

## Chloroplast-Like Organelles Were Found in Eucleate Sieve Elements of Transgenic Plants Overexpressing a Proteinase Inhibitor

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**SaPIN2a, a plant proteinase inhibitor from nightshade (*Solanum americanum*), was located to the eucleate sieve elements (SEs) of phloem. The expressed SaPIN2a in transgenic lettuce showed inhibition of plant endogenous trypsin- and chymotrypsin-like activities, suggesting that SaPIN2a can regulate proteolysis in plant cells. To further investigate the physiological role of SaPIN2a, we produced transgenic nightshade and lettuce plants overexpressing SaPIN2a from the cauliflower mosaic virus (CaMV) 35S promoter using *Agrobacterium*-mediated transformation. Overexpression of SaPIN2a in transgenic plants was demonstrated by northern blot and western blot analysis. SaPIN2a-overexpressing transgenic nightshade plants showed significantly lower height than wild-type plants. Transmission electron microscopy analysis showed that chloroplast-like organelles with thylakoids, which are not present in eucleate SEs of wild-type plants, were present in the eucleate SEs of SaPIN2a-overexpressing transgenic plants. This finding is discussed in terms of the possible role played by SaPIN2a in the regulation of proteolysis in SEs.**

**Key words:** chloroplast-like; phloem; proteinase inhibitor; sieve elements; *Solanum americanum*

Proteinase inhibitor II (PIN2) proteins are plant serine proteinase inhibitors<sup>1)</sup> that occur in many Solanaceous plants, including tomato,<sup>2)</sup> potato,<sup>1)</sup> tobacco<sup>3)</sup> and nightshade.<sup>4,5)</sup> The PIN2 protein might play an endogenous role in preventing uncontrolled proteolysis and/or serve a function in protecting against foreign proteolytic enzymes of pests or pathogens.<sup>6,7)</sup> Observations of their wound-inducible expression,<sup>3,8)</sup> however, have led to investigations focusing on their role in plant protection against insects.<sup>9–11)</sup> Nevertheless, reports on their developmental regulation and their tissue-specific accumula-

tion suggest that they have endogenous functions.<sup>5,12–17)</sup> Solomon *et al.* have shown that a cysteine proteinase inhibitor plays a role in modulating programmed cell death in soybean.<sup>18)</sup>

We have found that the nightshade (*Solanum americanum*) PIN2 gene *SaPIN2a* was highly expressed in phloem.<sup>17)</sup> The localization of SaPIN2a protein to eucleate sieve elements (SEs)<sup>17)</sup> and the inhibitory activities of the overexpressed SaPIN2a toward endogenous trypsin- and chymotrypsin-like proteases in transgenic lettuce<sup>19)</sup> suggest that SaPIN2a has a physiological role in the regulation of proteolysis in SEs. SaPIN2a has been found recently to be involved in flower and seed development.<sup>5,20)</sup> To further elucidate the possible role of SaPIN2a in the differentiation of SEs, in this study we produced transgenic nightshade and lettuce plants overexpressing SaPIN2a. We found that overexpression of SaPIN2a in transgenic plants caused significant reductions in the plant height of transgenic plants. The most interesting finding was that chloroplast-like organelles, which are not present in eucleate SEs of normal wild-type plants,<sup>21)</sup> were present in the eucleate SEs of SaPIN2a-overexpressing transgenic plants.

### Materials and Methods

*Plant material and growth conditions.* American black nightshade (*Solanum americanum* Mill.) and lettuce (*Lactuca sativa* L.) plants were grown in soil under natural conditions in a greenhouse.

*Generation of transgenic plants overexpressing SaPIN2a.* *SaPIN2a* cDNA<sup>17)</sup> was first cloned between the *Xho*I and *Hind*III sites of pHANNIBAL<sup>22)</sup> by replacing the sequence of the *pd*k intron, resulting in a construct, pHANSaf. The expression cassette containing Cauliflower Mosaic Virus (CaMV) 35S promoter,

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Abbreviations: CaMV, cauliflower mosaic virus; DAPI, 4',6-diamidino-2-phenylindole; OCS, octopaline synthase; PCR, polymerase chain reaction; PI, protease inhibitors; PIN2, proteinase inhibitor II; SE, sieve elements

*SaPIN2a* cDNA, and OCS terminator cut with *NotI* from pHANSaf was then inserted at the *NotI* site of binary vector pART27<sup>23</sup>) to obtain the *SaPIN2a* overexpressing construct pARTSaf. *Agrobacterium tumefaciens* strain LBA4404 containing pARTSaf was used in leaf disk transformation of nightshade<sup>24</sup>) and lettuce.<sup>19</sup>) Putative transformants were selected on MS medium containing kanamycin (100 mg/l). Kanamycin-resistant plantlets were transferred to soil and grown under natural conditions in a greenhouse.

**Northern blot analysis.** Total RNA was extracted from transgenic plants and wild-type plants and analyzed by northern blot analysis, as previously described.<sup>17</sup>) *SaPIN2a* cDNA was used in the generation of a random-primed <sup>32</sup>P-labelled probe.

**Western blot analysis.** Total plant protein was extracted according to the procedure of Wu *et al.*<sup>25</sup>) Protein concentration was determined following Bradford.<sup>26</sup>) Western blot analysis was performed as previously described.<sup>19</sup>)

**Plant growth assay.** Seeds of transgenic plants (T<sub>1</sub>) and wild-type plants were germinated on MS medium with or without kanamycin (100 mg/l), and then transferred to a green house. All transgenic plants were confirmed by PCR analysis. The heights of wild-type and transgenic plants were measured during flowering in the years 2004 and 2005 respectively. Statistical analysis was performed using software SPSS 12.0 for Windows (Chicago, IL). Data on plant heights from two independent experiments were analyzed by independent-sample T test. P values < 0.05 were considered statistically significant and were derived from two-sided statistical tests.

**Transmission electron microscopy.** Sample preparation was carried out according to the procedures described by Hayat.<sup>27</sup>) Samples were examined with transmission electron microscopes JEM-100CX and JEM-1230.

**4',6-Diamidino-2-phenylindole (DAPI) staining.** DAPI staining was performed as previously described.<sup>28</sup>) Sections were observed and photographed using an Olympus BH-2 fluorescence microscope.

## Results

### *Characterization of transgenic plants*

To overexpress *SaPIN2a*, a binary vector pARTSaf containing *SaPIN2a* cDNA, driven by the CaMV 35S promoter, was used to transform nightshade. The transgenic *SaPIN2a* mRNA in transgenic plants was examined by northern blot analysis. Total RNA, isolated from the stems and leaves of transgenic plants and wild-type plants, was hybridized to the *SaPIN2a* cDNA probe. As

shown in Fig. 1A, high levels of *SaPIN2a* mRNA were present in the RNA from the stems of transgenic lines. Consistently with a previous report,<sup>17</sup>) a faint band was observed in the stems of wild-type plants, whereas no signal was detected in the leaves of wild-type plants. Total proteins from the stems of wild-type and transgenic plants that showed abundant *SaPIN2a* mRNA accumulation in northern blot analysis (Fig. 1A) were then extracted and used in western blot analysis with *SaPIN2a*-specific antibodies. As shown in Fig. 1B, the amount of *SaPIN2a* protein significantly increased in the stems of transgenic nightshade plants as compared with wild-type plants. A comparison of Fig. 1A and B, however, shows a discrepancy between mRNA and protein levels, which has also been observed in transgenic plants by other investigators.<sup>29–31</sup>)

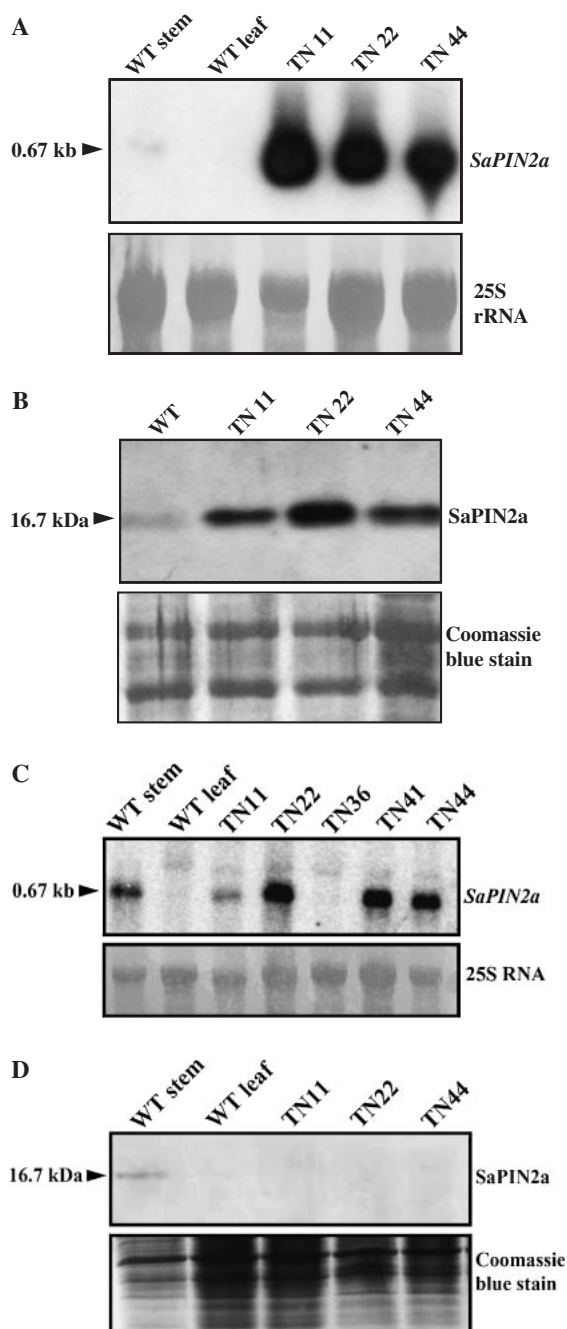
Although *SaPIN2a* mRNA expression was detected in the leaves of transgenic lines TN11, TN22, TN41, and TN44 (Fig. 1C), no *SaPIN2a* protein was found in the leaves of these transgenic plants in western blot analysis (Fig. 1D). The lack of *SaPIN2a* protein in the leaves of transgenic plants suggests that a post-transcriptional mechanism contributes to the regulation of *SaPIN2a* translation and/or accumulation. A failure of transgene protein accumulation despite high expression of its corresponding mRNA has also been reported in other transgenic plants.<sup>32–34</sup>) We also obtained transgenic lettuce plants with the same vector, pARTSaf, used for nightshade transformation, but only one transgenic line, P-11, showed *SaPIN2a* accumulation (data not shown). This was used for the subsequent analysis by transmission electron microscopy.

### *SaPIN2a overexpression caused a significant reduction in plant heights of transgenic nightshade*

Seedlings of transgenic nightshade plants grew more slowly than those of wild-type plants (Fig. 2A). The heights of mature wild-type and transgenic plants were measured in the years 2004 and 2005 respectively. Statistical analysis showed that *SaPIN2a*-overexpressing transgenic plants were significantly lower in height as compared with wild-type plants (Fig. 2B and C). The leaves of the transgenic plants were also considerably smaller than wild-type leaves (Fig. 2A). This observation suggests that overexpressed *SaPIN2a* might impair the differentiation of SEs in transgenic plant phloem, and resulting in growth retardation of transgenic plants.

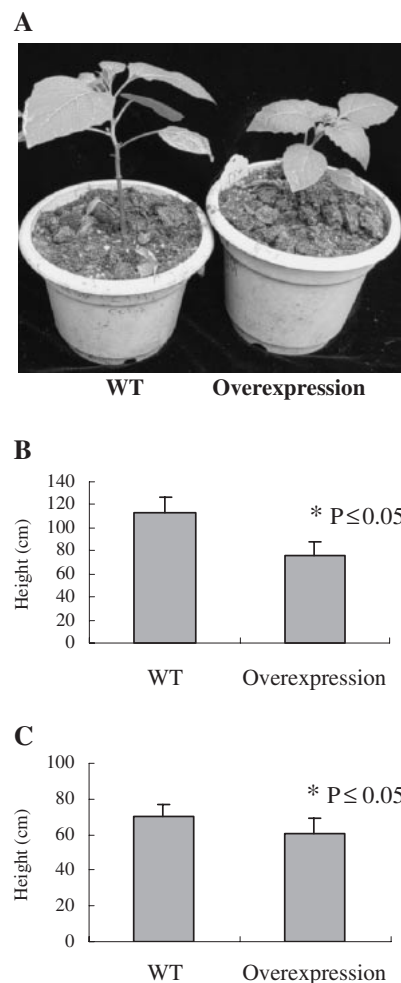
### *Chloroplast-like organelles were found in enucleate sieve elements of SaPIN2a-overexpressing transgenic plants*

To determine the cytohistological changes in the SEs of transgenic plants resulting from *SaPIN2a* overexpression, transverse sections of phloem in the stems of wild-type plants and transgenic plants were examined by transmission electron microscopy. Surprisingly, chloroplast-like organelles with thylakoids, which are not



**Fig. 1.** Northern Blot and Western Blot Analyses of Transgenic Nightshade Plants.

For northern blot analysis (A and C), the blots were probed with random-primed  $^{32}\text{P}$ -labelled *SaPIN2a* cDNA. Total RNA (20  $\mu\text{g}$ ) was isolated from the stems and leaves of wild-type plants (WT stem, WT leaf), and from the stems (A) and leaves (C) of transgenic nightshade plant lines. The hybridization bands corresponding to the *SaPIN2a* transcript (0.67 kb) are indicated by an arrowhead. The 25S rRNA bands stained with ethidium bromide are shown (bottom panel) to indicate the amount of total RNA loaded per lane. B and D, Western blot analysis of proteins from transgenic nightshade plants using *SaPIN2a*-specific antibodies. Total protein (50  $\mu\text{g}$ ) was isolated from the stems and leaves of wild-type plants (WT) and from the stems (B) and leaves (D) of transgenic nightshade plant lines TN11, TN22, and TN44. Cross-reacting bands (16.7 kDa) are indicated by an arrowhead. Coomassie blue stain of total proteins on a 15% SDS-PAGE gel is shown (bottom panel) to indicate the amount of proteins loaded per lane.

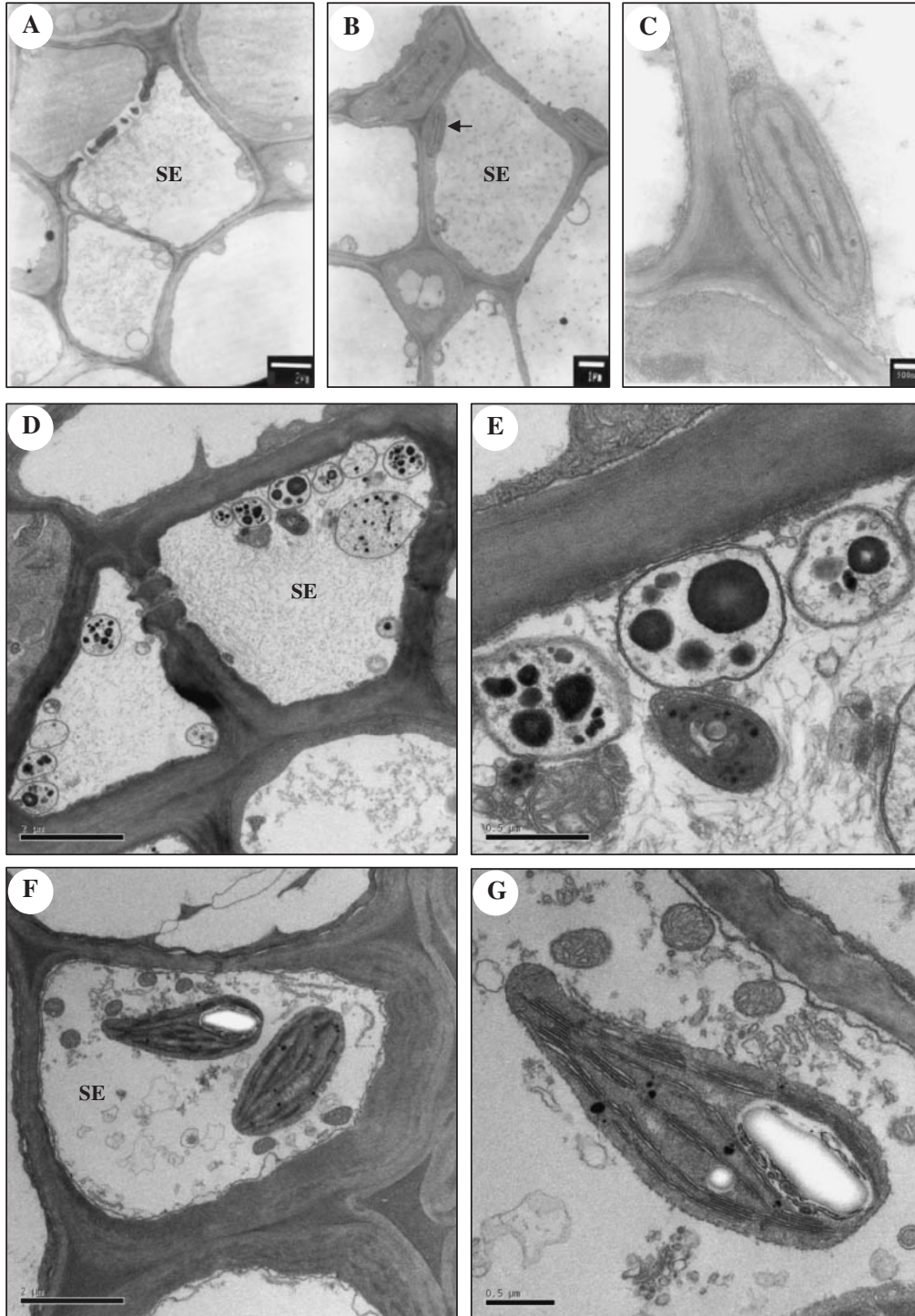


**Fig. 2.** Comparison of the Height of *SaPIN2a*-Overexpressing Transgenic Nightshade Plants with That of Wild-Type Plants.

A, Seedlings of the transgenic plant (overexpression) and the wild-type plant (WT). B, Statistical analysis of the height of mature wild-type and transgenic plants grown in 2004. The heights of a total of 19 transgenic plants and 30 wild-type plants were measured. C, Statistical analysis of the heights of mature wild-type and transgenic plants grown in 2005. The heights of 15 transgenic plants and 22 wild-type plants were measured. In B and C, data represent means  $\pm$  SD. \*P values < 0.05 were considered statistically significant (T-test.).

present in normal enucleate SEs of wild-type plants,<sup>21)</sup> were found in enucleate SEs of the *SaPIN2a*-overexpressing transgenic nightshade (Fig. 3B–C) and lettuce (Fig. 3F–G) plants. Only plastids with starch grains and mitochondria were present in the SEs of wild-type plants (Fig. 3A, D, and E). Usually about 1–3 chloroplast-like organelles can be found per section of phloem in stems of transgenic plants, but the chloroplast-like organelles found in the SEs of the *SaPIN2a*-overexpressing transgenic plants were different from typical chloroplasts in photosynthetic tissues, in which well-developed grana are present.

Transmission electron microscopy revealed that similarly to the wild-type plants (Fig. 3A and D), the nucleus was not present in the mature SEs of the

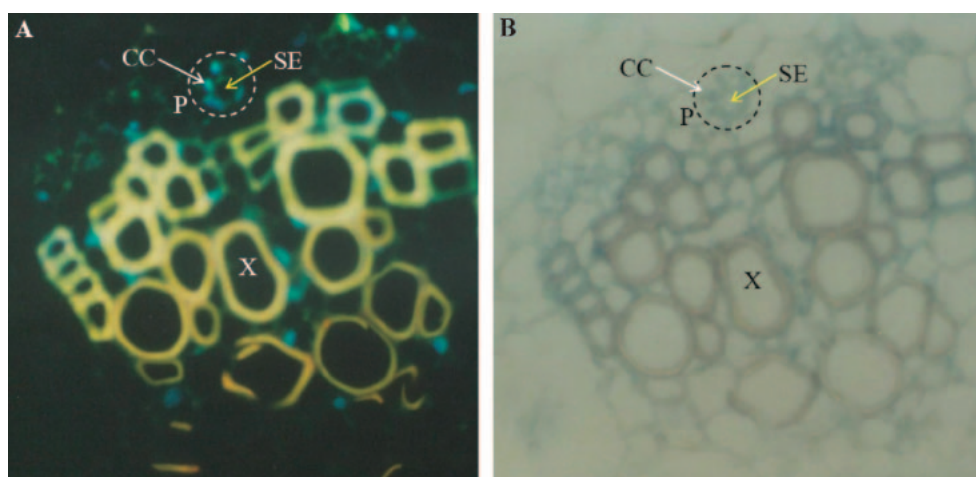


**Fig. 3.** Transmission Electron Micrograph of Phloem in Stems of SaPIN2a-Overexpressing Transgenic Nightshade (TN44) and Lettuce (P-11) and Wild-Type Plants.

A, Transverse section of phloem in stem of wild-type nightshade. Bar = 2  $\mu\text{m}$ . B, Transverse section of phloem in stems of SaPIN2a-overexpressing transgenic nightshade. Arrow indicates chloroplast-like organelle. Bar = 1  $\mu\text{m}$ . C, High magnification image of chloroplast-like organelle indicated by arrow in (B). Bar = 0.5  $\mu\text{m}$ . D, Transverse section of phloem in stem of wild-type lettuce. Bar = 2  $\mu\text{m}$ . E, High magnification image of plastids with starch grains shown in (D). Bar = 0.5  $\mu\text{m}$ . F, Transverse section of phloem in stem of transgenic lettuce. Bar = 2  $\mu\text{m}$ . G, High magnification image of chloroplast-like organelle shown in (F). Bar = 0.5  $\mu\text{m}$ . SE, sieve element.

SaPIN2a-overexpressing transgenic plants (Fig. 3B and F). This observation was further supported by 4',6-diamidino-2-phenylindole (DAPI) staining assay of stem sections from the SaPIN2a-overexpressing transgenic

plants (Fig. 4). DAPI-stained nuclei were found only in companion cells (CCs), not in mature sieve elements (SEs) in the phloem of the transgenic nightshade plants (Fig. 4A).



**Fig. 4.** Transverse Section of Stem of the SaPIN2a-Overexpressing Transgenic Nightshade Plant (line TN44) Stained with 4',6-Diamidino-2-phenylindole (DAPI).

A, Fluorescent image photographed using an Olympus BH-2 fluorescence microscope. B, Bright-field image of the section in (A). CC, companion cell; P, phloem; SE, sieve element, X, xylem.

## Discussion

Phloem, which is composed of SEs, companion cells, and parenchyma cells<sup>35</sup>) is responsible for the transport of various substances, especially photoassimilates, macromolecules, and signaling molecules, in plants.<sup>36–38</sup> Like mammalian erythrocytes, SEs undergo a unique selective autolysis during the differentiation process. Mature SEs eventually lose their nuclei and most of the cytoplasmic organelles,<sup>21,35,39,40</sup> creating an organelle-free pathway for the transport of various substances through the lumen of the cell.

No specific protease has been identified as responsible for the selective cytoplasmic degradation in SEs during differentiation, although proteases are thought to be involved in this process.<sup>41,42</sup> In contrast, many protease inhibitors (PIs) have been found in phloem.<sup>17,43–52</sup> Some of these PIs perhaps regulate selective autolysis during the maturation of SEs.<sup>19,44,48,51</sup>

In this study, we found that overexpression of SaPIN2a, a SE-localized PI, caused growth retardation in transgenic plants. This might have resulted from the occurrence of chloroplast-like organelles in the SEs of the transgenic plants. The ectopic presence of chloroplast-like organelles in the SEs of transgenic plants might impair the transport of various molecules through the phloem. We observed that some SaPIN2a-overexpressing transgenic plants died prematurely and that no seed was produced by these plants. The transgenic plant lines that survived and produced seeds showed less growth retardation. This observation might explain the fact that the difference in height between SaPIN2a-overexpressing and wild-type plants was more significant in the year 2004 than in 2005 (Fig. 2B and C).

It is well established that plastids in SEs lack thylakoid membranes and contain only grains of starch or protein crystals,<sup>53</sup> although no known function has so

far been attributed to this class of organelles in SEs. The results, reported here, suggest that starch plastids in the SEs of nightshade (Fig. 3D and E) are important to plant development and growth, since conversion of these starch plastids to the abnormal chloroplast-like organelles in the SEs of the transgenic plants (Fig. 3B, C, F and G) caused growth retardation in the transgenic plants. The occurrence of chloroplast-like organelles with thylakoid membranes in the SEs of the transgenic plants might have resulted from inhibition of protease activities, which are involved in plastid development and conversion processes,<sup>54,55</sup> by the overexpressed SaPIN2a. The isolation of a SaPIN2a-targeted protease(s) from nightshade phloem tissue will be necessary to determine the exact function of plant PIs in SE differentiation and phloem development.

## Note Added in Proof

Native SaPIN2a from nightshade stems and recombinant SaPIN2a expressed in *Escherichia coli* were recently purified and characterized.<sup>56</sup> The purified native SaPIN2a exists as three charge isomers of homodimers, and was weakly glycosylated. The native SaPIN2a significantly inhibited serine proteinases such as trypsin, chymotrypsin, and subtilisin.

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