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SERINE PROTEINASE INHIBITOR PROTEINS: EXOGENOUS AND ENDOGENOUS FUNCTIONS

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Summary

Proteinase inhibitor II (PIN2) proteins from the *Solanaceae* family have been previously used in plant transformation to acquire protection against caterpillars. Some of these PIN2 proteins have been shown to exhibit exogenous activities against trypsin and/or chymotrypsin *in vitro*. Despite their application in conferring insect resistance in transgenic plants, the endogenous roles of this family of proteins in various plant species have not been well defined. To investigate the exogenous and endogenous functions of PIN2 proteins, cDNAs encoding PIN2 proteins from the weed *Solanum americanum* (American black nightshade), designated SaPIN2a and SaPIN2b, were cloned and characterized. The localization of *S. americanum* SaPIN2a and SaPIN2b mRNAs and proteins in the reproductive tissues destined to undergo developmental programmed cell death subsequently led to investigations into their function during seed development. Using plant transformation of lettuce and *S. americanum*, it was evident that: (1) the expression of SaPIN2a in transgenic lettuce conferred resistance to cabbage looper (*Trichoplusia ni*) caterpillars; and (2) the expression of siRNAs from a PIN2–RNAi construct resulted in transgenic *S. americanum* that were impaired in seed development. These results suggest that *S. americanum* PIN2 proteins not only enhance resistance to caterpillars (when expressed exogenously) but they function in inhibiting endogenous proteases that are expressed during seed development. Specifically, the aborted seeds of PIN2–RNAi lines showed abnormal endothelium that subsequently affected endosperm and embryo development.

Key words: insect resistance; ovule RNA; interference; PIN2 proteins; seed abortion; seed coat endothelium; seed development; serine proteinase inhibitor; *Solanaceae*.

Introduction

It has been predicted from whole genome analyses that proteases are widespread in plants, animals, and microorganisms and comprise approximately 2% of encoded proteins (Barrett et al., 1998; Kenny, 1999). These proteases are assumed to assert physiological functions in the regulation of protein synthesis and turnover (Turk, 1999). Their corresponding protease inhibitors, too, are abundant in nature (Ryan, 1981; Fritz, 2000). Each protease inhibitor interacts with its target protease at the catalytic domain, forming a stable protease–inhibitor complex that thus renders the protease inactive (Laskowski and Kato, 1980; Norton, 1991).

In higher plants, proteinase inhibitor proteins are widespread in many species, ranging from legumes and cereals to solanaceous plants (Liener and Kakade, 1969; Brzin and Kidric, 1995). Historically, the soybean trypsin inhibitor was first characterized from a plant (Read and Haas, 1938). It was identified from soybean flour extract and was subsequently purified (Bowman, 1944; Ham and Sandstedt, 1944) and crystallized (Kunitz, 1945). Early investigations on the characterization of proteinase inhibitor protein focused mainly on their purification from plants and the determination of their physical and chemical properties, particularly the identification of enzymes to which they displayed inhibition (Liener and Kakade, 1969).

The size of plant proteinase inhibitor (PI) proteins ranges from 4 to 85 kDa, with a great proportion being small proteins of only 8–20 kDa (Ryan, 1990; Walsh and Strickland, 1993; Gatehouse, 1999). Their amino acid composition is enriched in cysteine residues that are significant in the formation disulfide bridges and in conferring stability to heat, pH changes, and proteolysis (Ryan, 1981; Greenblatt et al., 1989; Richardson, 1991). In plants, they are produced either as prepro-proteins (Graham et al., 1985a; Cleveland et al., 1987) or pre-proteins (Graham et al., 1985b), which are subsequently subjected to *in vivo* processing. Furthermore, they have been subcellularly localized to various compartments in the plant cell. In cotyledonary and embryonic cells, the soybean trypsin inhibitor was reported to be associated with the cell walls and, to a lesser extent, with protein bodies, cytosol, and nuclei (Horisberger and Tacchini-Vonlanthen, 1983a), while the soybean Bowman–Brik inhibitor has been localized to protein bodies, the nuclei, and the cytosol (Horisberger and Tacchini-Vonlanthen, 1983b). Also, a trypsin inhibitor has been reported to occur in the cytosol in mung bean cotyledonary cells (Baumgartner and Chrispeels, 1976). However, the

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wound-induced inhibitors of tomato (Wingate et al., 1991), potato (Hollaender-Czytko et al., 1985), and *Solanum americanum* (Sin and Chye, 2004) are located in vacuoles.

Serine Proteinase Inhibitors

The serine proteinase inhibitors in plants, which include the Kunitz (soybean trypsin inhibitor) family, the Bowman–Birk (soybean proteinase inhibitor) family, potato I inhibitor family, potato II inhibitor family, barley trypsin inhibitor family, and squash inhibitor family (Norton, 1991), have been particularly important to crop transformation because their over-expression has led to the generation of varieties that showed protection against insect larvae (Ryan, 1990). New members of this class of proteinase inhibitors are still being isolated and tested using inhibitor-incorporated artificial diets for insect larvae, e.g., a member of the Kunitz inhibitor family from chickpea was recently shown to be effective against podborer (*Helicoverpa armigera*) larvae (Srinivasan et al., 2005).

The proteinase inhibitor II (PIN2) proteins are serine proteinase inhibitors that show conservation to the potato II inhibitor family and they demonstrate trypsin and chymotrypsin inhibitor activities due to the presence of two reactive sites (Bryant et al., 1976). PIN2 proteins have been widely reported and characterized from *Solanaceae* plants, including eggplant (Richardson, 1979), potato (Bryant et al., 1976), tomato (Gustafson and Ryan, 1976), tobacco (Pearce et al., 1993), and *S. americanum* (Xu et al., 2001; Sin and Chye, 2004). The PIN2 proteins from *S. americanum*, designated SaPIN2a and SaPIN2b, are similar in structure to proteinase inhibitors (PI) from tomato TI-II (Graham et al., 1985b) and potato PI-IIk (Thornburg et al., 1987) by the conservation of two inhibitory domains and the presence of eight cysteine residues within each domain (Xu et al., 2001). However, it has been reported that some PIN2 proteins contain more than two inhibitory domains. Examples of PIN2 proteins with three reactive domains include tomato CEV157 (Gadea et al., 1996) and tobacco TobPI-II (Balandin et al., 1995). Further, four and six inhibitory domains have been reported in NaPI-IV and NaPI-II from *Nicotiana alata* (Atkinson et al., 1993; Miller et al., 2000) while NGPI-1 and NGPI-2 from *Nicotiana glutinosa* each possesses eight and six domains (Choi et al., 2000).

Roles of Serine Proteinase Inhibitor Proteins

The presence of inhibitory domains in proteinase inhibitor proteins prompted us to question their physiological functions. It was thought that many plant proteinase inhibitor proteins do not have endogenous functions against plant proteases but show specificities for animal or microbial enzymes (Laskowski and Sealock, 1971; Ryan, 1981; Sanchez-Serrano et al., 1986). Hence they could be applied to combat invasion by pest or pathogen by their action on foreign proteolytic enzymes (Ryan, 1989; Brzin and Kidric, 1995). Such conclusions may have culminated from studies that use commercially available proteases, e.g., trypsin, chymotrypsin, elastase, and subtilisin from animal or microbial sources, as test enzymes in activity assays with plant proteinase inhibitor proteins (Laskowski and Kato, 1980; Brzin and Kidric, 1995). Although it may be unlikely that such animal or microbial proteases are the true physiological targets for plant proteinase inhibitor proteins (Laskowski and Kato, 1980), their actions on insect gut proteases were nonetheless experimentally demonstrated using artificial diets and *in vitro* inhibition assays on insect gut proteases (Hilder et al., 1993; Felton and Gatehouse, 1996; Reeck et al., 1997; Gatehouse, 1999). As a result of such actions, they were designated a role in plant defense. Indeed the over-expression of plant proteinase inhibitor proteins in transgenic plants enhances protection against insects and pathogens (Hilder et al., 1987; Boulter et al., 1990; Gatehouse et al., 1997; Charity et al., 1999).

Subsequently, accumulating evidence from investigations on the developmental regulation and tissue-specific accumulation of plant proteinase inhibitor proteins suggests they possess endogenous functions (Rosahl et al., 1986; Sanchez-Serrano et al., 1986; Margossian et al., 1988; Hendriks et al., 1991; Peña-Cortés et al., 1991; Lorberth et al., 1992). Plant organs that express PIN2 proteins include flowers (Peña-Cortés et al., 1991; Atkinson et al. 1993; Pearce et al., 1993; Brandstädter et al., 1996; Sin and Chye, 2004), fruits (Richardson, 1979; Pearce et al., 1988), stems (Xu et al., 2001), tubers (Rosahl et al., 1986; Sanchez-Serrano et al., 1986; Stiekema et al., 1988), and roots (Taylor et al., 1993; Brandstädter et al., 1996). Their expressions are induced in leaves following attack by insects or wounding (Peña-Cortés et al., 1988; Ryan, 1990), thus implicating a role in defense. It has also been suggested that they can regulate cell proteolysis by their action on endogenous proteases, thereby controlling protein turnover and metabolism (Ryan, 1989). Hence, indications of their physiological functions are mainly based on reports on their developmental and tissue-specific expression patterns (Jofuku and Goldberg, 1989; Botella et al., 1996; Brandstädter et al., 1996). The function of some seed PIs are not just limited to being reserve proteins that are mobilized during germination and sprouting (Norton, 1991) because they show activity against endogenous seed proteases (Richardson, 1977; Ryan, 1981; Brzin and Kidric, 1995). A cysteine proteinase inhibitor from soybean has been shown to function in the modulation of programmed cell death (Solomon et al., 1999). Another from cucumber leaves that lack significant inhibitory activity against commercial proteases originating from animals or microorganisms was demonstrated to inhibit cucumber glutamyl endopeptidase, indicative of its role in regulating proteinase activity (Yamauchi et al., 2001). Further, *S. americanum* proteinase inhibitor SaPIN2a was shown to be highly expressed in phloem and in flower and seed (Xu et al., 2001; Sin and Chye, 2004). The localization of *SaPIN2a* mRNA and protein to the companion cells and sieve elements suggests its role in the regulation of proteolysis in phloem development/function. Accumulation of *SaPIN2a* and *SaPIN2b* mRNAs and proteins in *S. americanum* reproductive tissues destined to undergo developmental programmed cell death (PCD) implies the possible roles of these proteins in plant reproduction (Sin and Chye, 2004). The exogenous role of SaPIN2a was further investigated by its over-expression in transgenic lettuce to investigate whether it would confer resistance to caterpillars, and its endogenous role was addressed by the expression of siRNAs from a PIN2–RNAi construct in transgenic *S. americanum*.

Expression of *SaPIN2a* mRNA in Transgenic Lettuce Inhibits Plant Endogenous Protease Activity and Confers Protection to Caterpillars

To investigate if the heterologous expression of SaPIN2a would result in the inhibition of plant endogenous protease activity, transgenic lettuce that expressed SaPIN2a from the strong and

constitutive Cauliflower Mosaic Virus 35S promoter from a pBI121 (Clontech)-derivative carrying the selection marker gene *npt* II (conferring kanamycin resistance) were generated (Xu et al., 2004). Lettuce was used in *Agrobacterium*-mediated transformation because its transformation protocols are relatively well established (Michelmore et al., 1987; Curtis et al., 1994). Furthermore, lettuce neither possesses detectable trypsin inhibitory activity in its leaves nor responds to any treatments by accumulating inhibitors (Walker-Simmons and Ryan, 1977). Since the over-expression of potato and tomato PIN2 conferred insect resistance (Johnson et al., 1989; Duan et al., 1996; Klopfenstein et al., 1997), production of insect-resistant lettuce would be desirable as it is an economically important vegetable crop grown globally (Ryder, 1999).

Following *Agrobacterium*-mediated transformation and selection on kanamycin-containing media, stable integration and inheritance of the *SaPIN2a* cDNA in the genome of transgenic lettuce were demonstrated by Southern blot and segregation analysis of the regenerated R_1 progeny (Xu et al., 2004). *SaPIN2a* mRNA was detected in both the regenerated lines (R_0) and progeny from these regenerated lines (R_1) by Northern blot analysis (Xu et al., 2004). Despite an absence of significant inhibitory activity against bovine trypsin and chymotrypsin in extracts from transgenic lettuce, the endogenous trypsin-like activity in all transgenic lines analyzed was almost completely inhibited, and the endogenous chymotrypsin-like activity was moderately inhibited. Preliminary insect bioassays with R_0 transgenic plants (Fig. 1) showed that some of them had acquired enhanced resistance to cabbage looper (*Trichoplusia ni*), which is a very destructive lettuce pest (Barbour, 1999). Due to limited availability of the larvae at the same developmental stage, all of the feeding trials were not carried out concurrently. The experiments were performed separately, each trial having its own set of controls (Fig. 1). Larvae fed on leaves from transgenic line 1 (TL1) and transgenic line 11 (TL11) plants (Fig. 1*a*, *c*) grew significantly slower than those fed on control leaves, with a 49.8 and 37.7% reduction in larval weight, respectively, after feeding for 8 d. A moderate reduction (25.5% on day 9) in larval growth was observed with transgenic line 15 (TL15; Fig. 1*d*), while growth was not retarded when larvae were fed on transgenic line 7 (TL7) leaves (Fig. 1*b*). The growth of larvae fed on leaves of TL1 was arrested and these larvae consumed much less leaf tissue compared to those fed on wild-type leaves (Fig. 2). Although there was an absence in the significant inhibition against bovine trypsin and chymotrypsin activities in extracts from transgenic lettuce, the expressed SaPIN2a in transgenic lettuce showed inhibition of plant endogenous protease activity, suggesting that SaPIN2a can regulate proteolysis endogenously (Xu et al., 2004). The endogenous trypsin-like activity was almost completely inhibited, while the endogenous chymotrypsin-like activity was moderately inhibited (Xu et al., 2004). In comparison, protease inhibitors from soybean (Birk et al., 1963) and wheat (Applebaum and Konijn, 1966) inhibit larval gut proteolysis of *Tribolium castaneum* but are inactive towards either mammalian trypsin or chymotrypsin. The maize proteinase inhibitor MPI, which shows amino acid homology to the potato proteinase inhibitor I (PIN1) family (Cordero et al., 1994), effectively inhibits midgut chymotrypsin from *Spodoptera littoralis* larvae but only weakly inhibits bovine chymotrypsin, which is uncharacteristic of other PIN1 family members that are potent inhibitors of mammalian chymotrypsin (Tamayo et al., 2000). The demonstrated inhibition of trypsin-like and chymotrypsin-like activities on endogenous plant proteinases indicates that SaPIN2a could be potentially exploited for the protection of foreign protein production in transgenic plants since the yield and quality of foreign proteins such as antibodies in transgenic plants are subject to degradation by endogenous proteolytic activities (Stevens et al., 2000).

Endogenous Role of *Solanum americanum* Proteinase Inhibitors in Floral and Seed Development

Expression of SaPIN2a and SaPIN2b in tissues destined to undergo PCD during floral development. The endogenous function of proteinase inhibitors is best illustrated by recent studies using *S. americanum* as an experimental material. Northern blot analyses using *SaPIN2a*- and *SaPIN2b*-specific probes clearly show that the expressions of *SaPIN2a* and *SaPIN2b* mRNAs are regulated during floral development. These mRNAs decreased as the wild-type flower matured and senesced (Sin and Chye, 2004). Corresponding examination of total proteins from various stages during floral development by Western blot analyses using *SaPIN2a*- and *SaPIN2b*-specific polyclonal antibodies confirmed the greater accumulation of *SaPIN2a* and *SaPIN2b* in floral buds than in open flowers. Subsequently, *SaPIN2a* and *SaPIN2b* mRNAs were localized by *in situ* hybridization studies to the floral tissues that are destined to undergo developmental PCD (stigma, stylar transmitting tissue, vascular bundles, nucellar cells of the ovule, and the outermost cell layer of the placenta) in young floral buds and open flowers (Sin and Chye, 2004). Immunolocalization of SaPIN2a and SaPIN2b proteins corresponded well to their mRNA distribution patterns (Sin and Chye, 2004). *SaPIN2a* and *SaPIN2b* showed different expression profiles based on studies of *in situ* hybridization and immunolocalization, suggesting that their expressions are regulated differentially. These observations are indicative that they have overlapping and complementary roles in floral development (Sin and Chye, 2004). SaPIN2a and SaPIN2b expression during early floral development and in the stylar transmitting tissue suggests a their putative role in the regulation of PCD prior to pollination (Sin and Chye, 2004). Cell degeneration in the transmitting tissue following pollen tube elongation would facilitate space to accommodate growth of the pollen tubes (Wu and Cheung, 2000). The expression of SaPIN2a and SaPIN2b in the layer surrounding the transmitting tissue would confine PCD triggered by the onset of pollination to within the transmitting tissue (Sin and Chye, 2004).

The detection of PIN2 mRNAs has been previously reported in the floral parts of other Solanaceae plants, including tomato floral buds (Brandstädter et al., 1996) and tobacco stigmas (Atkinson et al., 1993). The patterns of *SaPIN2a* and *SaPIN2b* expression is not unlike that of *PIN2* in potato and tomato flowers as detected by northern blot analysis (Peña-Cortés et al., 1991). Further, investigations using the reporter gene encoding β-glucuronidase fused to the potato *PIN2* promoter revealed highest expression in the developing ovules and in the adjacent outermost cell layer of the placenta of young floral buds (Peña-Cortés et al., 1991). This expression pattern in the ovules and adjacent placenta cell layer was also observed for *SaPIN2a* and *SaPIN2b* mRNAs by *in situ* hybridization studies (Sin and Chye, 2004).

Detection of *in situ* nuclear DNA fragmentation during PCD was verified using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays on floral sections. When these assays and immunolocalization were carried out to further

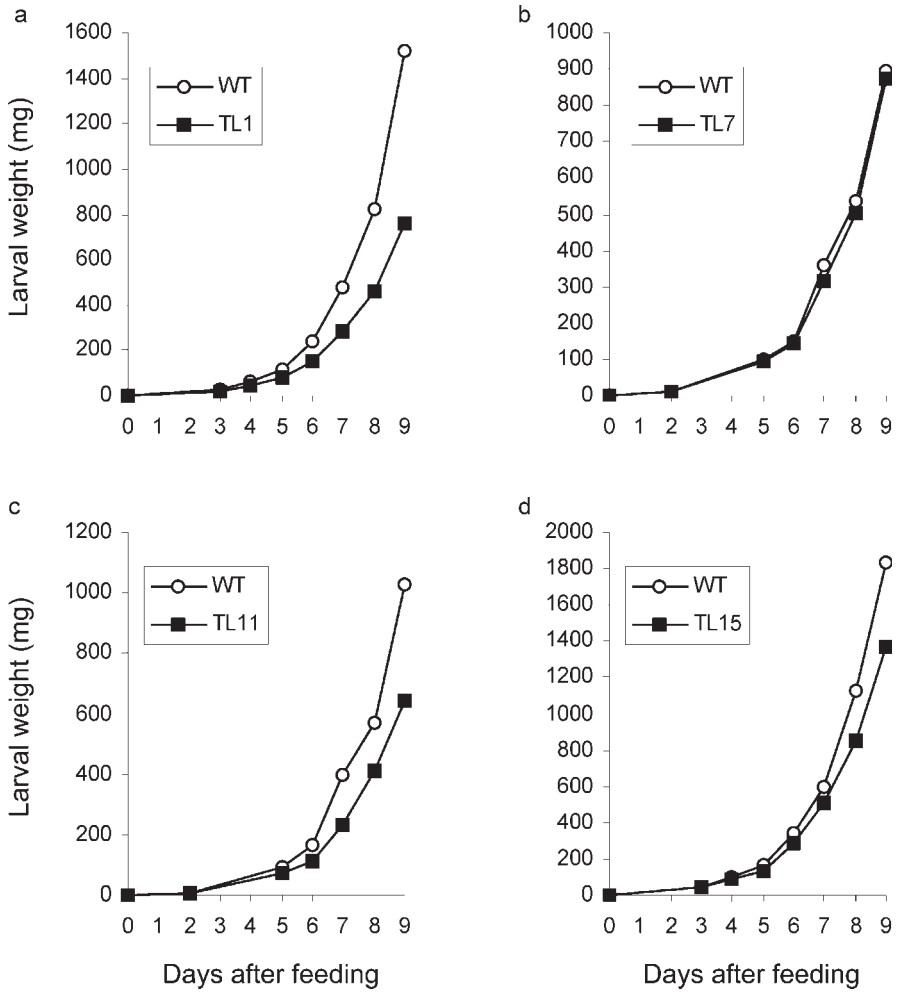

FIG. 1. Growth of cabbage loopers (*Trichoplusia ni*) fed on leaves of R_0 transgenic and wild-type lettuce. Newly hatched first-instar larvae were fed with daily fresh leaves from R_0 transgenic plants TL1 (*a*), TL7 (*b*), TL11 (*c*), and TL15 (*d*), each with leaves from wild-type control plants (WT). The points shown represent the total weight of 10 larvae. Insect feeding trials were carried out as described by Johnson et al. (1989). The larvae of cabbage looper (*T. ni*) were reared on lettuce grown in a growth chamber (16 h light, 20 ± 2°C) until pupation. Their pupae were collected and stored in a box covered with a net until adults emerged. The moths were fed with 5% honey obtained from the local market. The eggs were collected and hatched in Petri dishes. The first and second instar larvae were used for the experiments. Detached lettuce leaves from wild-type and transgenic plants were placed on top of three sheets of Whatman No.1 papers (125 mm diameter) wetted with 6 ml of distilled water in sterile Petri dishes (145 mm diameter). Ten first or second instar larvae of *T. ni* were added to each dish for further incubation in a plant growth chamber (20 ± 2 °C). Each d, the larvae were weighed and transferred with a brush to new dishes containing fresh leaves.

investigate a possible correlation of *SaPIN2a* and *SaPIN2b* expression with PCD during floral development (Sin and Chye, 2004), TUNEL-positive nuclei were detected in the nucellar cells in ovules of senescent flowers, with the exception of the innermost cell layer in the ovule. Corresponding ovules and the outermost layer of the placenta stained TUNEL-negative in young floral buds and open flowers. Interestingly, when examination of PCD in the style was carried out using transverse sections of the mid-style, *SaPIN2a* and *SaPIN2b* were detected in the style prior to pollination but their expression was restricted to a cell layer surrounding the transmitting tissue the day after pollination (Sin and Chye, 2004). In contrast, TUNEL-positive signals, absent in the unpollinated style, were located in the transmitting tissue of the pollinated style. Hence, investigations using TUNEL assays coupled with analyses by Northern blot, Western blot analyses, *in situ* mRNA hybridization and immunolocalization on the accumulation of *SaPIN2a* and *SaPIN2b* mRNAs, and proteins in floral tissues destined to undergo developmental PCD suggest that SaPIN2a and SaPIN2b may function endogenously in impeding PCD during flower development (Sin and Chye, 2004). The expression patterns of SaPIN2a and SaPIN2b resemble those of cysteine proteinases and their inhibitors (Solomon et al., 1999; Xu and Chye, 1999; Xu and Kermode, 2003). It has been shown that cysteine proteinase from eggplant (Xu and Chye, 1999) and an aspartic protease-like protein from barley (Chen and Foolad, 1997) are expressed in the nucellar cells, while wheat thiolprotease and carboxypeptidase are expressed in the degenerating nucellus (Domínguez and Cejudo, 1998). The expression of SaPIN2a and SaPIN2b in the nucellar

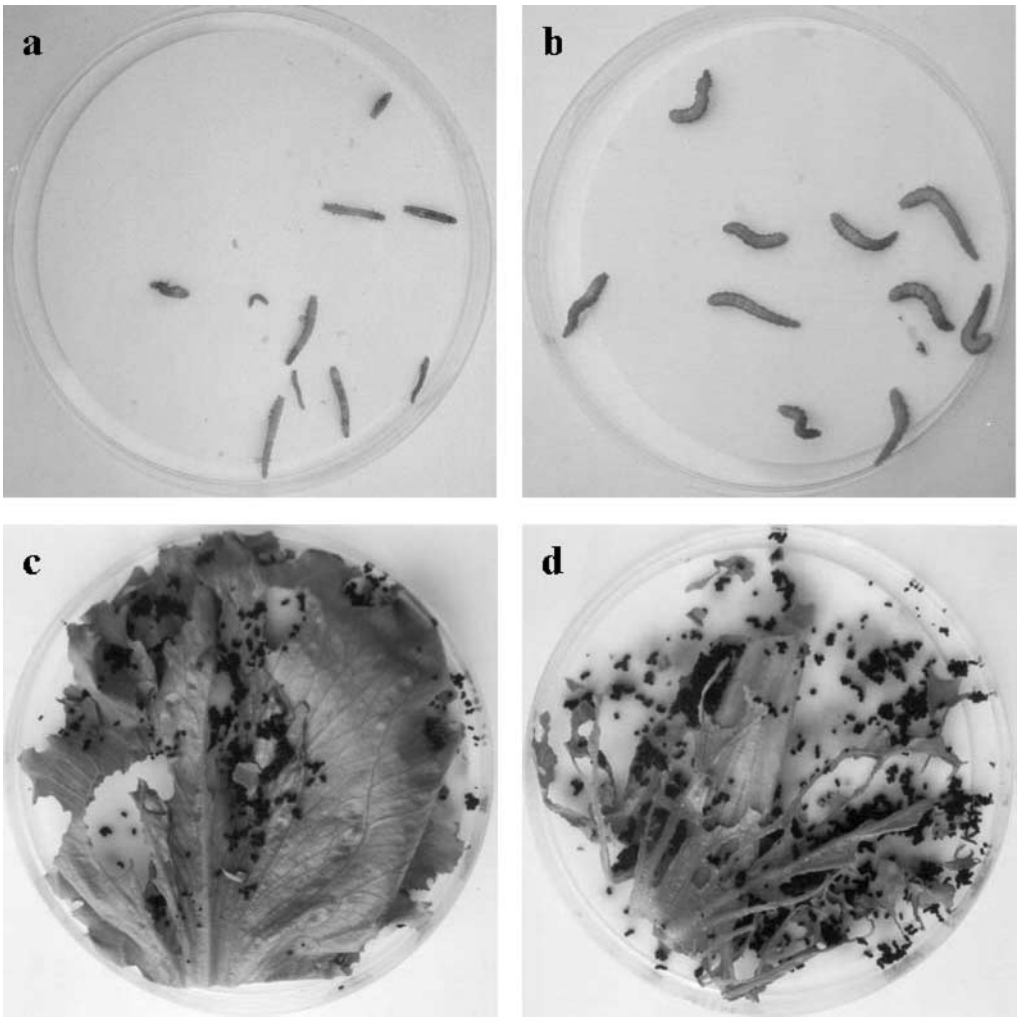

FIG. 2. Cabbage loopers (*T. ni*) fed on leaves of transgenic (TL1) and wild-type lettuce. *a*, *T. ni* larvae fed 8 d on leaves of transgenic lettuce (TL1); *b*, *T. ni* larvae fed 8 d on leaves of wild-type lettuce; *c*, resultant leaves of TL1 R_0 plants on day 8 of feeding trials; *d*, resultant leaves of wild-type lettuce on day 8 of feeding trials.

cells suggests that they may protect the embryo from the surrounding PCD-associated proteases. Also, the activities of these proteinases and their inhibitors regulate the release of protein reserves from the nucellar cells to the embryo.

Generation and analysis of SaPIN2a- *and* SaPIN2b-*silenced RNAi (PIN2–RNAi) plants in the study of seed development*. The endogenous functions of SaPIN2a and SaPIN2b in flower and seed development were further studied by transforming *S. americanum* with a hairpin double-stranded RNA construct to target degradation of endogenous *SaPIN2a* and *SaPIN2b* transcripts (Sin et al., 2006). Agrobacterium-mediated transformation of *S. americanum* was carried out according to Horsch et al. (1985) using small pieces of detached cotyledons from germinated 2-wk-old *S. americanum* seedlings (Sin et al., 2006). RNA of ~23 nucleotides was detected by Northern blot analysis on four independent PIN2–RNAi transformed lines following confirmation by PCR and Southern blot analyses (Sin et al., 2006). Also, Northern blot analyses of total RNA from flowers and seeds from these transformed lines using *SaPIN2a*- and *SaPIN2b*-specific probes showed that *SaPIN2a* and *SaPIN2b* mRNAs were reduced in flowers compared to wild-type (Sin et al., 2006).

SaPIN2a and *SaPIN2b* mRNAs and their corresponding protein were not expressed in ovules of senescent flowers from PIN2–RNAi lines using *in situ* hybridization studies and immunostains (Sin et al., 2006). In comparison, *SaPIN2a* mRNA and protein were detected in the innermost layer of the wild-type ovule and the developing endothelium, while *SaPIN2b* mRNA and protein were detected in the layers immediately adjacent to the developing endothelium. The inner cell layers of ovules consist of the inner integument which differentiates into the seed coat during seed development. SaPIN2b protein was not detected during wild-type seed maturation while SaPIN2a expression was strong and was present in the embryo and endothelium. In the endosperm, neither SaPIN2a nor SaPIN2b proteins were detected (Sin et al., 2006).

Phenotypic differences include the occurrence of fewer seeds in the young and mature fruits from PIN2–RNAi lines compared to those from wild-type, and approximately 80% of total seeds per fruit were aborted in three PIN2–RNAi lines (Sin et al., 2006). Examination of histological sections of young fruit from PIN2–RNAi lines indicated a lack of normal development of the seed coat and endosperm in the aborted seeds of these PIN2–RNAi lines

(Sin et al., 2006). As a consequence, embryos were lacking in the aborted seeds. During normal seed development, the integumentary tapetum becomes the endothelium of the seed coat but, in the PIN2–RNAi lines, vacuolation occurred in this tissue and this continued throughout the early stages of seed development. It has been reported that observations of such vacuolation are indicative of PCD (Wu and Cheung, 2000). As a consequence, the seed coat endothelium, which normally differentiates from the integumentary tapetum, was defective in phenotypically abnormal PIN2–RNAi seeds (Sin et al., 2006). The anomalous expansion of the endothelial cells encroached into the endosperm cavity and prevented further endosperm development (Sin et al., 2006). The absence of a proper seed coat endothelium in PIN2–RNAi lines could have adversely affected the nutritional support to the endosperm and embryo because normal embryogenesis in these PIN2–RNAi lines was absent (Sin et al., 2006). In wild-type *S. americanum* seeds, SaPIN2a is expressed in the developing embryo and seed coat endothelium in seeds (Sin et al., 2006). The lack of seed set in PIN2–RNAi lines could not have been due to pollen viability or pollen tube germination because further investigations proved that greater than 90% of pollen from both wild-type and PIN2–RNAi lines were viable (Sin et al., 2006).

The study by Sin et al. (2006) also suggested that proper development of the endosperm, embryo, and maternal tissues are interdependent events during embryogenesis and seed formation. Other studies have also demonstrated that a proper coordination in the development of the embryo, the endosperm, and the maternal seed coat is a prerequisite to normal seed formation (Vielle-Calzada et al., 1999). It has been reported that a reduction in the petunia floral binding proteins FBP7 and FBP11 in sporophytic tissues of ovules caused endothelium degeneration that impaired endosperm development due to the lack of nutrient distribution from the seed coat to the developing endosperm and embryo (Colombo et al., 1997).

The majority of the seeds were aborted due to defective seed coat endothelium formation that led to abnormal endosperm development (Sin et al., 2006). Also, embryos were lacking in the aborted seeds (Sin et al., 2006). Hence, proteinase inhibitors present in the developing seeds of *S. americanum* could play a role in the protection of the endosperm and embryo by regulating the proteases generated within the seed (Sin et al., 2006). Expression of SaPIN2a and SaPIN2b in the inner cell layers of the ovules (the inner integument) would prevent the occurrence of PCD. Such innermost cell layers also lacked TUNEL-positive signals, in contrast to the nucellar cells, suggesting that SaPIN2a and SaPIN2b are strategically located in protecting the embryo sac from PCD-associated proteases found in the surrounding nucellar cells (Sin and Chye, 2004). Wan et al. (2002) have reported a PCD-associated cysteine proteinase present in the inner integument of developing *Brassica napus* seeds. The presence of proteinases may signify the occurrence of PCD within this tissue. Taken together, it appears that inhibitors to these proteinases would function in regulating the action of these proteinases generated from the seed coat during seed development and in protecting the developing endosperm and embryo. It appears that SaPIN2a is the major proteinase inhibitor in ovule and subsequent seed development, while SaPIN2b functions mainly during ovule development (Sin et al., 2006).

While much progress has been made in the area of embryo and endosperm development (Meinke, 1995; Yeung et al. 2001), less is known on the development of the seed coat. Normal embryogenesis is dependent on the proper development of the endosperm, which acts as a nutrient source, since embryo development is known to proceed rapidly following the enlargement and differentiation of the endosperm (Lopes and Larkins, 1993; Costa et al., 2004). The seed coat develops from the integuments of an ovule and its initial development is rapid with active mitosis, followed by tissue differentiation and cell expansion (Yeung, 1983; Boesewinkel and Bouman, 1984). The innermost layer of the seed coat, the endothelium, originates from the integumentary tapetum of the ovule (Boesewinkel, 1980; Yeung and Cavey, 1990). This layer of cells serves as the boundary between the maternal tissues and the developing embryo and structural specializations have been noted. The cells of the inner region of the seed coat begin to degenerate following the establishment of the tissue pattern (Yeung and Cavey, 1990). The seed coat protects the embryo during its development and germination. Since there is no direct vascular connection between the embryo and the maternal tissues, the seed coat has to play a nutritive role and may even control the development of the embryo. It has been demonstrated that the seed coat transports metabolites into the developing embryo (Murray, 1988; Walker et al., 1995). Proteinases may play a role in embryo nutrition by participating in the breakdown and/or modification of macromolecules. That degenerative structural changes do occur within the seed coat during seed maturation (Yeung and Cavey, 1990) is not surprising, since PCD occurs within the seed coat in seed development. The presence of proteinase inhibitors in *S. americanum* seeds suggests their involvement in modulating endogenous proteolysis and hence, in protecting the developing embryo from proteinase action.

Concluding Remarks

Using a transgenic system, the exogenous function of SaPIN2a was demonstrated by observations of enhanced protection to cabbage looper (*T. ni*) caterpillars in transgenic lettuce (Xu et al., 2004). Furthermore, the endogenous functions of SaPIN2a and SaPIN2b were also investigated by observations of inhibition of endogenous trypsin- and chymotrypsin-like activities in these transgenic lettuce lines as well as by the generation of PIN2–RNAi *S. americanum* lines (Sin et al., 2006). These PIN2–RNAi transgenic lines were down-regulated for both *SaPIN2a* and *SaPIN2b* and showed abnormal seed development (Sin et al., 2006). Hence, serine proteinase inhibitors are endogenously essential in seed development. Other proteinase inhibitors may have similar important roles to play in plant development.

The expression of PIN2 proteins not only confers insect resistance in transgenic plants, due to its exogenous activities on gut enzymes of caterpillars, but can now be extended, as demonstrated in transgenic lettuce by SaPIN2a expression, to protect against chymotrysin- and trypsin-like activities of plant endogenous proteases (Xu et al., 2004). Such applications of PIN2 need further optimization as plant transformation techniques (Birch, 1997) are progressively exploited in molecular farming for the production of desirable proteins, including biopharmaceuticals (Ma et al., 2003). New evidence on the role of PIN2 proteins in modulating protease activities during seed development and embryogenesis will lead to investigations on the effects of PIN2 expression in the regeneration process, which is often the stumbling

block in further transgenic plant generation. *In vitro* systems may well serve as excellent model systems to study the endogenous functions of PIN2 proteins and how these proteins are regulated.

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