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A proteinase inhibitor II of *Solanum americanum* is expressed in phloem

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Key words: black nightshade, companion cell, immunolocalization, *in situ* hybridization, sieve-area pore, sieve element

Abstract

Although proteinase inhibitor proteins are known to confer insect resistance in transgenic plants, their endogenous roles remain undefined. Here, we describe the expression of a proteinase inhibitor II (PIN2) protein from *Solanum americanum* in phloem of stems, roots and leaves suggesting a novel endogenous role for PIN2 in phloem. The phloem consists of parenchyma cells, sieve elements (SE), and companion cells (CC) which are in close association with SE. We isolated two cDNAs encoding PIN2, *SaPIN2a* and *SaPIN2b*, from a *S. americanum* cDNA library using a tomato *PIN2* cDNA as hybridization probe. SaPIN2a shows 73.6% identity to SaPIN2b. Southern blot analysis confirmed that two genes occur in *S. americanum*. Northern blot analysis showed that both are wound-inducible and are expressed in flowers. Unlike *SaPIN2b* and other previously characterized plant PIN2 proteins, *SaPIN2a* is abundantly expressed in stems. *In situ* hybridization studies on stem sections showed that *SaPIN2a* mRNA is expressed in CC and some SE, likely the immature developing SE, of external and internal phloem. Western blot analysis using SaPIN2a-specific antibodies showed SaPIN2a accumulation in stems, leaf midribs and fruits. Immunohistochemical localization, using these antibodies, revealed SaPIN2a expression in external and internal phloem of stem. Immunoelectron microscopy of stem, root and leaf sections further localized SaPIN2a to the CC and predominantly to the SE, particularly the parietal cytoplasm adjacent to the cell wall, the lumen and the sieve-area pores. These results suggest that, other than a possible role in plant defense, SaPIN2a could be involved in regulating proteolysis in the SE.

Abbreviations: CC, companion cells; PIN2, proteinase inhibitor II; SE, sieve elements

Introduction

Proteinase inhibitor II (PIN2), a serine proteinase inhibitor with trypsin and chymotrypsin inhibitory activities (Bryant *et al.*, 1976), occurs in many Solanaceae plants including tomato (Gustafson and Ryan, 1976), potato (Bryant *et al.*, 1976) and tobacco (Pearce *et al.*, 1993). PIN2 proteins could serve an endogenous role in preventing uncontrolled proteolysis and/or a function in protecting against foreign proteolytic enzymes of pest or pathogen (Ryan, 1989; Brzin and Kidric, 1995). Observations of their wound-inducible expression (Pena-Cortes *et al.*, 1988; Pearce *et al.*, 1993) have led to investigations focusing on their role in plant protection against insects (Johnson *et al.*, 1989; Duan *et al.*, 1996; Klopfenstein *et al.*, 1997). Nevertheless, reports on their developmental regulation and their tissue-specific accumulation (Rosahl *et al.*, 1986; Sanchez-Serrano *et al.*, 1986; Hendriks *et al.*, 1991; Pena-Cortes *et al.*, 1991; Lorberth *et al.*, 1992) suggest that they have endogenous functions. Recently, a

The nucleotide sequence data reported will appear in the EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AF174381 (*SaPIN2a*) and AF209709 (*SaPIN2b*).

different class of plant proteinase inhibitor protein, the cysteine proteinase inhibitor, was designated a novel role in modulating programmed cell death in soybean (Solomon *et al.*, 1999).

One of the aims of our laboratory is to enhance pest/pathogen protection in transgenic crops by the expression of plant defense proteins. To this end, we have cloned cDNAs encoding PIN2 from *Solanum americanum*, a weed belonging to the Solanaceae family which is a rich source of proteinase inhibitors (Brzin and Kidric, 1995). From an evolutionary viewpoint, this weed would have evolved to resist insects endemic to this region and evokes our interest in cloning its cDNA encoding PIN2. Here, we report the differential expression of two cDNAs encoding PIN2 from *S. americanum*, one of which shows abundant expression in the phloem, suggesting a novel endogenous role for PIN2 in phloem development/function.

Materials and methods

Plant materials and wounding treatment

American black nightshade (*Solanum americanum* Mill.) growing in the University of Hong Kong was identified according to the criteria of Schilling *et al.* (1992) by Dr R.T. Corlett, Department of Ecology and Biodiversity, University of Hong Kong. *S. americanum* plants were grown in soil in the greenhouse under natural light. Leaves were wounded with scissors as described by Duan *et al.* (1996). The sampled plant tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use.

Construction and screening of a S. americanum *cDNA library*

Total RNA was extracted (Nagy *et al.*, 1988) from *S. americanum* wounded leaves for a cDNA library construction by means of the Great Length cDNA Synthesis Kit (Clontech), the Predigested λZAP II/*Eco*RI/CIAP Cloning Kit (Stratagene) and Gigapack II Gold Packaging Extract (Stratagene) following the manufacturers' instructions. The cDNA library was screened by *in situ* plaque hybridization with a random-primed ^{32}P-labeled probe of tomato *PIN2* cDNA (Graham *et al.*, 1985).

DNA fragments containing the sequences of interest were sequenced with the T7 Sequenase Version 2.0 DNA Sequencing Kit (Amersham). The DNA sequence data were analyzed with Wisconsin Package Version 10.0 of the Genetics Computer Group.

Southern blot analysis

DNA (20 μg), isolated (Dellaporta *et al.*, 1983) from *S. americanum* leaves, was digested with restriction endonucleases, separated by electrophoresis in 0.8% agarose gel and blotted onto Hybond-N membrane (Amersham) according to Sambrook *et al.* (1989). The blot was pre-hybridized in 30% deionized formamide, 6× SSC, 5× Denhardt's solution, 1% SDS, 500 μg/ml denatured, sonicated salmon sperm DNA at 42 °C for 4 h. The random-primed ^{32}P-labeled *SaPIN2a* cDNA probe was added and hybridized at 42 °C overnight. The blot was washed in 0.1× SSC, 0.1% SDS at room temperature.

In vitro *transcription/translation*

The TNT T3 Coupled Wheat Germ Extract System (Promega) was used for *in vitro* transcription/translation. L-[^{35}S]cysteine (600 Ci/mmol at 11 mCi/ml, NEN Life Science) was used for labeling the expressed protein. For co-translational processing of translation products, 1.5 μl of canine pancreatic microsomal membranes was included in the 25 μl transcription/translation reaction. Of the translation product 2 μl was subjected to 12% SDS-PAGE (Laemmli, 1970). The gel was dried and visualized by autoradiography.

Northern blot analysis

Total RNA (20 μg), extracted (Nagy *et al.*, 1988) from *S. americanum* tissues, was denatured at 50 °C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and blotted onto Hybond-N membrane. Blots were pre-hybridized in 50% deionized formamide, 1× Denhardt's solution, 6× SSPE, 0.1% SDS, 100 μg/ml denatured, sonicated salmon sperm DNA and 10% dextran sulfate at 42 °C for 12 h. Either the random-primed ^{32}P-labeled *SaPIN2a* cDNA probe or the *SaPIN2b* cDNA probe was added to the blot for hybridization at 42 °C overnight. The blot was washed at 65 °C in 0.1× SSC, 0.1% SDS. The hot SDS procedure (Amersham) was adopted for stripping blots. The RNA blots were stained with methylene blue (Sambrook *et al.*, 1989).

Western blot analysis

A synthetic peptide (GESDPRNPKDC) corresponding to amino acids 77–87 of SaPIN2a (Figure 1a) was used for raising antibodies in rabbit. The peptide, coupled to Keyhole Limpet Hemocyanin (KLH), was used for immunization. Antibodies were purified using Protein A-Sepharose CL-4B (Pharmacia) affinity column and SaPIN2a peptide-Sepharose 4B (Pharmacia) immunoaffinity column. Total plant protein was extracted according to the procedure of Wu *et al.* (1997). SaPIN2a was partially purified by KCl extraction, heat treatment and ammonium sulfate precipitation following the method used for tomato PIN2 purification (Gustafson and Ryan, 1976). Protein concentration was determined by the method of Bradford (1976). *S. americanum* total protein was separated by SDS-PAGE (Laemmli, 1970) with 15% w/v acrylamide gels and blotted onto Hybond-C membrane (Amersham) for western blot analysis (Sambrook *et al.*, 1989) with affinity-purified antibodies against SaPIN2a at a 1:10 000 (v/v) dilution. The amplified alkaline phosphatase goat anti-rabbit immuno-blot assay kit (Bio-Rad) was used to detect cross-reacting bands.

In situ *hybridization analysis*

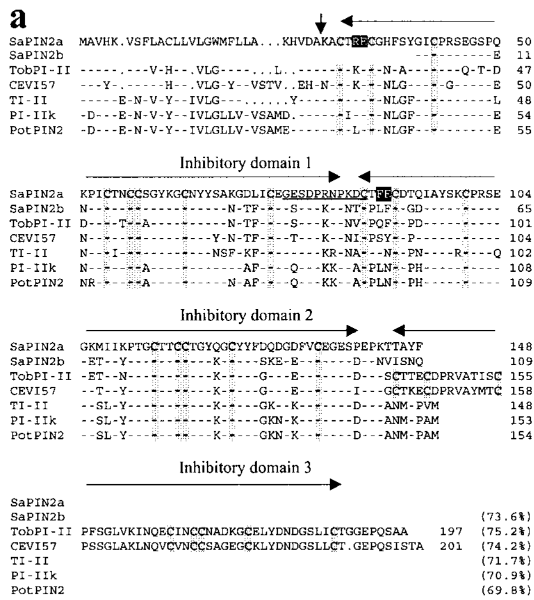
In situ hybridization studies were carried out following the procedures of Cox and Goldberg (1988). The 0.55 kb *SaPIN2a* cDNA, cloned in pBluescript SK (Stratagene), was cleaved with either *Bam*HI or *Kpn*I to generate antisense and sense RNA probes that were synthesized *in vitro* with T7 or T3 RNA polymerase, respectively. The antisense and sense RNA probes were hybridized with 10 μm stem sections overnight at 42 °C. Sections were washed and digoxigenin-labeled RNA probes were detected by an alkaline phosphatase-linked immunoassay (Boehringer Mannheim). This assay uses a color reaction with the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NBT) which produces a purple precipitate.

Immunohistochemical localization of SaPIN2a using light microscopy

S. americanum stems were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 16 h. The fixed tissues were dehydrated through a series of ethanol solutions (30%, 50%, 70%, 80%, 90%, 100%, 100%) for 30 min each, infiltrated with a xylene/Paraplast (Oxford) mixture and then embedded in Paraplast. Transverse 10-μm thick sections were cut on a Leica microtome (Jung RM 2035) and mounted on poly-L-lysine-coated slides. Slides were dried overnight and incubated in xylene to remove the Paraplast from the sections. The sections were rehydrated by a series of ethanol solutions (100%, 100%, 90%, 80%, 70%, 50%, 30%) and washed twice with water. The sections were then incubated in blocking solution (0.1% saponin, 1% BSA, 2% goat serum and 0.3 mM PMSF in PBS) for 1.5 h at room temperature and washed with blocking solution. Subsequently, they were incubated with antibodies against SaPIN2a, diluted 1:100 (v/v) in blocking solution, at 4 °C overnight, and subsequently with the secondary antibody, biotinylated alkaline phosphatase-conjugated goat anti-rabbit antibody (BioRad) diluted 1:1500 (v/v) in TTBS, for 2 h at room temperature. The alkaline phosphatase reaction was carried out as instructed by the supplier (BioRad). The slides were mounted with GelTol aqueous mounting medium (Immunon), examined and photographed under a Leica DMRXA microscope. Controls included the replacement of rabbit anti-SaPIN2a antibodies with either pre-immune rabbit serum or blocking solution.

Immunogold localization of SaPIN2a by transmission electron microscopy

Sample preparation and immunodetection were carried out according to the procedures previously described by Chye *et al.* (1999) with modifications as described. Ultra-thin sections (70–90 nm) of roots, stems and leaves were cut and mounted on nickel grids (150 or 200 mesh; Sigma). Grids were incubated in a blocking solution consisting of 1% fish skin gelatin and 1% BSA in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 30 min and subsequently in anti-SaPIN2a antibody solution at 1:5 (v/v), dilution in blocking solution, for 1 h at room temperature. After three washes with TTBS, each for 5 min, the grids were incubated in 10 nm gold-conjugated goat anti-rabbit IgG secondary antibody (Sigma), diluted 1:20 with blocking solution, for 40 min. Grids were rinsed three times in TTBS followed by two rinses in distilled water. After staining with 2% uranyl acetate for 15 min at room temperature, the grids were visualized and photographed under a Jeol 100 SX electron microscope operating at 80 kV. Controls included the replacement of rabbit anti-SaPIN2a antibodies with either pre-immune rabbit serum or blocking solution.



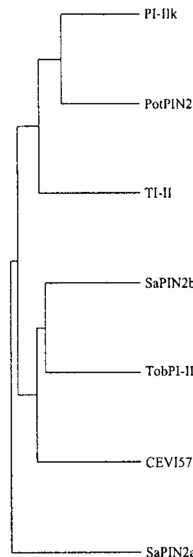


Figure 1. Analysis of SaPIN2a and SaPIN2b. a. Comparison of PIN2 proteins from *S. americanum* (SaPIN2a and SaPIN2b), tobacco (TobPI-II, Balandin *et al.*, 1995), tomato (TI-II, Graham *et al.*, 1985; CEVI57, Gadea *et al.*, 1996) and potato (PotPIN2, Sanchez-Serrano *et al.*, 1986; PI-IIk, Thornburg *et al.*, 1987). Dotted gaps are introduced to optimize alignment. Dashes denote positions of identity. Percent identities to SaPIN2a are shown at the end of each sequence. Horizontal arrows indicate inhibitory domains (Gadea *et al.*, 1996), shaded boxes indicate conserved cysteines and black boxes indicate putative reactive sites (Graham *et al.*, 1985) in SaPIN2a. The predicted signal peptide cleavage site is marked with a vertical arrow. The peptide (G^{77}-C^{87}) in SaPIN2a used for generating anti-peptide antibodies is underlined. b. Dendrogram plotted by means of the PileUp program of the Wisconsin Package Version 10.0, based on the amino acid sequences alignment shown in a.

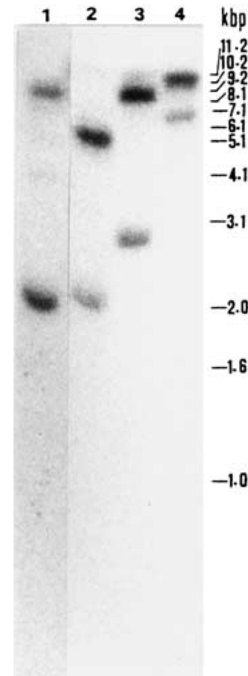


Figure 2. Southern blot analysis. *S. americanum* DNA (20 μg) digested with *Acc*I (lane 1), *Hind*II (lane 2), *Hind*III (lane 3) or *Xba*I (lane 4) was separated by agarose gel electrophoresis, blotted onto Hybond-N membrane and hybridized to ^{32}P-labeled *SaPIN2a* cDNA.

Results

Isolation and characterization of S. americanum *cDNAs encoding PIN2*

Two cDNAs, designated *SaPIN2a* and *SaPIN2b*, were isolated by screening a *S. americanum* cDNA library prepared from wounded leaves using a tomato *PIN2* cDNA (Graham *et al.*, 1985) as a heterologous hybridization probe. The 529 bp *SaPIN2a* cDNA (GenBank accession number AF174381) consists of 4 bp 5′-untranslated region, 444 bp coding region and 81 bp 3′-untranslated region, while the 532 bp *SaPIN2b* cDNA (GenBank accession number AF209709) is incomplete at the 5′ end, with only 328 bp deduced coding region and 204 bp 3′-untranslated region. These two cDNAs share 76.5% nucleotide sequence identity and 73.6% deduced amino acid sequence identity (Figure 1a). A dendrogram (Figure 1b) shows SaPIN2b is closely related to previously characterized tobacco PI-II (TobPI-II; Balandin *et al.*, 1995) and tomato CEVI57 (Gadea *et al.*, 1996), while SaPIN2a occupies a well-resolved branch separate from others, suggesting that it could be functionally distinct. Southern blot

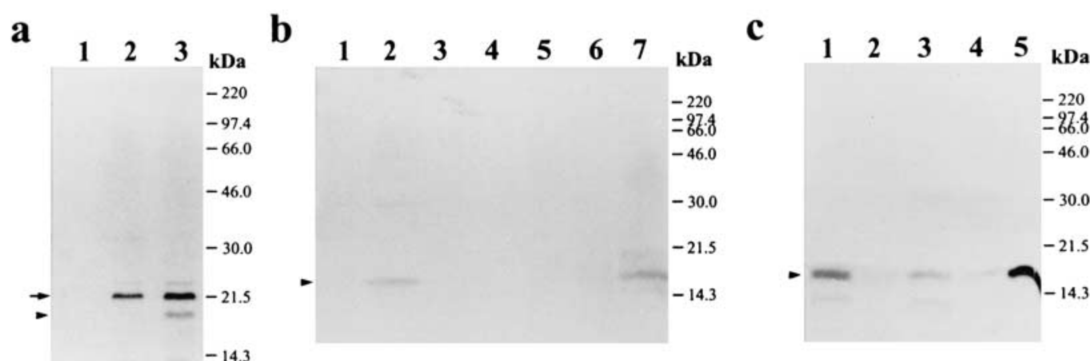


Figure 3. SaPIN2a expression *in vitro* and in *S. americanum*. a. *In vitro* transcription/translation products analyzed on 12% SDS-PAGE. Lane 1, control reaction with vector pBluescript II SK; lane 2, reaction with plasmid pSa2, a pBluescript II SK-derivative containing the full-length *SaPIN2a* cDNA; lane 3, reaction with pSa2 in the presence of canine pancreatic microsome membranes. Arrow indicates the 22 kDa SaPIN2a precursor and arrowhead indicates the 19.4 kDa mature protein. Molecular mass standards denoted on right. b. Western blot analysis of total proteins from various organs of mature *S. americanum* plants. Total protein samples (20 μg) were separated by 15% SDS-PAGE. The gel was blotted onto Hybond-C membrane (Amersham) and the blot cross-reacted with antibodies against the SaPIN2a peptide. Arrowhead indicates the 16.7 kDa SaPIN2a cross-reacting bands. Lane 1, roots; lane 2, stems; lane 3, developing leaves; lane 4, senescing leaves; lane 5, mature flowers; lane 6, developing green fruits; lane 7, ripened purple fruits. c. Western blot analysis of total leaf and stem proteins. Total proteins (60 μg) from *S. americanum* stems (lane 1), leaves without midribs (lane 2), leaf midribs (lane 3), leaves (lane 4) and 18 μg of partially purified SaPIN2a from stem (lane 5). Arrowhead indicates the 16.7 kDa SaPIN2a cross-reacting bands.

analysis (Figure 2) of *S. americanum* DNA with a ^{32}P-labeled *SaPIN2a* cDNA probe detected two hybridizing bands in the *Acc*I (lane 1), *Hin*dII (lane 2), *Hin*dIII (lane 3) and *Xba*I (lane 4) digests confirming two genes encode PIN2 in *S. americanum*, as these enzymes are not known to cleave the cDNAs. Small multigene families have been reported to encode PIN2 in haploid (Rosahl *et al.*, 1986) and in tetraploid (Stiekema *et al.*, 1988) potato.

SaPIN2a consists of 148 amino acids (M_r 16 300) with a putative trypsin-reactive site (R^{32}-E^{33}) within inhibitory domain 1 and a putative chymotrypsin-reactive site (F^{89}-E^{90}) within inhibitory domain 2 (Figure 1a). A comparison of SaPIN2a and SaPIN2b with homologues from potato, tobacco and tomato shows conservation of eight cysteines within each inhibitory domain (Figure 1a). Potato chymotrypsin inhibitor-1 has similar cysteine residues which form four disulfide bridges (Greenblatt *et al.*, 1989). SaPIN2a and SaPIN2b structurally resemble tomato TI-II (Graham *et al.*, 1985), potato proteinase inhibitor IIk (PI-IIk; Thornburg *et al.*, 1987) and potato PIN2 (PotPIN2; Sanchez-Serrano *et al.*, 1986), by the presence of two inhibitory domains (Figure 1a). Despite having higher identities to SaPIN2a, both tobacco PI-II (TobPI-II; Balandin *et al.*, 1995) and tomato CEVI57 (Gadea *et al.*, 1996) have a third inhibitory domain (Figure 1a). Here, a putative trypsin reactive site (K^{147}-E^{148}) resides in CEVI57 (Gadea *et al.*, 1996) while in TobPI-II, there lies a putative active site (T^{144}-E^{145}) not previously identified in plant PIN2 proteins but which occurs in avian ovomucoid serine proteinase inhibitors (Balandin *et al.*, 1995). In addition to these two- and three-domain PIN2 proteins, *Nicotiana alata* NA-PI-II and NaPI IV contain six and four inhibitory domains, respectively (Atkinson *et al.*, 1993; Miller *et al.*, 2000), while *Nicotiana glutinosa* NGPI-1 and NGPI-2 contain eight and six domains, respectively (Choi *et al.*, 2000).

A putative signal peptide (von Heijne, 1983) with a predicted cleavage site after A^{27} was identified at the N-terminus using the SignalP program (Nielsen *et al.*, 1997) (Figure 1a). By SDS-PAGE analysis, the *in vitro* transcription/translation product of the *SaPIN2a* cDNA is a precursor protein with an apparent molecular mass of 22 kDa (Figure 3a, lane 2). In the presence of canine pancreatic microsome membranes, the signal peptide (calculated mass 3 kDa) was cleaved and a smaller mature protein (estimated 19.4 kDa) was also seen (Figure 3a, lane 3).

Differential expression of SaPIN2a *and* SaPIN2b

Like in tomato *TI-II* (Graham *et al.*, 1985) and potato *PotPIN2* (Sanchez-Serrano *et al.*, 1986), northern blot analysis showed wound-inducible expression of *SaPIN2a* and *SaPIN2b* in leaves (Figure 4a). In a mature plant, *SaPIN2a* mRNA (Figure 4b, top panel) is more highly expressed in stems (lane 2) than roots (lane 1), mature flowers (lane 4) or developing green

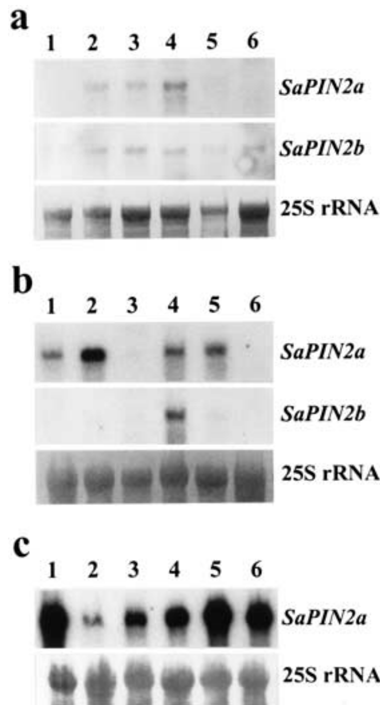


Figure 4. Northern blot analysis of *SaPIN2a* and *SaPIN2b* mRNA. The blots were probed with a ^{32}P-labeled *SaPIN2a* cDNA (a–c), stripped and reprobed with ^{32}P-labeled *SaPIN2b* cDNA (a, b). The 25S rRNA bands stained with methylene blue are at the bottom panel. Each lane contains 20 μg of total RNA extracted from: (a) leaves collected at 0 h (lane 1), 2 h (lane 2), 4 h (lane 3), 7 h (lane 4), 10 h (lane 5) and 24 h (lane 6) after wounding; (b) roots (lane 1), mature stems (lane 2), leaves (lane 3), mature flowers (lane 4), developing green fruits (lane 5) and seeds (lane 6); (c) developing green stems (lane 1) and senescing stems (lane 2) from mature plant, hypocotyls (lane 3) from 2-week old seedlings, developing stems from 3-week old (lane 4), 4-week old (lane 5) and 5-week old (lane 6) seedlings.

fruits (lane 5). Very faint expression and no expression were observed in leaves (lane 3) and seeds (lane 6), respectively. In contrast, *SaPIN2b* mRNA (Figure 4b, middle panel) is expressed in mature flowers (lane 4) but is lacking in roots (lane 1), stems (lane 2), leaves (lane 3), developing green fruits (lane 5) and seeds (lane 6).

To investigate the developmental regulation of *SaPIN2a* in stems, RNA was extracted from *S. americanum* stems at various stages of development. Young green stems (Figure 4c, lane 1) show higher *SaPIN2a* expression than senescing stems (Figure 4c, lane 2). In 2- to 5-week old seedlings (Figure 4c, lanes 3–6), *SaPIN2a* mRNA accumulates more in stems of 3- to 5-week-old seedlings (lane 4–6) than in hypocotyls (lane 3) of 2-week old seedlings. These results suggest some correlation between SaPIN2a expression and stem development.

Localization of SaPIN2a *mRNA and protein in phloem*

Since our study first reports stem-abundant PIN2 expression, we further investigated the distribution of *SaPIN2a* mRNA in stem by *in situ* hybridization analysis (Figure 5). Our results from light microscopy suggest that *SaPIN2a* mRNA is expressed in both internal (Figure 5a, c, e, g) and external (Figure 5a, f, h) phloem, particularly in companion cells (CC, indicated with arrows in Figure 5c, e–h) and in some sieve elements (SE, indicated with arrowheads in Figure 5c, e–h), likely the immature developing SE which are capable of transcription.

Immunolocalization of SaPIN2a was carried out using polyclonal antibodies raised against a peptide consisting of residues G^{77}-C^{87} in SaPIN2a (Figure 1a). The peptide was selected based on its antigenicity index as predicted according to Jameson and Wolf (1988); only seven of these eleven residues also occur in SaPIN2b (Figure 1a). Using these antibodies in western blot analysis of *S. americanum* protein, a strong SaPIN2a cross-reacting band with an apparent molecular mass of 16.7 kDa was detected in stems (Figure 3b, lane 2) and mature purple fruits (Figure 3b, lane 7). This value lies between the calculated mass of the mature protein (13.3 kDa) and that of the *in vitro* translated product after removal of signal peptide (Figure 3a, lane 3). The cross-reacting band could be enriched after partial purification of PIN2 protein from *S. americanum* stems following the procedure previously used in the purification of tomato PIN2 (Gustafson and Ryan, 1976); Figure 3c (lane 5) shows a stronger cross-reacting band in partially purified SaPIN2a than in unpurified stem protein (lane 1).

In a transverse section of stem, immunohistochemical analysis with the SaPIN2a-specific antibodies shows accumulation of SaPIN2a, as depicted by the purple stain, in external and internal phloem (Figure 6a), which did not occur in the control section stained with pre-immune serum (Figure 6b). By immunoelectron microscopy of young stem sections, with the SaPIN2a-specific antibodies, SaPIN2a was localized to CC and to both immature developing SE and mature enucleated SE, particularly to the parietal cytoplasm adjacent to the cell wall (Figure 7a, b). These results correspond with detection of *SaPIN2a* mRNA in these cells (Figure 5) and of SaPIN2a in external and internal phloem of stem (Fig-

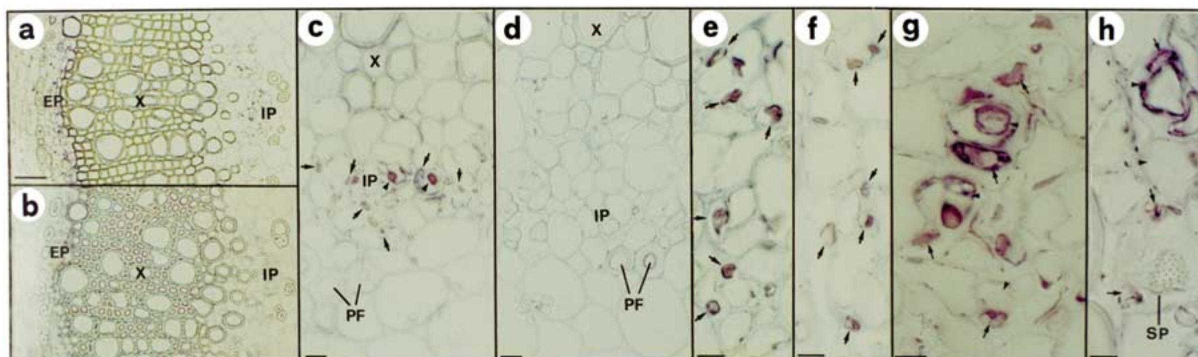


Figure 5. Localization of *SaPIN2a* mRNA in *S. americanum* stem by *in situ* hybridization analysis. Transverse sections of stem hybridized to either digoxigenin-labeled antisense *SaPIN2a* RNA probe (a, c, e–h) or sense *SaPIN2a* RNA probe (b, d). Arrows indicate companion cells. Arrowheads indicate sieve elements. EP, external phloem; IP, internal phloem; PF, phloem fiber; SP, sieve plate; X, xylem. a and b. Vascular bundles of stem. Bar = 50 μm. c and d. Portion of internal phloem and surrounding areas. Bar = 10 μm. e. Portion of internal phloem. Bar = 5 μm. f. Portion of external phloem. Bar = 5 μm. g. Higher magnification of internal phloem showing labelling in some SE. Bar = 5 μm. h. Higher magnification of external phloem showing labelling in some SE. Bar = 5 μm.

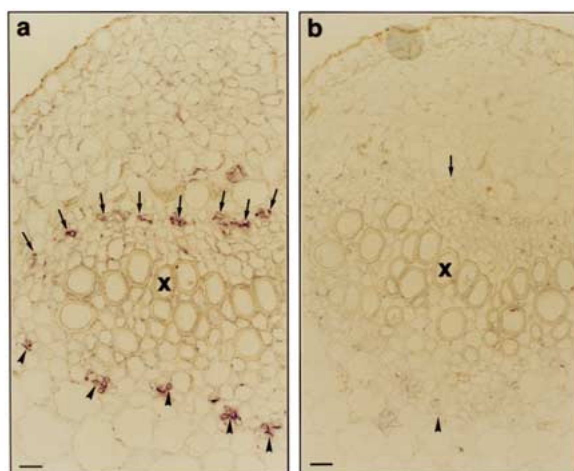


Figure 6. Immunolocalization of SaPIN2a in *S. americanum* stem by light microscopy. Transverse sections of stem stained with either affinity-purified SaPIN2a-specific antibodies (a) or pre-immune serum (b). Arrows indicate external phloem; Arrowheads indicate internal phloem. X, xylem. Bar = 20 μm.

ure 6). SaPIN2a was also immunolocalized to SE in hypocotyl (Figure 7d, e) and mature stem (Figure 7f, g), at the parietal cytoplasm adjacent to the cell wall of the SE (Figure 7d, f and g). Some SaPIN2a was detected in the SE lumen (Figure 7d and e). Also, SaPIN2a was localized to the zone of sieve-area pores (Figure 7d, e) that develop from plasmodesmata (Schulz, 1990) and which interconnect the protoplasts of contiguous SE (Esau, 1977).

Since the SE forms a major component of phloem, we investigated the expression of SaPIN2a in phloem from organs other than stem. SaPIN2a was again localized to the parietal cytoplasm adjacent to the cell wall of the SE in root (Figure 7h–j) and leaf (Figure 7m) and the SE lumen of root (Figure 7h and k) and leaf (Figure 7m). Controls with pre-immune serum did not show immunogold labeling (Figure 7c and l). Initially, we had detected only faint levels of *SaPIN2a* mRNA in leaves (Figure 4b, lane 3) and did not detect SaPIN2a in roots (Figure 3b, lane 1) and leaves (Figure 3b, lanes 3 and 4) when 20 μg of protein was used in western blot analysis as there is relatively lower abundance of phloem in them than in stem. Subsequently, western blot analysis using 60 μg of protein (Figure 3c) showed a stronger cross-reacting SaPIN2a band in midribs (Figure 3c, lane 3) than leaf lacking midribs (Figure 3c, lane 2) or total leaf protein (Figure 3c, lane 4) because midribs contain most phloem.

Discussion

PIN2 occurs in eggplant fruits (Richardson, 1979), potato tubers (Bryant *et al.*, 1976), wounded leaves and flowers of tobacco (Pearce *et al.*, 1993) and unripe fruits of wild tomato (Pearce *et al.*, 1988). In tomato, *PIN2* mRNA was found in wounded leaves (Graham *et al.*, 1986), auxin-induced roots (Taylor *et al.*, 1993), flowers (Pena-Cortes *et al.*, 1991), floral buds, shoot apices and root tips (Brandstadter *et al.*, 1996). Potato *PIN2* is expressed in wounded leaves (Pena-Cortes *et al.*, 1988), flowers (Pena-Cortes *et al.*, 1991) and tubers (Rosahl *et al.*, 1986; Sanchez-Serrano *et al.*, 1986; Stiekema *et al.*, 1988). *In situ*

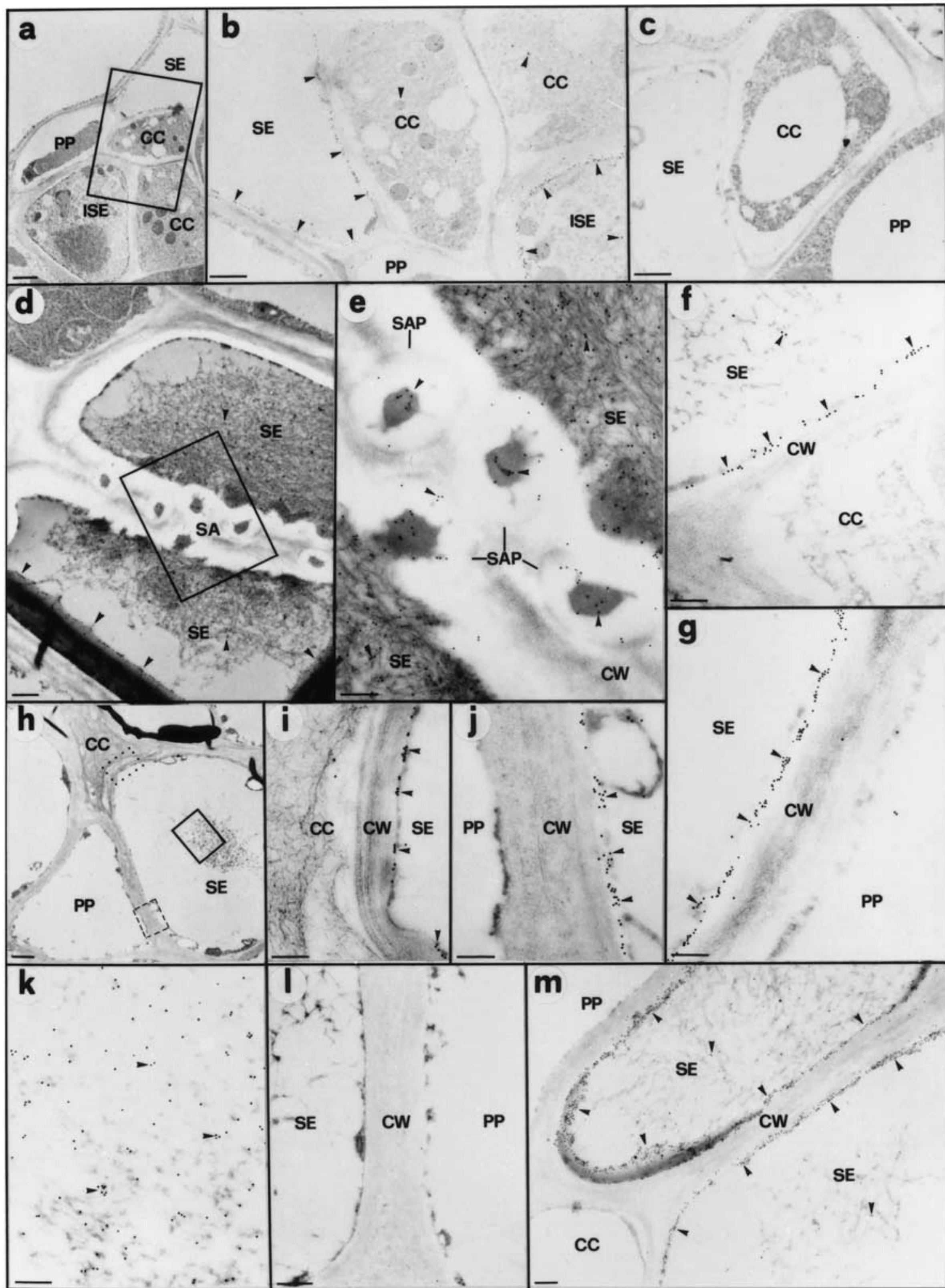

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Figure 7. Immunogold localization of SaPIN2a using transmission electron microscopy. Transverse sections of 3-week old stem (a–c), hypocotyl (d, e), mature stem (f, g), root (h–l) and leaf (m) were stained with affinity-purified SaPIN2a-specific antibodies or pre-immune serum from the same rabbit (c, l). Arrowheads indicate gold particles; CC, companion cell; CW, cell wall; ISE, imature sieve element; PP, phloem parenchyma; SA, sieve area; SAP, sieve-area pore; SE, sieve element. a. Transverse section of phloem in 3-week old stem. The box indicates the zone magnified in b. Bar = 1 μm. b. High-magnification image of the boxed area in a showing localization of SaPIN2a to both immature and mature SE and to the CC. Bar = 0.5 μm. c. Pre-immune control for b, replacement of anti-SaPIN2a with pre-immune serum. Bar = 0.5 μm. d. Oblique section through sieve area (SA) that links two adjacent SE of 3-week old hypocotyl. The box indicates the area magnified in e. Bar = 0.5 μm. e. High-magnification image of the boxed area shown in d showing localization of SaPIN2a in the zone of sieve-area pores (SAP) and in the SE lumen. Bar = 0.2 μm. f and g. Transverse sections of mature stem. Localization of SaPIN2a to the parietal cytoplasm adjacent to the cell wall of the SE. Bar = 0.2 μm. h. Transverse section of phloem in root. The region within the dotted box is magnified in i. The region within the dashed box is magnified in j. The box in the SE lumen is magnified in k. Bar = 1 μm. i. High-magnification image within the dotted box of h. Bar = 0.2 μm. j. High-magnification image within the dashed box of h. Bar = 0.2 μm. k. High-magnification image within box in SE lumen of h. Bar = 0.2 μm. l. Control for h, replacement of anti-SaPIN2a with pre-immune serum. Bar = 0.3 μm. m. Transverse section of phloem in midrib of leaf. Bar = 0.3 μm.

hybridization experiments demonstrated that *N. alata NA-PI-II* is specifically expressed in stigma (Atkinson *et al.*, 1993). Here, we show that *SaPIN2a* and *SaPIN2b* are differentially expressed and that localization of *SaPIN2a* mRNA and protein in phloem suggests it could have a novel role in controlling proteolysis in these cells. Phylogenetically, SaPIN2a is less related to other previously reported PIN2 proteins. In contrast, SaPIN2b appears more closely related to these reported homologues and by its floral-abundant expression, it resembles tomato PIN2.

The phloem, particularly in stem, is not a common target of chewing insects, notably caterpillars to which protection is conferred by heterologous PIN2 expression in transgenic plants, so why is SaPIN2a expressed in phloem? SaPIN2a is only one of several plant defense proteins identified in phloem. Possibly, from a defense perspective, expression here could enable their quick translocation to other parts of the plant. They include *Pinus sabiniana* lectin-like protein (Schulz *et al.*, 1989), pumpkin phloem lectin PP2 (Smith *et al.*, 1987; Bostwick *et al.*, 1992; Dannenhoffer *et al.*, 1997), winged bean Kunitz proteinase inhibitor (Habu *et al.*, 1996), *Ricinus communis* cystatin (Balachandran *et al.*, 1997), pumpkin phloem filament protein PP1 that contains structural motifs common to cysteine proteinase inhibitors (Clark *et al.*, 1997), and pumpkin fruit trypsin inhibitors (PFTIs, Dannenhoffer *et al.*, 2001). Phloem lectins have been proposed to protect the phloem from pathogen invasion or to anchor P-protein filaments to glycoproteins/glycolipids on the plasma membranes of the SE (Sabnis and Hart, 1978). The developmental-regulated expression of PP1 (Clark *et al.*, 1997) together with PP2 (Dannenhoffer *et al.*, 1997), in the vascular tissue of pumpkin hypocotyl, suggests that they interact in P-filament formation (Clark *et al.*, 1997). The phloem-specific expression of PFTIs and their translocation within the transport phloem indicate a possible role in regulating protein turnover during SE differentiation (Dannenhoffer *et al.*, 2001).

Our observations in this study suggest the role of SaPIN2a may not be limited to plant defense and it could well have endogenous roles. *SaPIN2a* expression in stems, from seedling to maturity, and the localization of *SaPIN2a* mRNA and SaPIN2a to the CC and SE, imply it could also play a role regulating proteolysis in phloem development/function. The high accumulation of SaPIN2a in the SE, as detected by immunoelectron microscopy, suggests it could modulate SE development by regulating the activity of endogenous proteinases involved in the selective autophagy of the protoplasmic contents of SE, a process resembling programmed cell death (Esau, 1977; Evert, 1990; Schulz, 1998; Oparka and Turgeon, 1999). This incomplete autolysis process culminating in the degeneration of the nucleus and disappearance of the tonoplast, dictysomes, Golgi bodies, cytoplasmic ribosomes, and microtubules (Esau, 1977; Evert, 1990; Oparka and Turgeon, 1999) must involve proteinases. The immunolocalization of SaPIN2a to the parietal cytoplasm adjacent to the cell wall of the SE implies it protects proteins essential for SE integrity and function, particularly during the selective autophagy process, after which the functional components are retained. Unlike proteinases related to seed germination and seedling development (Ryan and Walker-Simmons, 1981), proteinases involved in the proteolysis of the protoplasmic contents of SE have received little attention. To date, no specific proteinase has been identified responsible for this proteolysis.

We detected *SaPIN2a* mRNA in some SE (likely the immature SE) of external and internal phloem and, consistently, SaPIN2a in immature and mature SE by immunoelectron microscopy. Since mature SE are incapable of transcription and translation (Oparka

and Turgeon, 1999; Thompson and Schulz, 1999), SaPIN2a in mature SE may have been synthesized by immature SE. Alternatively, as *PP1* (Clark *et al.*, 1997) and *PP2* (Bostwick *et al.*, 1992; Dannenhoffer *et al.*, 1997) mRNAs are CC-specific and their proteins, confined to CC and SE, are likely derived from CC (Smith *et al.*, 1987; Clark *et al.*, 1997; Dannenhoffer *et al.*, 1997), SaPIN2a may have been transported from CC to mature SE. Transport from CC to SE is consistent with the observation that *SaPIN2a* mRNA is more abundant in CC than in SE, while the reverse is true of SaPIN2a. CC are known to maintain adjacent mature SE by the export of macromolecules through the plasmodesmata (Oparka and Turgeon, 1999). Indeed, mRNAs encoding PP1, PP2, SUT1, thioredoxin h and CmPP16, and their proteins show differential distribution in CC and SE (reviewed in Crawford and Zambryski, 1999). Like *SaPIN2a* mRNA, *CmPP16* mRNA (Xoconostle-Cazares *et al.*, 1999) accumulates predominantly in CC and immature SE. The distribution of SaPIN2a within the parietal cytoplasm adjacent to the cell wall in the SE is reminiscent of that for SUT1 (Kuhn *et al.*, 1997), CmPP16 (Xoconostle-Cazares *et al.*, 1999) and PFTIs (Dannenhoffer *et al.*, 2001). The immunolocalization of SaPIN2a to the sieve-area pores and to the SE lumen further implies that SaPIN2a could be translocated between protoplasts of contiguous SE. Our observations of *SaPIN2a* induction upon wounding, which causes exudation of phloem sap due to positive pressure (Esau, 1977) is consistent with increased transcription to balance this loss. Further investigations using microinjections with fluorescent-tagged SaPIN2a and transgenic *S. americanum* plants expressing antisense *SaPIN2a* RNA would be required to further elucidate the role of SaPIN2a in the phloem.

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