain to the end of the catalytic domain. The rooted tree represents a consensus tree generated by 10 000 bootstrap replicates, each inferred from parametric distances by the neighbor joining method. (Saitou and Nei, 1987). The numbers represent bootstrap support (in percentages) and branches with less than 50% support are collapsed. The corresponding MAtDB gene names for the PERK family members are: AtPERK1: [*At3g24550*]; AtPERK2: [*At3g24400*]; AtPERK3: [*At3g24540*]; AtPERK4: [*At2g18470*]; AtPERK5: [*At4g34440*]; AtPERK6: [*At3g18810*]; AtPERK7: [*At1g49270*]; AtPERK8: [*At5g38560*]; AtPERK9: [*At1g68690*]; AtPERK10: [*At1g26150*]; AtPERK11: [*At1g10620*]; AtPERK12: [*At1g23540*]; AtPERK13: [*At1g70460*]; AtPERK14: [*At4g32710*]. The GenBank accession number for BnPERK1 is AY028699. Databases searches to identify *Arabidopsis* PERK family members were conducted through MIPS (http://mips.gsf.de/proj/thal/) and TIGR (http://www.tigr.org/tdb/agi/). B. Chromosomal map locations of the individual PERK family members were determined using the AGI maps available at http://arabidopsis.org/home.html. Chromosome numbers are indicated in roman numerals.

(Figure 6A). Of the four *Arabidopsis* PERK members found on chromosome III, three (AtPERK1–3) show a high degree of relatedness and cluster with the *B. napus* PERK1 sequence (Figure 6A and 6B). The fourth chromosome III member, AtPERK6, also maps to the same general location, but shows less sequence relatedness and clusters in a different position in the protein similarity tree (Figure 6A and 6B). In fact, AtPERK6 clusters with AtPERK7 (located on chromosome I), and these two genes may have their origins in an ancestral duplication event between these regions of chromosomes I and III (Arabidopsis Genome Initiative, 2000).

Duplication events may also explain some of the relationships between the *Arabidopsis* PERK members, AtPERK9 to AtPERK13, which are located on chromosome I, and are also found to cluster tightly in the similarity tree (Figure 6B). Although the tree illustrates a greater degree of sequence relatedness between AtPERK9 and AtPERK10, these genes map to distant locations on chromosome I (Figure 6A and 6B). Interestingly, the genes located next to AtPERK9 and AtPERK10 (AtPERK12 and AtPERK13) share a similar relationship. In fact, the regions where these AtPERK genes are located have been identified as duplicated sections of chromosome I (Arabidopsis Genome Initiative, 2000).

Arabdopsis databases (MIPS, TIGR and TAIR) were also searched for ESTs corresponding to the *Arabdopsis* PERK members. Six ESTs for AtPERK1 were identified from root and floral bud libraries. Three ESTs for AtPERK10 were also identified in root, rosette and above-ground libraries. In addition, one EST for AtPERK9 was identified from a root library, and three ESTs for AtPERK8 were identified from root, green silique and rosette libraries. EST abundance can vary significantly for different members of a gene family (Mekhedov *et al.*, 2000). The lack of ESTs identified for most of the PERK family members may reflect low expression levels or an inducible nature of their expression.

Changes in levels of PERK1 mRNA in response to mechanical stresses and infection by a fungal pathogen

Given the proposed role of extensins in wound healing and plant defense (Wilson and Fry, 1986; Showalter, 1993) and the similarity of PERK1's extracellular domain to extensins, we investigated the effects of wounding on PERK1 gene expression. *B. napus* leaf, stem and root tissues were wounded and the abundance of PERK1 mRNA was determined at different time points by RNA blot analysis. In wounded leaf tissue, PERK1 steady-state mRNA levels began to increase as early as 5 min after wounding and reached highest levels at 15 min corresponding to an 11.6-fold induction (Figure 7A). Steady-state PERK1 mRNA levels remained fairly high 30 min after wounding corresponding to a 5-fold increase; however, basal levels of PERK1 mRNA were re-established within 2 h of injury (Figure 7A). We also found that this rapid increase in PERK1 steady-state mRNA levels was a local response restricted to wounded tissue. When the first leaf of each plant was wounded, and the adjacent unwounded leaf was harvested, no increase in PERK1 mRNA levels was detected in the unwounded tissue (data not shown).

For wounded stem tissue, an increase in PERK1 steady-state mRNA levels was clearly apparent within 5 min after wounding; however, maximum steady-state mRNA levels were reached at 30 min after injury corresponding to a 5.9-fold increase (Figure 7A). Increases in PERK1 steady-state mRNA levels were also observed in root tissue with a 2.3-fold increase in mRNA levels at 30 min after wounding (data not shown). Basal levels of PERK1 mRNA were re-established in the roots within 1 h after treatment (data not shown).

We then tested two other mechanical stresses, rubbing the undersides of leaves and stems with abrasive sandpaper, and gentle rubbing of the leaves. The abrasive sandpaper treatment was found to cause a rapid increase in PERK1 mRNA levels corresponding to a 4-fold increase in the leaves and a 3.2-fold increase in the stems 30 min after treatment (data not shown). As shown in Figure 7B, the gentle rubbing of the leaves elicited a very pronounced effect on PERK1 steady-state mRNA levels. A 4-fold increase was detected within 5 min, with maximal levels corresponding to a 14.6-fold increase detected at 30 min after rubbing (Figure 7B). Therefore, it is clearly evident from the various mechanical stresses that the kinetics of PERK1 mRNA accumulation is both rapid and transient.

In order to address the potential role of PERK1 in a plant's defense response against pathogen attack, an experiment was performed to determine whether PERK1 mRNA levels accumulate in response to invasion by the fungal pathogen *Sclerotinia sclerotiorum*, a ubiquitous phytopathogenic ascomycete fungus. *Sclerotinia sclerotiorum* is the fungal pathogen responsible for *Sclerotinia* stem rot (syn. white mold) disease of soybean, but is capable of infecting a wide range of other plants including *Brassica* (Kim *et al.*, 2000). The fungal bioassay was performed essentially as described by Kim *et al.* (2000); however, basal PERK1 mRNA expression levels were re-established by incubating excised tissue for 2 h prior to fungal inoculation. Figure 8 illustrates the averaged results obtained from two independent fungal bioassays. An accumulation in PERK1 mRNA levels was observed at 8.5 h after fungal inoculation corresponding to a 3.4-fold increase (Figure 8). Interestingly, just prior to the increase in the PERK1 mRNA levels, it was observed (at 7–7.5 h) that the fungal agar plugs were beginning to adhere to the leaf tissue, due to the early stages of infection. The first visible signs of necrosis were observed at 13–15 h after inoculation. A control experiment was simultaneously performed where leaves were inoculated with non-colonized agar plugs and no increase in the levels of PERK1 mRNA was observed (Figure 8). Interestingly, in a separate experiment, the application of the defense-related compounds, methyl jasmonate and salicylic acid, had no significant effect on the steady-state levels of PERK1 mRNA (data not shown). Thus, the increase in PERK1 steady-state mRNA levels following *Sclerotinia* infection may re-

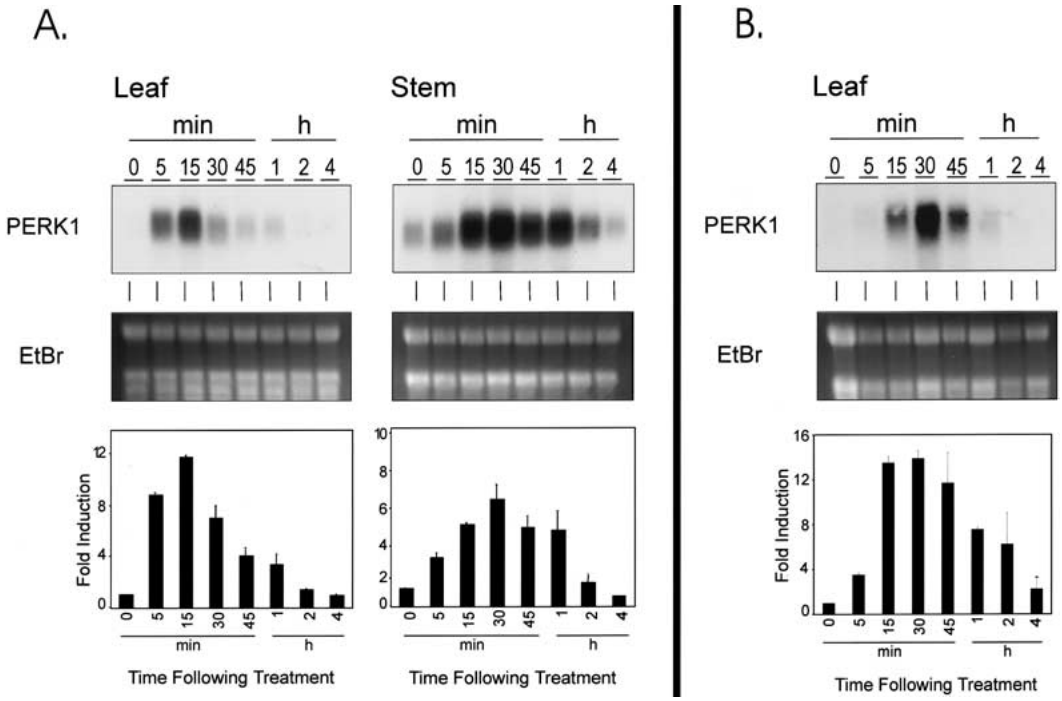

Figure 7. Wound-inducible accumulation of PERK1 mRNA in *B. napus* leaf and stem tissues. A. Time-course analysis of PERK1 mRNA accumulation in wounded leaf and stem tissues. Fully expanded leaves were wounded by punching out disks around the perimeter of the leaf; blade and stems were wounded by cutting into segments. B. Time course analysis of PERK1 mRNA accumulation in leaves in response to a rubbing stimulus. To investigate whether the accumulation of PERK1 mRNA levels was responsive to a different mechanical stimulus, fully expanded leaves were rubbed with gloved hands and total RNA was extracted for each time point. For both A and B, 40 μg of total RNA extracted for each time interval was subjected to RNA blot analysis and probed with full-length [^{32}P]α-ATP-labeled PERK1 cDNA (representative blots are shown in the upper panels). The corresponding ethidium bromide-stained gel images in the lower panels show the relative levels of total RNA loaded in each lane. The RNA blots were reprobed with cyclophilin as a control to correct for any differences in loading (data not shown), and this data was used to generate the graphs showing changes in PERK1 mRNA levels. The graphs represent the average of two experiments with the PERK1 mRNA levels corrected against cyclophilin mRNA levels. Error bars indicate $\pm$ standard error. EtBr; ethidium bromide

sult directly from cell wall damage caused by fungal penetration.

Discussion

In this study, we report the isolation and molecular characterization of the *B. napus* PERK1 cDNA that is predicted to encode a novel receptor-like kinase. We have shown that like other plant RLKs, the kinase domain of PERK1 has serine/threonine kinase activity. In addition, the localization of a PERK1-GFP fusion protein to the plasma membrane supports the prediction that PERK1 is an integral membrane protein. Plant RLKs are classified into several classes based on characteristics of their extracellular domains, and using this criterion, PERK1 represents a novel class of plant RLKs. The extracellular domain of PERK1 is proline-rich and shares sequence similarity to extensins, a family of abundant cell wall proteins. Extensins are proposed to have important structural and developmental roles associated with the cell wall and have also been implicated in wounding and pathogen defense responses where they are synthesized in response to physical damage and thereby act to reinforce and repair the cell wall (Showalter, 1993; Tiré *et al.*, 1994; Merkouropoulos *et al.*, 1999). While PERK1 shares sequence similarity to extensins, PERK1 is unique in that it also contains a transmembrane domain and a kinase domain. In addition, Southern blot analysis on *B. napus* genomic DNA and database searches of the *Arabidopsis* genome indicate that PERK1 is one member of a large family of related plant receptor kinases which we have called the PERK family. It is conceivable, therefore, that the PERK family may be involved in the transduction of extracellular stimuli, such as per-

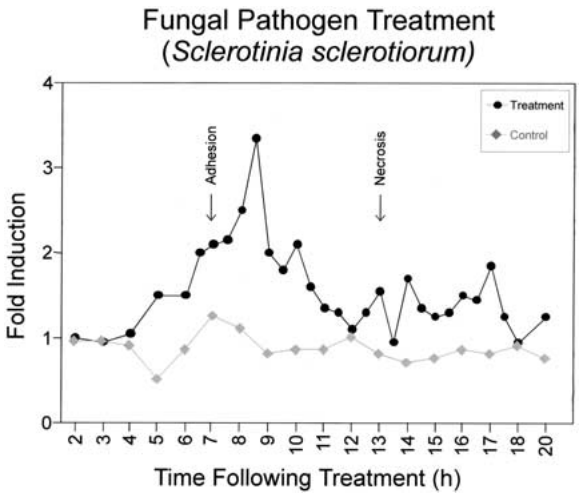

Figure 8. Effects on PERK1 mRNA accumulation in *B. napus* leaf tissue inoculated with the fungal pathogen *Sclerotinia sclerotiorum* (O-CEF-34). The fungal bioassays were performed essentially as described by Kim *et al.* (2000). Leaves were infected with *Sclerotinia* by placing several agar plugs colonized with *Sclerotinia* on each leaf (circles). Uninfected agar plugs were placed on the control leaves (diamonds). The graph represents the average of two independent experiments.

turbations to the cell wall, into intracellular responses through their cytoplasmic kinase domains.

The similarity of PERK1's extracellular domain to extensin proteins prompted us to investigate whether PERK1 may play a role in some aspect of plant defense. Different types of mechanical wounding resulted in a rapid and dramatic increase in PERK1 steady-state mRNA levels, which was most pronounced in the leaf. This is in contrast to the conventional extensin proteins in which an increase in transcript levels resulting from mechanical injury is delayed as late as 12 h after the wound stimulus (Sauer *et al.*, 1990; Showalter *et al.*, 1992; Shirsat *et al.*, 1996). It is also interesting to note that of the various mechanical stresses tested, the rubbing treatment caused a greater induction in levels of PERK1 mRNA perhaps due to the fact that a larger surface area of the leaf was affected. The inducibility of the PERK1 gene in response to treatment with the fungal pathogen *Sclerotinia sclerotiorum* was also investigated. After a time course treatment, increased PERK1 mRNA levels were detected slightly after the onset of adhesion and secretion of crystal deposits, possibly oxalic acid, on the surface of the leaf, but well before the first signs of necrosis appeared 13 h after inoculation. Among the enzymes and substances produced by *Sclerotinia* during infection are polygalacturonases, pectinases and oxalic acid (Fraissinet-Tachet and Fevre, 1996; Cessna *et al.*, 2000). Given PERK1's proposed function in sensing perturbations to the cell wall, the increase in PERK1 mRNA levels correlated well with the stage of *Sclerotinia* infection where physical and perhaps chemical events associated with fungal penetration appeared to be occurring.

Gene activation in response to various mechanical stresses has been well documented (Ichimura *et al.*, 2000b; Hirt, 2000). Mechanical wounding and stimulation (touch) can activate a variety of plant responses that occur when plants have been exposed to adverse weather conditions (strong winds) or herbivory. For example, several *Arabidopsis* TCH genes have been characterized which are markedly up-regulated in response to several environmental stimuli such as wind, touch, and water spray (Braam and Davis, 1990). One of the TCH genes encodes a xyloglucan endotransglycosylase and is thought to be involved in cell wall biogenesis while other TCH genes are implicated in signaling (Braam *et al.*, 1997). Plants react to mechanical injury by expressing a subset of proteins that facilitate wound healing and provide protection against further herbivory or pathogen invasion (Bowles, 1990; Léon *et al.*, 2001). Plants begin to express wound-responsive (WR) genes not only at or within the vicinity of the wound site but in some cases systemically in distant, undamaged tissues of the wounded plant. WR gene products are involved in the synthesis of antimicrobial compounds; in the production of proteinase inhibitors and lytic enzymes such as chitinases and glucanases; and in the reinforcement of the cell wall by deposition of callose, lignin and hydroxyproline rich glycoproteins (Hildmann *et al.*, 1992; Yang *et al.*, 1997; Léon *et al.*, 2001).

A subset of genes activated by mechanical stress encode signaling proteins which may play a role in mediating the plant's response to these stresses (Hirt, 2000; Léon *et al.*, 2001). Mechanical stimuli such as wind or touch lead to the up-regulation of TCH genes encoding calmodulin and calmodulin-related proteins (Braam *et al.*, 1997). The *Arabidopsis* calcineurin B-like calcium sensor gene, AtCBL1, is rapidly up-regulated in response to wounding and other abiotic stresses (Kudla *et al.*, 1999). Transcripts of the SFR2 RLK, a member of the *Brassica S*-domain receptor kinase family, accumulate in response to wounding and infection by a bacterial pathogen (Pastuglia *et al.*, 1997). Another group of receptor kinase genes, the cell wall-associated WAK1 and WAK2 genes, have recently been shown to have their expression environ-

mentally regulated and inducible by wounding (Wagner and Kohorn, 2001). Genes encoding components of the MAP kinase cascade have also been found to be up-regulated by a variety of stresses. For example, transcripts for the tobacco WIPK (MAP kinase) and alfalfa MMK4 (MAP kinase) genes rapidly accumulate following wounding (Seo *et al.*, 1995; Bögre *et al.*, 1997). The gene encoding the tobacco SIPKK (MAP kinase kinase), which was isolated following an interaction screen with SIPK (MAP kinase), was found to be up-regulated at the mRNA level following wounding, and tobacco mosaic virus infection (Liu *et al.*, 2000). Finally, the *Arabidopsis* ATMPK3 (MAP kinase) and ATMEKK1 (MAP kinase kinase kinase) genes have been found to be up-regulated at the RNA level by touch and other abiotic stresses (Mizoguchi *et al.*, 1996). Recently, Jonak *et al.* (2000) have shown that the mRNA levels for WIG, a novel GSK-3 kinase gene also increase rapidly after wounding. Of particular interest with these studies is the kinetics of the mRNA accumulation where the TCH genes, the AtCBL1 gene, the MAP kinase cascade genes and the WIG gene show a very rapid increase in mRNA levels following the wound/touch stimulus that is reminiscent of what we observed for PERK1.

The activity of several kinases has also been demonstrated to be rapidly activated by mechanical stresses. For example, the tobacco WIPK and SIPK MAP kinases, and the alfalfa WIG GSK-3 kinase are rapidly activated upon wounding (Seo *et al.*, 1995; Zhang and Klessig, 1998; Jonak *et al.*, 2000). The alfalfa MAP kinase, MMK4, is rapidly activated by mechanical stimulation and wounding (Bögre *et al.*, 1996, 1997). The *Arabidopsis* MAP kinases, ATMPK4 and ATMPK6, are rapidly activated by touch and wounding as well as other abiotic stresses (Ichimura *et al.*, 2000a). In general, the kinase activities were shown to rapidly and transiently increase within a few minutes after the mechanical stress.

Since several signaling genes such as the MAP kinase cascade genes and WIG show a rapid increase in mRNA levels after the wound/touch stimulus, as we have detected for PERK1, and these kinases have been implicated in early stages of the wound response due to associated rapid increases in kinase activity, it is tempting to speculate that PERK1 may also have such a role in the wound response. The very rapid increase in PERK1 mRNA levels could result from a positive feedback loop as part of the PERK1 signaling pathway as described for other signaling genes (Yamamoto *et al.*, 1998). However, the activation of PERK1's kinase domain upon mechanical stress still needs to be demonstrated. If this does prove to be the case and given the fact that PERK1 is predicted to be a receptor kinase, then PERK1 will be an excellent candidate for a signaling molecule functioning upstream of these cytosolic kinases. Thus, it will be of interest to study the activation of the PERK1 kinase, with respect to changes in levels of PERK1 phosphorylation, in relation to these cytoplasmic kinases upon wounding.

Acknowledgements

We thank Dr Frans Tax, Shinhan Shiu and Mark Gagliardi for critical reading and valuable comments on the manuscript. We are also grateful to Maria Mazzurco for some technical assistance; Dr Robert Mullen and Sophia Stone for advice with the PERK1-GFP experiments; Dr Klaus Palme for providing the GFP-AtROP6Q64L construct; Dr Mark Cock for providing the degenerate oligonucleotides; and Dr Linda Kohn for providing the fungal inoculum and experimental advice. N.F.S. is supported by a graduate scholarship from the Ontario Graduate Scholarships in Science & Technology, and research in this lab is supported by grants from the Natural Sciences and Engineering Research Council (NSERC) to D.R.G.

References

Arabidopsis Genome Initiative 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815.

Becraft, P.W., Stinard, P.S. and McCarty, D.R. 1996. CRINKLY4: a TNFR-like receptor kinase involved in maize epidermal differentiation. Science 273: 1406–1409.

Bischoff, F., Vahlkamp, L., Molendijk, A. and Palme K. 2000. Localization of AtROP4 and AtROP6 and interaction with the guanine nucleotide dissociation inhibitor AtRhoGDI1 from *Arabidopsis*. Plant Mol. Biol. 42: 515–530.

Bögre, L., Ligterink, W., Heberle-Bors, E., Hirt, H. 1996. Mechanosensors in plants. Nature 383: 489–490.

Bögre, L., Ligterrink, W., Meskiene, I., Barker, P.J., HeberieBors, E., Huskisson, N.S. and Hirt, H. 1997. Wounding induces the rapid and transient activation of a specific MAP kinase pathway. Plant Cell 9: 75–83.

Bowles, K.J. 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. 59: 873–907.

Boyle, W.J., van der Geer, P. and Hunter, T. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Meth. Enzymol. 201: 110–149.

Braam, J. and Davis, R.W. 1990. Rain-, wind- and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. Cell 60: 357–364.

Braam, J., Sistrunk, M.L., Polisensky, D.H., Xu, W., Purugganan, M.M., Antosiewicz, D.M., Campbell, P. and Johnson, K.A. 1997. Plant responses to environmental stress: regulation and functions of the *Arabidopsis TCH* genes. Planta 203: S35–S41.

Braun, D.M. and Walker, J.C. 1996. Plant transmembrane receptors: new pieces in the signalling puzzle. Trends Biochem. Sci. 21: 70–73.

Cassab, G.I. 1998. Plant cell wall proteins. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 49: 281–309.

Cessna, S.G., Sears, V.E., Dickman, M.B. and Low, P.S. 2000. Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. Plant Cell 12: 2191–2199.

Chuck, G., Lincoln, C. and Hake S. 1996. KNAT1 induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. Plant Cell 8: 1277–1289.

Clark, S., Williams, R.W. and Meyerowitz, E.M. 1997. The *CLAVATA1* gene encodes a putative receptor-kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89: 575–585.

Cock, J.M., Swarup, R. and Dumas, C. 1997. Natural antisense transcripts of the *S* locus receptor kinase gene and related sequences in *Brasscia oleracea*. Mol. Gen. Genet. 255: 514–524.

Dwyer, K.G., Kandasamy, M.K., Mahosky, D.I., Acciai, J., Kudish, B.I., Miller, J.E., Nasrallah, M.E. and Nasrallah, J.B. 1994. A superfamily of *S* locus-related sequences in *Arabidopsis*: diverse structures and expression patterns. Plant Cell 6: 1829–1843.

Dyer, J.M. and Mullen, R.T. 2001. Immunocytological localization of two plant fatty acid desaturases in the endoplasmic reticulum. FEBS Lett. 247: 1–4.

Feinberg, A.P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6–13.

Fraissinet-Tachet, L, and Fevre, M. 1996. Regulation of galacturonic acid and pectinolytic enzyme production by *Sclerotinia sclerotiorum*. Curr. Microbiol. 33: 49–53.

Friedrichsen, D.M., Joazeiro C.A.P., Li, J., Hunter T. and Chory J. 2000. Brassinosteroid-Insensitive-1 is a ubiquitously expressed leucine-rich receptor serine/threonine kinase. Plant Physiol. 123: 1247–1255.

Gasser, C.S., Gunnin, D.A., Budelier, K.A. and Brown, S.M. 1990. Structure and expression of cytosolic cyclophilin peptidyl-proyl-*cis-trans* isomerase of higher plants and the production of active tomato cyclophilin in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 87: 9519–9523.

Goring, D.R. and Rothstein, S.J. 1992. The *S*-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. Plant Cell 4: 1273–1281.

Hanks, S.K. and Quinn, A.M. 1991. Protein kinase catalytic domain sequence database: identification of conserved features of 1° structure and classification of family members. Meth. Enzymol. 200: 38–62.

He, Z.H., Fujuki, M. and Kohorn, B.D. 1996. A cell wall-associated, receptor-like kinase. J. Biol. Chem. 271: 19789–19793.

He, Z.H., He, D. and Kohorn, B.D. 1998. Requirement for the induced expression of a cell wall-associated receptor kinase for survival during the pathogen response. Plant J. 14: 55–63.

He, Z.H., Cheeseman, I., He, D. and Kohorn, B.D. 1999. A cluster of five cell wall-associated receptor kinase genes, *Wak1–5*, are expressed in specific organs of *Arabidopsis*. Plant Mol. Biol. 39: 1189–1196.

Hervé, C., Dabos, P., Galaud, J.P., Rougé, P. and Lescure, B. 1996. Characterization of an *Arabidopsis thaliana* gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain. J. Mol. Biol. 258: 778–788.

Hildmann, T., Ebneth, M., Peña-Cortés, H., Sánchez-Serrano, J.J., Willmitzer, L. and Prat, S. 1992. General roles for abscisic acid and jasmonic acid in gene activation as a result of mechanical damage Plant Cell 4: 1157–1170.

Hirt, H. 2000. MAP kinases in plant signal transduction. In: H. Hirt (Ed.), MAP Kinases in Plant Signal Transduction: Results, Problems and Cell Differences, Springer-Verlag, Heidelberg, Germany, pp. 1–9.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. 2000a. Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. Plant J. 24: 655–665.

Ichimura, K., Mizoguchi T., Yoshida, T., Yuasa, T. and Shinozaki, K. 2000b. Protein phosphorylation and dephosphorylation in environmental stress responses in plants. In: J. Callow (Ed.) Plant Protein Kinases: Advances in Botanical Research, Academic Press, San Diego, CA, pp. 355–370.

Jinn, T.L., Stone, J.M. and Walker, J.C. 2000. HAESA, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev. 14: 108–117.

Jonak, C., Beisteiner, D., Beyerly, J., and Hirt, H. 2000. Wound-induced expression and activation of WIG, a novel glycogen synthase kinase 3. Plant Cell 12: 1467–1475.

Jones, J.D.G., Dunsmuir, P. and Bedbrook, J. 1985. High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. 4: 2411–2418.

Kim, H.S., Hartman, G.L., Manandhar, J.B., Graef, G.L., Steadman, J.R., and Diers, B.W. 2000. Reaction of soybean cultivars to *Sclerotinia* stem rot in filed, greenhouse, and laboratory examinations. Crop Sci. 40: 665–669.

Kohorn, B.D., Lane, S. and Smith, T.A. 1992. An *Arabidopsis* serine/threonine kinase homologue with an epidermal growth factor repeat selected in yeast for its specificity for a thylakoid membrane protein. Proc. Natl. Acad. Sci. USA 89: 10989–10992.

Kudla, J., Xu, Q., Harter, K., Gruissem, W. and Luan, S. 1999. Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc. Natl. Acad. Sci. USA. 96: 4718–4723.

Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105–132.

Lally, D., Ingmire, P., Tong, H.Y. and He Z.H. 2001. Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. Plant Cell 13: 1317–1331.

Lease, K., Ingham, E. and Walker, J.C. 1998. Challenges in understanding RLK function. Curr. Opin. Plant Biol. 1: 388–392.

Lee, J.K., Suh, M.C., Kim, S., Kwon, J.K., Kim, M., Paek, K.H., Choi, D. and Lom B.D. 2001. Molecular cloning of a novel pathogen-inducible cDNA encoding a putative acyl-CoA synthetase from *Capsicum annuum* L. Plant Mol. Biol. 46: 661–671.

Léon, J., Rojo E. and Sánchez-Serrano, J. 2001. Wound signaling in plants. J. Exp. Bot. 52: 1–9.

Li, J. and Chroy, J. 1997. A putative leucine rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90: 929–938.

Lippuner, V., Chou, I.T., Scott, S.V., Ettinger, W.F., Theg, S.M. and Gasser, C.S. 1994. Cloning and characterization of chloroplast and cytosolic forms of cyclophilin from *Arabidopsis thaliana*. J. Biol. Chem. 269: 7863–7868.

Liu, Y., Zhang, S. and Klessig, D.F. 2000. Molecular cloning and characterization of a tobacco MAP kinase kinase that interacts with SIPK. Mol. Plant-Microbe Interact. 13: 118–124.

Lutcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F. and Scheele, G.A. 1987. Selection of AUG initiation codons differs in plants and animals. EMBO J. 6: 43–48.

McCarty, D.R. and Chory, J. 2000. Conservation and innovation in plant signalling pathways. Cell 103: 201–209.

Mekhedov, S., De Ilárduya, O.M. and Ohlrogge, J. 2000. Toward a functional catalog of the plant genome. A survey of genes for lipid biosynthesis. Plant Physiol. 122: 389–401.

Memelink, J., Swords, K.M.M., de Kam, R.J., Schilperoort, R.A., Hoge, J.H.C. and Staehelin, L.A. 1993 Structure and regulation of tobacco extensin. Plant J. 4: 1011–1022.

Merkouropoulos, G., Barnett, D.C. and Shirsat, A.H. 1999 The *Arabidopsis* extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic acid and salicylic acid, and codes for a protein with unusual motifs. Planta 208: 212–219.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. 1996. A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 93: 765–769.

Pastuglia, M., Roby, D., Dumas, C. and Cock, M. 1997. Rapid induction by wounding and bacterial infection of an *S* gene family receptor-like kinase gene in *Brassica oleracea*. Plant Cell 9: 49–60.

Rodríguez-Concepción, M., Alicia Pérez-García, A. and Béltrán J.P. 2001. Up-regulation of genes encoding novel extracellular proteins during fruit set in pea. Plant Mol. Biol. 46: 373–382.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Sanger, F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463–5467.

Sauer, N., Corbin, D.R., Keller, B. and Lamb, C.J. 1990. Cloning and characterization of a wound specific hydroxyproline-rich glycoprotein in *Phaseolus vulgaris*. Plant Cell Environ. 13: 257–266.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H. and Ohashi, Y. 1995. Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science 270: 1988–1992.

Shirsat, A.H., Wieczoreky, D. and Kozibal, P. 1996. A gene for *Brassica napus* extensin is differentially expressed on wounding. Plant Mol. Biol. 30: 1291–1300.

Shiu, S.H. and Bleecker, A.B. 2001. Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc. Natl. Acad. Sci. USA 98: 10763-10768.

Showalter, A.M. 1993. Structure and function of plant cell wall proteins. Plant Cell 5: 9–23.

Showalter, A.M., Butt, A.D. and Kim, S. 1992. Molecular details of tomato extensin and glycine rich protein gene expression. Plant Mol. Biol. 19: 205–215.

Singer, S.J. 1990. The structure and insertion of integral proteins in the membrane. Annu. Rev. Cell Biol. 6: 247–296.

Silva, N.F., Stone, S.L., Christie, L.N., Sulaman, W., Nazarian, K.A., Burnett, L.A., Arnoldo, M.A., Rothstein, S.J. and Goring, D.R. 2001. Expression of the *S* receptor kinase in self-compatible *Brassica napus* cv. Westar leads to the allele-specific rejection of self-incompatible *Brassica napus* pollen. Mol. Genet. Genomics 265: 552–559.

Sommerville, C.R. and Ogren, W.L. 1982. Isolation of photorespiration mutants in *Arabidopsis thaliana*. In: M. Edelman, R.B. Hallick and N.H. Chua (Eds) Methods of Chloroplast Biology, Elsevier Biomedical Press, Amsterdam, pp. 129–138.

Song, W.Y., Wang, G.L., Chen, L., Kim, H.S., Pi, L.Y., Gardner, J., Wang, B., Holsten, T., Zhai, W.X., Zhu, L.H., Fauquet, C. and Ronald, P.C. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. Science 270: 1804–1806.

Stein, J.C., Howlett, B., Boyes, D.C., Nasrallah, M.E. and Nasrallah, J.B. 1991. Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc. Natl. Acad. Sci. USA 88: 8816–8820.

Stone, J.M. and Walker, J.C. 1995. Plant protein kinase families and signal transduction. Plant Physiol. 108: 451–457.

Swarup, R., Dumas, D. and Cock, J.M. 1996. A new class of receptor-like protein kinase gene from *Arabidopsis thaliana* possessing a domain with similarity of plant lectin genes. Plant Physiol. 111: 347.

Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A. and Hinata, K. 2000. The *S* receptor kinase determines self-incompatibility in *Brassica* stigma. Nature 403: 913–916.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22: 4673–4680.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24: 4876–4882.

Tiré, C., De Rycke, R., De Loose, M., Inzé, D., Van Montagu, M. and Engler, G. 1994. Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotania plumbaginifolia*. Planta 195: 175–181.

Tobias, C.M. and Nasrallah, J.B. 1996. An *S*-locus-related gene in *Arabidopsis* encodes a functional kinase and produces two classes of transcripts. Plant J. 10: 523–531.

Tobias, C.M., Howlett, B. and Nasrallah, J.B. 1992. An *Arabidopsis thaliana* gene with sequence similarity to the *S*-locus receptor kinase of *Brassica oleracea*. Plant Physiol. 99: 284–290.

Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R.F. and Komeda, Y. 1996. The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell 8: 735–746.

Trotochaud, A.E., Jeong, S. and Clark, S.E. 2000. CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. Science 289: 613–617.

Wagner, T.A. and Kohorn, B.D. 2001. Wall-associated kinases are expressed throughout plant development and are required for cell expansion. Plant Cell 13: 303–318.

Walker, J.C. 1994. Structure and function of the receptor-like protein kinases of higher plants. Plant Mol. Biol. 26: 1599–1609.

Walker, J.C. and Zhang, R. 1990. Relationship of a putative receptor protein kinase from maize to the *S*-locus glycoproteins of *Brassica*. Nature 345: 743–746.

Wang, X., Zafian, P., Choudhary, M. and Lawton, M. 1996. The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins. Proc. Natl. Acad. Sci. USA 93: 2598–2602.

Weinstein, J.N., Blumenthal, R., Van Renwoude, J., Kempf, C. and Mauser, R.O. 1982. Charge clusters and the orientation of membrane proteins. J. Membr. Biol. 66: 203–212.

Wilson, L.G. and Fry, J.L. 1986. Extensins: a major cell wall glycoprotein. Plant Cell Environ. 9: 239–260.

Yamamoto, Y.Y., Matsui, M. and Deng, X.W. 1998. Positive feedback in plant signalling pathways. Trend Plant Sci. 3: 374–375.

Yang, Y., Shah, J. and Klessig, D.F. 1997. Signal perception and transduction in plant defense responses. Genes Dev. 11: 1621–1639.

Zhang, S. and Klessig, D.F. 1998. The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. Proc. Natl. Acad. Sci. USA 95: 7225–7230.

Zhao, Y., Feng, X.H., Watson, J.C., Bottino, P.J. and Kung, S.D. 1994. Molecular cloning and biochemical characterization of a receptor-like serine/threonine kinase from rice. Plant Mol. Biol. 26: 791–803.