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ORIGINAL ARTICLE

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The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling

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Abstract The systemin precursor, prosystemin, has been previously shown to be sequestered in vascular bundles of tomato (*Lycopersicon esculentum* Mill.) plants, but its subcellular compartmentalization and association with a specific cell type has not been established. We present in situ hybridization and immunocytochemical evidence at the light, confocal, and transmission electron microscopy levels that wound-induced and methyl jasmonate-induced prosystemin mRNA and protein are exclusively found in vascular phloem parenchyma cells of minor veins and midribs of leaves, and in the bicollateral phloem bundles of petioles and stems of tomato. Prosystemin protein was also found constitutively in parenchyma cells of various floral organs, including sepals, petals and anthers. At the subcellular level, prosystemin was found compartmentalized in the cytosol and the nucleus of vascular parenchyma cells. The cumulative data indicate that vascular phloem parenchyma cells are the sites for the synthesis and processing of prosystemin as a first line of defense signaling in response to herbivore and pathogen attacks.

Keywords Defense response · *Lycopersicon* · Phloem transport · Systemic wound signaling · Systemin

Abbreviations *IgG*: immunoglobulin · *TEM*: transmission electron microscope

Introduction

Prosystemin is the 200-amino-acid precursor of systemin, an 18-amino-acid polypeptide hormone that plays a central role in the systemic wound induction of defensive proteins in several members of the Solanaceae (Ryan 2000). Prosystemin is a non-glycosylated, highly charged protein, synthesized without a putative signal sequence, suggesting that it is synthesized on free ribosomes in the cytoplasm. Low constitutive levels of prosystemin mRNA are found in leaves, but rapidly increase upon wounding. Previous studies using tissue printing and the expression of a prosystemin–β-glucuronidase reporter gene have shown that in response to wounding, prosystemin protein accumulates rapidly in vascular bundles of tomato plants (Jacinto et al. 1997), but the specific cell type(s) in which prosystemin is synthesized and its subcellular location were not known.

The movement of systemin through the plants from wound sites has been associated with phloem (Ryan, 2000), a complex system for long-distance transport of both small molecules and macro-molecules that are essential for developmental and physiological processes (Thompson and Schulz 1999; Haywood et al. 2002). The movement of ^{14}C-labeled systemin is blocked by *p*-chloromercuribenzene sulfonic acid (PCMBS), a known inhibitor of phloem loading of sucrose in plants, including tomatoes (Nárvaez-Vásquez et al. 1994, 1995). Tomato plants transformed with an antisense prosystemin gene are incapable of signaling distal leaves in response to wounding (Ryan 2000). Conversely, transgenic tomato plants that constitutively overexpress the prosystemin gene in the sense orientation cause the constitutive expression of defense genes throughout the plants (Ryan 2000). Grafting wild-type plants on transgenic rootstalks that constitutively express the prosystemin sense gene causes the wild-type scions to behave as if they are wounded, and the leaves accumulate large amounts of defense proteins (Ryan 2000). This suggests that systemin may be an important component of the mobile signal. A combined role for both systemin and jasmonic acid has been proposed (Li et al. 2002; Ryan and Moura 2002; Lee and Howe 2003; Stenzel et al. 2003), as the two signals mutually amplify the systemic signaling process as they are translocated through the plant.

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In the study reported here, we use in situ hybridization and immunocytochemical techniques to seek the specific cells in which prosystemin mRNA and protein are synthesized and accumulate. The data define a major role for phloem parenchyma cells as the sites of synthesis and processing of prosystemin as part of the amplification of wound signaling.

Materials and methods

Plant material

Wild-type (*Lycopersicon esculentum* Mill. cv. Castlemart) and transgenic tomato plants overexpressing the prosystemin cDNA in the sense and antisense orientation (McGurl et al. 1992, 1994) were grown from seeds in a growth chamber with 18-h days (300 μmol photons m^{-2} s^{-1}) at 28 °C and 6-h nights at 18 °C. Potato (*Solanum tuberosum* L. cv. Desiree) plants were grown from tubers under greenhouse conditions.

Wounding and methyl jasmonate treatments

The lower leaf of 2-week-old tomato plants was wounded twice across the midvein of its terminal leaflet. Leaf tissues were collected for immuno-cytochemical analyses from undamaged tissues of wounded and unwounded leaves at times indicated in the text. Fourteen-day-old tomato plants, and detached leaves from 6- to 8-week-old potato plants, were exposed to methyl jasmonate vapors by applying 2 μl of absolute methyl jasmonate (Bedoukian Research, Danbury, CT, USA) to a cotton wick inside a Plexiglas box (Farmer et al. 1992). The plants or leaves were incubated for up to 24 h under constant light at 300 μmol photons m^{-2} s^{-1} at 28 °C.

Affinity purification of prosystemin antisera

Rabbit polyclonal antibodies and pre-immune sera were obtained using pure recombinant full-length prosystemin (Delano et al. 1999), and a truncated, biologically inactive form of prosystemin (Δ-prosystemin), which lacks the last C-term 22 amino acids that contain the 18-amino-acid sequence of systemin (Dombrowski et al. 1999). The anti-prosystemin antisera specifically cross-reacted with full-length prosystemin in ELISA (enzyme-linked immunosorbent assay) tests and immunoblot analyses of tomato plant extracts and tissue prints (Jacinto et al. 1997; Delano et al. 1999; Dombrowski et al. 1999). However, to minimize the appearance of artifacts in the immunocytochemical analyses due to non-specific labeling (Tavares et al. 2002), specific immunoglobulins (IgGs) from each antiserum were purified by affinity chromatography as explained below.

To purify the IgGs specific for prosystemin by affinity chromatography, pure prosystemin protein was first cross-linked to magnetic beads (Dynabeads M-280 Tosylactivated; Dynal Inc.), following the manufacturer's recommendations. For the cross-linking, about 300 μg of HPLC-purified recombinant prosystemin (Delano et al. 1999; Dombrowski et al. 1999) was mixed with 10^9 beads in 0.5 ml of 0.1 M phosphate buffer and incubated in a rotary lab quake at 37 °C for 24 h. The beads were washed twice with phosphate-buffered saline (PBS, pH 7.8) containing 0.1% (w/v) BSA at room temperature and blocked with 0.2 M Tris (pH 8.5), with 0.1% (w/v) BSA for 4 h at 37 °C. The beads were washed twice with PBS–BSA at room temperature, and finally stored at 4 °C in the PBS–BSA solution.

Immunoglobulins of the prosystemin antisera and their corresponding pre-immune sera were affinity-purified using a HiTrap Protein A–Sepharose column (Pharmacia Biotech). To affinity-purify specific anti-prosystemin IgGs, the cross-linked Dynabeads prepared as described above were incubated for 2 h at room temperature in a blocking solution consisting of 10 mM Tris, 500 mM NaCl, 0.05% (v/v) polyoxyethylenesorbitan monolaureate (Tween 20), pH 7.2 (TBST-1 buffer), containing 0.5% (w/v) gelatin and 0.02% (w/v) NaN_3 and washed 3 times with TBST-1. Protein A-affinity-purified IgGs (0.2 ml) diluted with 0.3 ml of the blocking solution were applied to the beads and incubated for 24 h in a rotary lab quake at room temperature. Thereafter, the beads were pelleted and rinsed 3 times with TBST-1, and once with 0.01 M phosphate buffer, to wash out unbound IgGs. IgGs were eluted with 200 μl of 50 mM glycine–HCl (pH 2.5). The recovered solutions of specific IgGs were neutralized with the addition of a few microliters of 1 M Tris–HCl (pH 9), and stored at −20 °C until they were employed for the immunolocalization studies of prosystemin. The titers and specificities of the affinity-purified IgGs were confirmed by ELISA and immunoblot analyses against pure prosystemin.

In situ hybridization

Tissue samples were dissected with an scalpel from leaves (including minor veins and the midrib), petioles and stems of 2-week-old tomato plants, and fixed in 10% (v/v) formalin, 5% (v/v) glacial acetic acid, 50% (v/v) ethanol, at 4 °C overnight. Tissues were then sequentially dehydrated in an ethanolic series in a stepwise manner, and the tissue was slowly infiltrated with paraffin over a period of a week at 62 °C. Tissue samples were cut into 10-μm sections and mounted on silane-coated slides. The sections were deparaffinized with xylene, rehydrated and air-dried, treated with 1 μg ml^{-1} proteinase K in TE buffer (50 mM Tris–HCl, 5 mM EDTA, pH 7.4) for 30 min at 37 °C, washed twice with TE buffer at room temperature, and then incubated for 2 h at room temperature in a prehybridization solution consisting of 50% (v/v) formamide, 300 mM NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1× Denhardt's solution [0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) BSA], 10% (v/v) dextran sulfate, 100 mM DTT, 500 μg ml^{-1} denatured salmon sperm DNA and 150 μg ml^{-1} yeast tRNA. Digoxigenin-labeled antisense and sense riboprobes were synthesized using an in vitro transcription kit (Roche) from pBluescript SK^- template containing the prosystemin cDNA insert (McGurl et al. 1992). The plasmid was linearized with either *Xho*I or *Eco*RI, and digoxigenin-11-dUTP was incorporated using either T7 or T3 polymerase, respectively. RNA transcripts were hydrolyzed to about 150 bases with 0.2 M $NaHCO_3/N_2CO_3$ (pH 10.2) for 35 min at 60 °C. Each section was incubated in 50 μl of the prehybridization solution containing 1 ng μl^{-1} of riboprobe in a humid chamber overnight at 37 °C. After hybridization, slides were washed sequentially with 4× SSC (1× SSC: 300 mM NaCl, 30 mM sodium citrate, pH 7.2), 2× SSC, 1× SSC and 0.1× SSC for 30 min each at room temperature and quickly rinsed with water. Hybridization of the riboprobes was detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche) and visualized by color development according to the manufacturer's instructions. After development, sections were air-dried, DPS mounting medium (EMC) and a coverslip added, and then analyzed and photographed with an Olympus BH2 light microscope.

Tissue preparation for immuno-cytochemistry

Tissue samples were obtained from leaves, petioles, and stems of 2-week-old tomato plants; as well as from leaves, petioles and flowers of 6- to 8-week-old tomato and potato plants. Tissue was immediately fixed in 2% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde in 50 mM 1,4-piperazine diethanesulfonic acid (Pipes; pH 7.2), and incubated overnight at 4 °C. Thereafter, tissues were washed three times with the Pipes buffer alone, dehydrated in an ethanolic series and embedded in L.R. White resin, as described previously (Narváez-Vásquez et al. 1993). Thick (0.5–1.0 μm) and

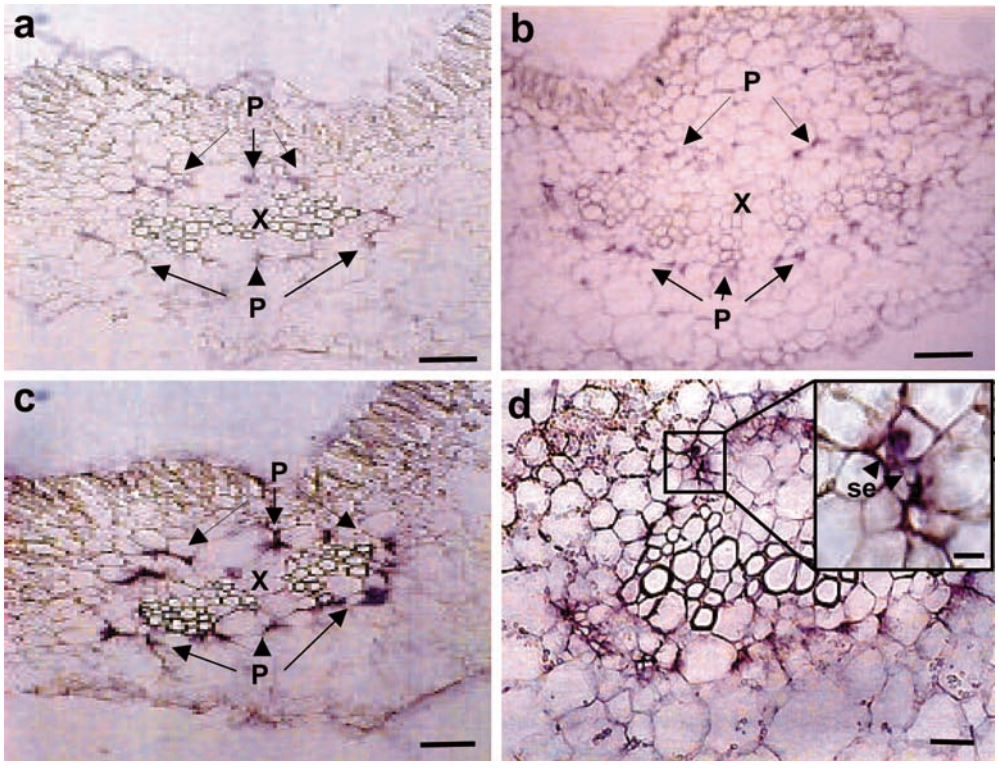


Fig. 1a–d Localization of prosystemin mRNA in vascular bundles of tomato (*Lycopersicon esculentum*) leaves. Sections were hybridized with digoxigenin-labeled prosystemin sense or anti-sense riboprobes. The probes were detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase (see Materials and methods). **a** Cross-section through the mid vein of a wounded leaf hybridized with a digoxigenin-labeled prosystemin mRNA sense probe, used as a negative control. **b** Section from an unwounded leaf, hybridized with the prosystemin antisense-labeled probe. **c** Wound-induced prosystemin mRNA accumulation in phloem bundles of a mid vein 6 h after wounding of the leaf. **d** Methyl jasmonate-induced prosystemin mRNA expression in phloem bundle cells of a mid vein, 6 h after treatment. Region within the *square* is shown at a higher magnification in the *inset*. *X* Xylem, *P* phloem, *se* sieve elements. Bars = 50 μm (**a–d**), 5 μm (inset in **d**)

ultra-thin (0.1 μm) sections were obtained using a diamond knife (Delaware Diamond Knives, Wilmington, DE, USA) and mounted respectively on gelatin-coated slides or 200-mesh nickel grids.

Immunocytochemical staining

Sections were bathed for 2 h in a blocking solution consisting of 10 mM Tris, 500 mM NaCl, 0.3% (v/v) Tween 20 (pH 7.2; TBST-2), containing 1% (w/v) BSA, 0.5% (w/v) polyvinylpolypyrrolidone (M_r 10,000), 0.5% (w/v) donkey serum, and 0.02% (w/v) NaN_3. Sections were incubated overnight with the anti-prosystemin IgGs diluted with blocking solution to a final concentration of 5–50 ng μl^{-1}. A comparable dilution of the corresponding pre-immune serum IgGs was always used as a negative control. Sections were washed 4 times with TBST-2, incubated 2 h in blocking solution containing a 1:20 dilution of 18-nm gold-labeled donkey anti-rabbit polyclonal antibodies (Jackson ImmunoResearch Labs), washed again 4 times with TBST-2 and 3 times with distilled water. Thick sections on glass slides were further incubated with a silver-enhancement solution (Ted Pella), for about 10–15 min. After several washes with distilled water, sections were dried and post-stained with 1% (w/v) aqueous safranin, and then DPS mounting medium and a coverslip were added. Ultra-thin sections were stained for 5 min in a 1:3 mixture of 1% (w/v) potassium permanganate and 1% (w/v) aqueous uranyl acetate, and examined with a transmission electron microscope (TEM; model JEM 1200EX; JEOL).

Confocal microscopy

Silver-enhanced sections on glass slides were analyzed by a two-channel method using reflection (silver grains) and transmission (section) imaging on a confocal microscope (Bio-Rad MRC-1024). Image processing was done using the Adobe Photoshop (version 5.5) and Microsoft PowerPoint computer programs.

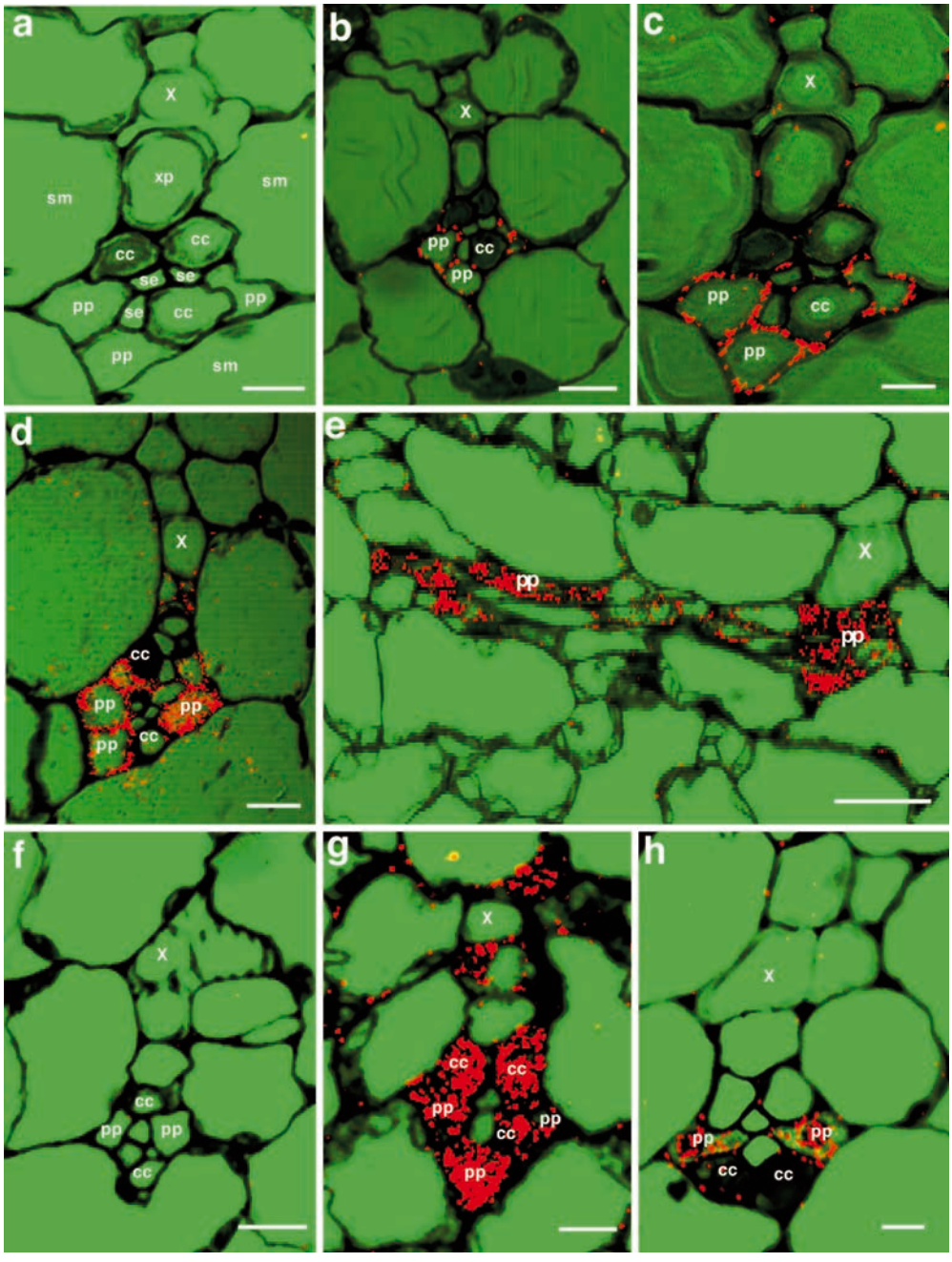
►

Fig. 2a–h Immunocytochemical localization of prosystemin in phloem parenchyma (*pp*) cells of leaf minor veins of tomato plants. Affinity-purified prosystemin immune serum (approx. 50 ng IgG μl^{-1}) and corresponding pre-immune serum negative control were applied to serial cross-sections and longitudinal sections of leaves. Silver-enhanced, immuno-gold-labeled prosystemin was visualized with a BioRad MRC 1024 laser scanning confocal microscope operated in transmitted/reflected mode. Localization of prosystemin in the vascular parenchyma cells is indicated by shades of *red*. Large *green* empty areas within the cells correspond to the vacuoles, and the darker areas to the cell walls and the cytoplasm. **a** Cross-section through a minor vein treated with pre-immune serum. **b** Specific localization of basal, constitutive levels of prosystemin in phloem parenchyma of an untreated plant. **c** Accumulation of prosystemin in phloem parenchyma 18 h after wounding of the leaf. **d** Methyl jasmonate-induced accumulation of prosystemin in phloem parenchyma 18 h after treatment of whole plants. **e** Longitudinal section through a leaf treated as in **d**. **f** Lack of the prosystemin signal in leaves of prosystemin antisense tomato plants. **g** Constitutive overexpression of prosystemin in all leaf cell types of prosystemin sense plants. **h** Methyl jasmonate-induced accumulation of prosystemin in phloem parenchyma of a potato (*Solanum tuberosum*) leaf. *X* Xylem vessel, *xp* xylem parenchyma cells, *cc* companion cells, *se* sieve elements, *pp* phloem parenchyma cells, *sm* spongy mesophyll cells. Bars = 5 μm (**a, c, g, h**), 10 μm (**b, d, f**), 20 μm (**e**)

Results

Prosystemin mRNA expression pattern in leaf tissue, visualized by in situ hybridization

The localization of prosystemin mRNA in cells of tomato leaf tissues was analyzed using in situ hybridization in 10-μm paraffin-embedded cross-sections. The sections were hybridized with digoxigenin-labeled prosystemin riboprobes, which in turn were detected with anti-digoxigenin antibodies conjugated with alkaline phosphatase followed by color development. Prosystemin mRNA is present at very low levels in unwounded tomato leaves, but the levels increase upon wounding or treatment of the plants with methyl jasmonate (Ryan 2000). Serial sections of wounded tomato leaves from vascular bundles containing minor veins and mid veins, when hybridized with a sense digoxigenin-labeled prosystemin riboprobe, as a negative control, showed very low, non-specific cross-hybridization of prosystemin mRNA (Fig. 1a). Cross-sections of leaf mid veins from unwounded plants, hybridized with the prosystemin antisense-labeled probe (Fig. 1b), showed only a mild increase in signal intensity compared with the negative control section (Fig. 1a), indicating the presence of very low, constitutive levels of prosystemin mRNA. However, sections from wounded leaves



hybridized with the prosystemin antisense-labeled probe exhibited an intense pink color reaction concentrated around the phloem bundles of the mid vein, evidencing the presence of high levels of prosystemin mRNA (Fig. 1c). Tomato plants exposed to methyl jasmonate vapors also showed the accumulation of high levels of prosystemin mRNA, especially in the cells surrounding the sieve elements of the phloem in vascular bundles of mid veins (Fig. 1d).

Immunocytochemical localization of prosystemin in phloem parenchyma cells of vascular bundles

To assess whether the accumulation of prosystemin mRNA was accompanied by the accumulation of prosystemin protein in specific cell types, cross-sections of resin-embedded tissues of wounded and unwounded tomato plants were reacted with affinity-purified anti-prosystemin IgGs, followed by immuno-gold labeling and silver enhancement. Figure 2 shows confocal images of cross-sections and longitudinal sections through the vascular bundles of leaf minor veins. Prosystemin was detected by shades of red color over a dark-green background (Fig. 2b–e, g). Little background labeling was observed in the sections treated with the pre-immune serum used as a negative control (Fig. 2a). In sections from leaves of unwounded tomato plants, a low level of the prosystemin signal could be detected in the phloem parenchyma cells (Fig. 2b), but the signal was elevated in leaves of wounded plants (Fig. 2c) and in leaves of methyl jasmonate-treated plants (Fig. 2d, e). The prosystemin label appears to be almost exclusively confined within the phloem parenchyma cells of the minor veins, with very low background labeling present in the surrounding companion cells, spongy parenchyma cells or sieve elements. This cell-type-specific expression pattern of prosystemin is the same in both wounded or methyl jasmonate-treated leaves (Fig. 2c–e). Prosystemin was not detected in sections from leaves of transgenic tomato plants overexpressing the prosystemin gene in the antisense orientation (McGurl et al. 1992; Fig. 2f). Conversely, in tomato plants overexpressing the prosystemin gene in the sense orientation (McGurl et al. 1994), a high level of prosystemin protein was found in all cell types of the leaves, but was more noticeable in the vascular bundle cells (Fig. 2g). In cross-sections from leaves of potato plants, the minor veins exhibited the same pattern of prosystemin accumulation in the phloem parenchyma cells as tomato leaves (Fig. 2h).

The expression of prosystemin in phloem parenchyma cells was also observed in the vascular bundles of other tissues and organs of the tomato plant (Figs. 3, 4). Figure 3a shows the vasculature of a leaf midrib of a control tomato leaf treated with pre-immune serum. In Fig. 3b is shown the midrib of a leaf which had been wounded 18 h earlier and labeled with the prosystemin antibody. The parenchyma cells of the bicollateral phloem bundles exhibited an elevated level of prosystemin protein. In plants treated with methyl jasmonate, the phloem bundles from a midrib also contained high levels of prosystemin in phloem parenchyma, but not in the neighboring companion cells or sieve elements (Fig. 3c). A similar localization was observed in petioles (Fig. 3d) and stems of both wounded (Fig. 3e) and methyl jasmonate-treated (Fig. 3f) plants.

High constitutive levels of prosystemin mRNA are present in various floral organs of tomato plants (McGurl et al. 1992). To address whether prosystemin protein is also present in phloem parenchyma of tomato flower parts, cross-sections of sepals, petals and anthers were analyzed by immunolocalization using confocal microscopy (Fig. 4). Prosystemin was observed in phloem parenchyma cells near the sieve elements and companion cells of the vascular bundles of sepals (Fig. 4b), petals (Fig. 4c) and the center vascular cylinder of developed anthers (Fig. 4f). Control sections of sepals (Fig. 4a) and anthers (Fig. 4d, e) treated with pre-immune serum showed very low background labeling.

Subcellular immunolocalization of prosystemin in phloem parenchyma cells

The subcellular localization of prosystemin protein in phloem parenchyma was observed in ultrathin sections through leaf vascular bundles of plants exposed to methyl jasmonate vapors (Fig. 5). Cross-sections of leaf veins were visualized using affinity-purified anti-prosystemin IgGs, followed by immuno-gold labeling and examination with a TEM. Selected areas from the cells shown in Fig. 5a are magnified in Fig. 5b–d, which confirmed the localization of prosystemin in the phloem parenchyma cells of a first-order minor vein. The micrographs revealed profuse labeling in the cytosol, with low background label in the companion cells, sieve element, and surrounding mesophyll cells. A similar labeling pattern was observed in the phloem bundles of the mid vein, where phloem parenchyma cells show high levels of labeled prosystemin compared with little or no label in the companion cells or sieve elements (Fig. 5e, f).

In addition to its presence in the cytosol, immunogold labeled prosystemin was also observed in the nucleus of phloem parenchyma cells of wounded (Fig. 6b) and methyl jasmonate-treated tomato leaves (data not shown). This nucleo-cytoplasmic location of prosystemin was re-confirmed using affinity-purified IgGs that recognize a truncated Δ-prosystemin in which the systemin sequence has been deleted from the C-terminus (Fig. 6d, see Materials and methods). Sections treated with the respective pre-immune sera showed little background labeling in the cytosol or in the nucleus of any cell type (Fig. 6a, c). The nucleo-cytoplasmic localization of prosystemin induced by wounding or methyl jasmonate was observed in both the cytoplasm and nucleus of phloem parenchyma cells of floral

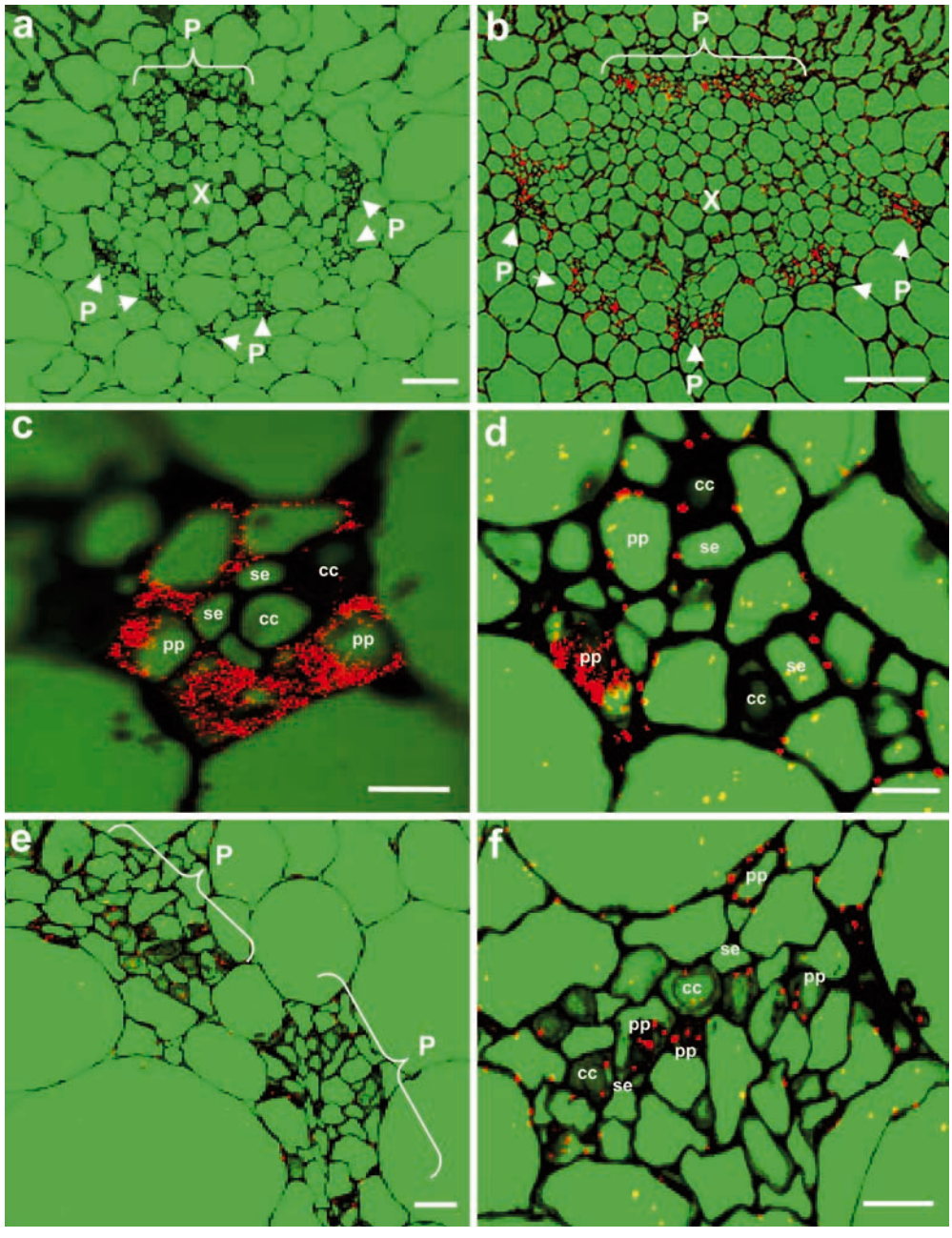


Fig. 3a–f Immunocytochemical localization of prosystemin in phloem parenchyma cells of leaf mid veins, petioles and stems of tomato plants. Tissue sections were treated and visualized as in Fig. 2. **a** Cross-section through a mid vein treated with pre-immune serum. **b** Wound-induced accumulation of prosystemin in phloem bundle cells of the midrib, 18 h after wounding of the leaf. **c** Methyl jasmonate-induced accumulation of prosystemin in midrib phloem parenchyma 18 h after treatment of whole plants. **d** Accumulation of prosystemin in phloem parenchyma of a petiole 18 h after wounding of the leaf. **e** Wound-induced accumulation of prosystemin in phloem parenchyma cells of the stem 18 h after wounding of the leaves. **f** Methyl jasmonate-induced accumulation of prosystemin in phloem parenchyma cells of the stem 18 h after treatment of whole plants. *P* Phloem, *X* xylem, *cc* companion cells, *se* sieve elements, *pp*, phloem parenchyma cells. Bars = 5 μm (**c, d**), 10 μm (**e, f**), 25 μm (**a**), 50 μm (**b**)

organs, where prosystemin is constitutively accumulated in the absence of wounding (data not shown). Immunogold-labeled prosystemin was found in the nucleus and cytosol of all cell types of leaves from transgenic tomato plants over-expressing prosystemin, including the guard cells of stomata (Fig. 6f). Guard cells from wild-type wounded plants did not show any cross-reactivity with prosystemin antibodies (Fig. 6e).

Discussion

The expression of the prosystemin gene in tissues of tomato plants is known to occur in the vascular bundles of minor and mid veins of leaves, petiolules, petioles and stems (Jacinto et al. 1997). However, the specific cell types within the vascular bundles in which the prosystemin transcript and protein were accumulating have not been established. We describe here the examination of prosystemin synthesis at the cellular and subcellular levels, using in situ hybridization and immunocytochemical techniques.

In situ hybridization of tomato prosystemin mRNA with digoxigenin-labeled riboprobes indicated that the prosystemin mRNA is present in phloem tissues within the vascular bundles of minor veins and the midrib of leaves (Fig. 1), where high levels of prosystemin transcripts were readily detected in the phloem bundles of leaves from wounded and methyl jasmonate-treated plants.

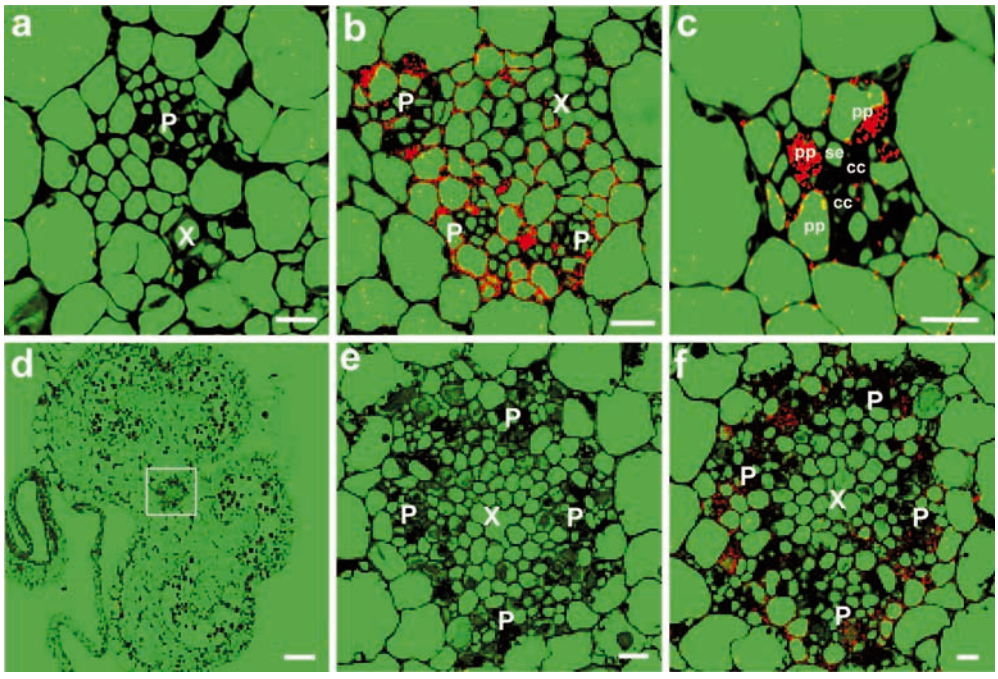


Fig. 4a–f Developmental accumulation of prosystemin in phloem parenchyma cells of tomato floral organs. Tissue sections were treated and visualized as in Fig. 2. **a** Cross-section through a vascular bundle of a sepal treated with prosystemin pre-immune serum, used as a negative control. **b** Localization of prosystemin in vascular bundle phloem parenchyma cells of a sepal. **c** Localization of prosystemin in vascular bundle phloem parenchyma cells of a petal. **d** Low-magnification view of an anther cross-section showing the location of the central filament vascular bundle (*within white square*). **e** Cross-section through the central filament vascular bundle of an anther treated with the prosystemin pre-immune serum, used as a negative control. **f** Localization of prosystemin in phloem parenchyma cells of the central vascular bundle of an anther. *X* Xylem, *P* phloem, *cc* companion cells, *se* sieve elements, *pp* phloem parenchyma cells. Bars = 10 μm (**c, e, f**), 15 μm (**a**), 20 μm (**b**), 100 μm (**d**)

Immunocytochemical analysis of the cellular location of prosystemin protein in leaf minor veins indicated that the protein was localized in the vascular phloem parenchyma cells, and increased in response to wounding or exposure to methyl jasmonate vapors (Fig. 2). These results were confirmed by examining the localization of prosystemin in transgenic tomato plants in which the prosystemin gene was expressed constitutively in both the antisense and sense orientations (Ryan 2000). Prosystemin protein could not be detected in vascular bundles of antisense plants (Fig. 2f). However, in tomato plants constitutively expressing the prosystemin gene, high levels of prosystemin were observed in all cell types of the leaves, but predominantly within the vascular parenchyma and companion cells (Fig. 2g). These experiments revealed the association of phloem parenchyma cells with the wound defense response in tomato plants.

Prosystemin was also localized in leaf phloem parenchyma cells of potato (Fig. 2h), another member of the family Solanaceae in which prosystemin is functional (Constabel et al. 1999), suggesting that the prosystemin gene is under the same cell-type-specific regulation in other solanaceous plant species where it is present.

Immunocytochemical analyses of the prosystemin protein in midribs, petioles and stems showed the same localization in phloem parenchyma as in the leaf minor veins (Fig. 3). However, the intensity of the prosystemin labeling signal after wounding or methyl jasmonate treatments was higher in the first-order veins of the leaf blade and in the midribs (Figs. 2c–e, 3a, b), compared with the petioles and the stems (Fig. 3d–f), suggesting that the systemin signal is more actively produced in the leaves.

The prosystemin transcript is also found in high levels in flower organs of wild-type plants (McGurl et al. 1992), suggesting that in flowers prosystemin expression may be regulated developmentally. We therefore immunochemically analyzed floral organs for the localization of prosystemin in sepals, petals, and anthers. In each organ the protein was found associated with the vascular parenchyma cells (Fig. 4). Thus, while regulated developmentally, prosystemin synthesis was localized to the same cell types as it is in leaves, petioles and stems.

Ultrastructural immunocytochemical analysis at the TEM level of leaf minor veins and midribs confirmed the localization of prosystemin in phloem parenchyma cells (Fig. 5). High levels of immuno-gold-labeled prosystemin were identified in the phloem parenchyma cells of methyl jasmonate-treated leaves, with a very low background labeling in the companion cells, sieve elements, xylem vessels or neighboring spongy mesophyll cells (Fig. 5).

With the use of two different affinity-purified anti-prosystemin IgGs, capable of recognizing different epitopes of the systemin precursor protein, it was found

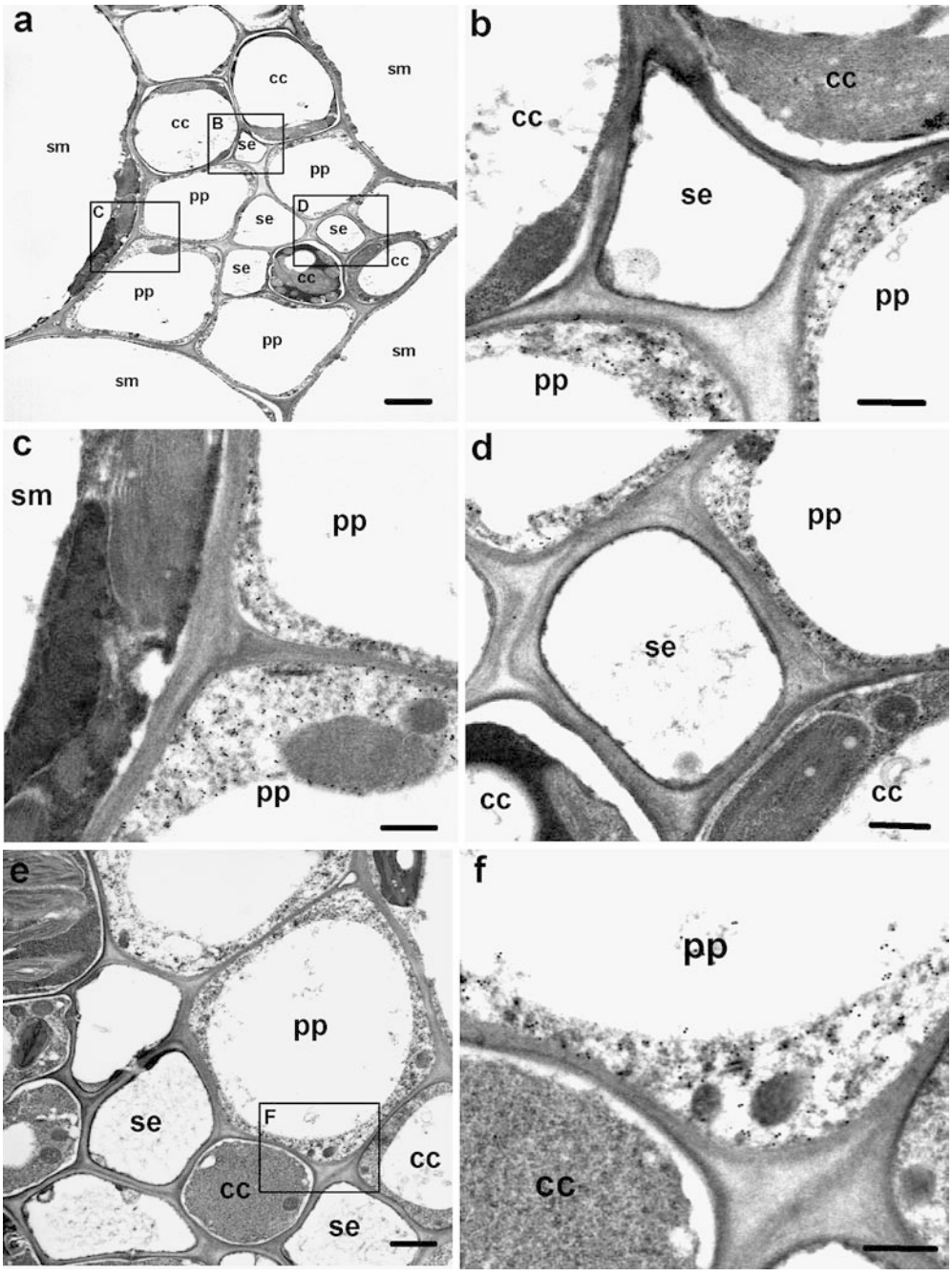


Fig. 5a–f Electron micrographs showing immuno-gold labelling of prosystemin in phloem parenchyma cells of a first-order minor vein and the midrib of a tomato leaf. Leaf tissue was obtained from methyl jasmonate-treated (18 h) tomato plants and processed for immunocytochemical analysis under the TEM as described in Materials and methods. **a** A low-magnification view of a minor vein treated with affinity-purified anti-prosystemin IgGs (approx. 50 ng μl^{-1}). **b–d** Higher-magnification views of the regions selected in **a**, showing companion cell-sieve element complexes (**b, d**) and the minor-vein right border with a spongy mesophyll cell (**c**); immuno-gold-labeled prosystemin is present exclusively in the cytosol of phloem parenchyma cells. **e** Low-magnification view of a cross-section through a phloem strand of a mid vein showing several companion cells, sieve elements and parenchyma cells. **f** Higher-magnification view of the region selected in **e**, showing immuno-gold-labeled prosystemin in the cytosol of a phloem parenchyma cell, and the absence of label in two neighboring companion cells. Symbols are as in Fig. 2. Bars = 0.5 μm (**b–d, f**), 1 μm (**e**), 2 μm (**a**)

that full-length prosystemin was compartmentalized in both the cytosol and the nucleus of the phloem parenchyma cells of leaves from wounded and methyl jasmonate-treated tomato plants (Fig. 6b, d). Furthermore, prosystemin was distributed between the nucleus and the cytosol of all cell types of the leaves from transgenic sense tomato plants overexpressing prosystemin (Figs. 2g, 6f). The nucleo-cytoplasmic transport of regulatory proteins has been suggested to contribute to the regulation of signal-transduction pathways in plants (Merkle 2001). However, changes in the nucleo-cytoplasmic partitioning of the prosystemin protein upon elicitation by wounding or methyl jasmonate treatments could not be detected in this work. Due to its small size (approx. 23 kDa), prosystemin could possibly diffuse in and out of the nucleus.

Several wound signaling components have been identified that are associated with the vascular bundles of tomato plants, including systemin and prosystemin (Ryan 2000), H_2O_2 (Orozco-Cárdenas et al. 2001), allene oxide cyclase (AOC; Hause et al. 2000; Stenzel et al. 2003), and jasmonic acid (Stenzel et al. 2003). AOC, an enzyme required for wound-induced jasmonic acid biosynthesis during systemin signaling, and a rate-limiting enzyme of the octadecanoid pathway, is specifically localized in vascular parenchyma cells (Hause et al.

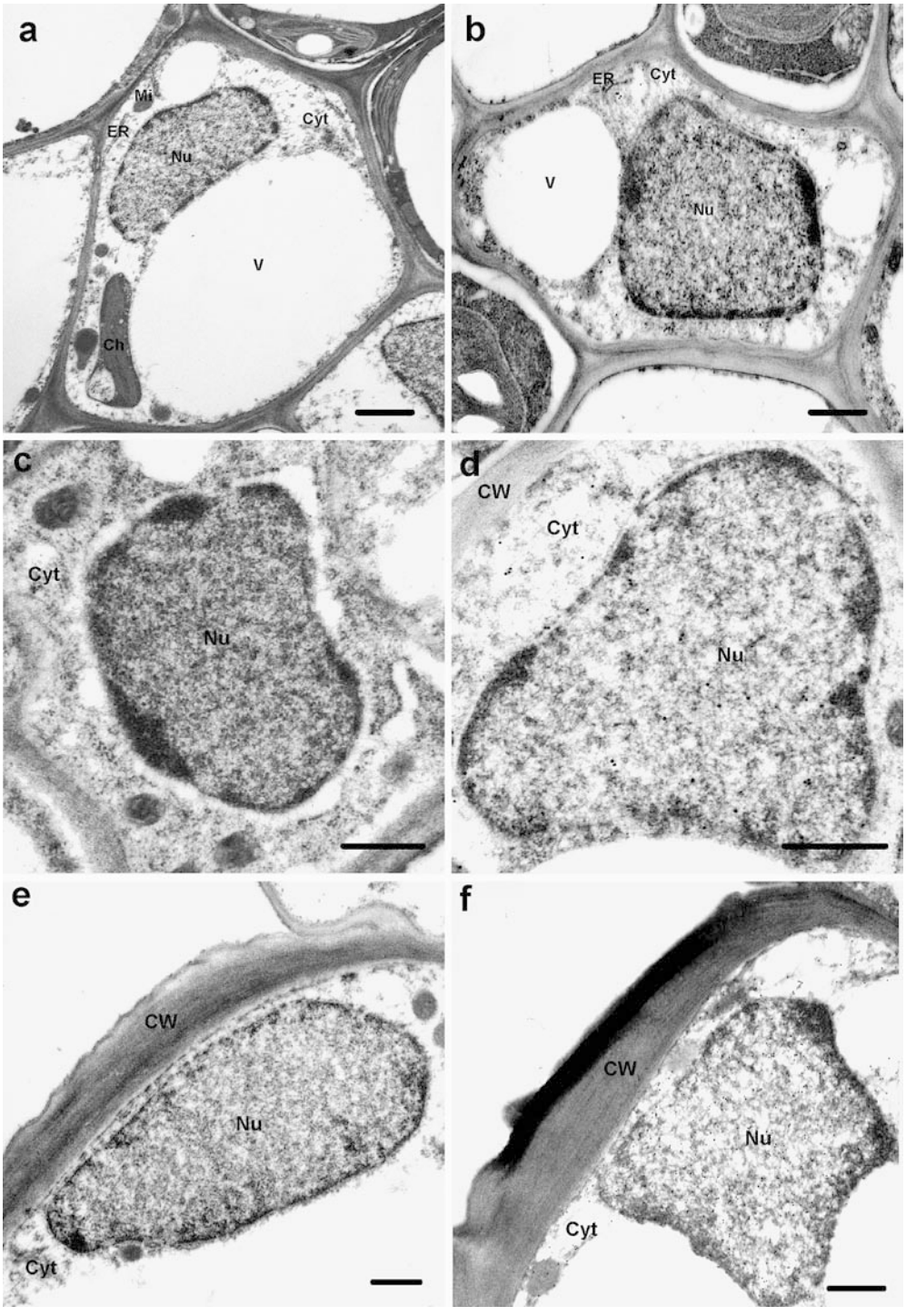


Fig. 6a–f Nucleo-cytoplasmic localization of prosystemin in phloem parenchyma cells. Undamaged tissue from wounded tomato leaves was processed for immunocytochemical detection and examined under the TEM as described in Materials and methods. **a** Cross-section through a phloem parenchyma cell of a leaf minor vein obtained from wounded leaves (12 h after wounding), and incubated with affinity-purified prosystemin pre-immune serum. **b** Section as in **a** treated with anti-prosystemin IgGs (approx. 25 ng μl^{-1}), showing immuno-gold-labeled prosystemin in the cytosol and the nucleus of the phloem parenchyma cell. **c** Section as in **a** treated with affinity-purified Δ-prosystemin pre-immune serum. **d** Section as in **a** treated with anti-Δ-prosystemin IgGs (approx. 15 ng μl^{-1}), showing immuno-gold-labeled prosystemin in the nucleus and the cytosol of the phloem parenchyma cell. **e** Cross-section through a stomatal guard cell from a wounded leaf treated with anti-prosystemin IgGs (approx. 25 ng μl^{-1}), showing the absence of label in the nucleus and cytosol. **f** Cross-section through a stomatal guard cell from a leaf of a transgenic sense tomato plant treated with anti-prosystemin IgGs (approx. 25 ng μl^{-1}), showing abundant labeling in both the nucleus and the cytosol. *Nu* Nucleus, *Cyt* cytosol, *ER* endoplasmic reticulum, *CW* cell wall, *Mi* mitochondria, *Ch* chloroplast. Bars = 0.5 μm (**b–f**), 1 μm (**a**)

2000; Stenzel et al. 2003). Therefore, both systemin synthesis and jasmonic acid synthesis have now been associated with the vascular bundle parenchyma cells.

In summary, prosystemin mRNA and protein are synthesized in phloem parenchyma cells of vascular bundles of leaves, stems, and flowers of the tomato plants. Upon wounding, systemin is processed from prosystemin by a still unknown mechanism and somehow is translocated into the apoplast of the vascular bundles where it is perceived by the systemin receptor (Scheer and Ryan 2002). Within the vascular bundles, systemin initiates a positive amplification loop, in which systemin and jasmonic acid, or another oxylipin signal, are self-induced as a wave through the plant vasculature for signaling the systemic wound response (Li et al. 2002; Ryan and Moura 2002; Lee and Howe 2003; Stenzel et al. 2003). The results presented here support a major defense role for phloem parenchyma cells in systemic wound signaling. This finding provides a new framework to further explore the details of prosystemin

synthesis in phloem parenchyma cells and their role in the systemic wound response. Major questions still remain, including how prosystemin maintains its stability in the cytoplasm, the mechanism of prosystemin processing and transport of systemin to interact with its membrane receptor, and the role of systemin in the phloem in amplifying systemic signaling.

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References

Constabel CP, Yip L, Ryan CA (1998) Prosystemin from potato, black nightshade, and bell pepper: primary structure and biological activity of predicted systemin polypeptides. Plant Mol Biol 36:55–62

Délano PJ, Dombrowski JE, Ryan CA (1999) The expression of tomato prosystemin in *Escherichia coli*: a structural challenge. Protein Expr Purif 17:74–82

Dombrowski JE, Pearce G, Ryan CA (1999) Proteinase Inhibitor-inducing activity of the prohormone prosystemin resides exclusively in the C-terminal systemin domain. Proc Natl Acad Sci USA 96:12947–12952

Farmer EE, Johnson RJ, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol. 98:995–1002

Hause B, Stenzel I, Miersch O, Maucher H, Kramel R, Ziegler J, Wasternack C (2000) Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J 24, 113–126

Haywood V, Kragler F, Lucas WJ (2002) Plasmodesmata: pathways for protein and ribonucleoprotein signaling. Plant Cell [Suppl] 14:303–325

Jacinto T, McGurl B, Franceschi V, Delano-Freier J, Ryan CA (1997) Tomato prosystemin promoter confers wound-inducible, vascular bundle-specific expression of the β-glucuronidase gene in transgenic tomato plants. Planta 203:406–412

Lee GI, Howe GA (2003) The tomato mutant *spr1* is defective in systemin perception and the production of a systemic wound signal for defense gene expression. Plant J 33:567–576

Li L, Li C, Lee GI, Howe GA (2002) Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc Natl Acad Sci USA 99:6416–6421

McGurl B, Pearce G, Orozco-Cárdenas ML, Ryan CA (1992) Structure, expression and antisense inhibition of the systemin precursor gene. Science 255:1570–1573

McGurl B, Orozco-Cárdenas ML, Pearce G, Ryan CA (1994) Overexpression of a CaMV–prosystemin gene in transgenic tomato plants generates a systemic signal that induces proteinase inhibitor synthesis. Proc Natl Acad Sci USA 91:9799–9802

Merkle T (2001) Nuclear import and export of proteins in plants: a tool for the regulation of signalling. Planta 213:499–517

Narváez-Vásquez J, Franceschi VR, Ryan CA (1993) Proteinase inhibitor synthesis in tomato plants: evidence for extracellular deposition in roots through the secretory pathway. Planta 189:257–266

Narváez-Vásquez J, Orozco-Cárdenas ML, Ryan CA (1994) A sulfhydryl reagent modulates systemic signaling for wound-induced and systemin-induced proteinase inhibitor synthesis. Plant Physiol 105:725–730

Narváez-Vásquez J, Pearce G, Orozco-Cárdenas ML, Franceschi VR, Ryan CA (1995) Autoradiographic and biochemical evidence for the systemic translocation of systemin in tomato plants. Planta 195:593–600

Orozco-Cárdenas ML, Narváez-Vásquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell 13:179–191

Ryan CA (2000) The systemin signaling pathway: differential activation of plant defensive genes. Biochim Biophys Acta 1477:112–121

Ryan CA, Moura DS (2002) Systemic wound signaling in plants: a new perception. Proc Natl Acad Sci USA 99:6519–6520

Scheer JM, Ryan CA (2002) The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. Proc Natl Acad Sci USA 99:9585–9590

Stenzel I, Hause B, Maucher H, Pitzschke A, Miersch O, Ziegler J, Ryan CA, Wasternack C (2003) Allene oxide cyclase dependence of the wound response and vascular bundle specific generation of jasmonates in tomato — amplification in wound-signalling. Plant J 33:577–589

Tavares R, Vidal J, Lammeren AV, Kreis M (2002) Non-purified anti-peptide sera generate tissue specific artefacts in immunohistochemical staining of *Arabidopsis thaliana*. Plant Sci 162:309–314

Thompson GA, Schulz A (1999) Macromolecular trafficking in the phloem. Trends Plant Sci 4:354–360