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ORIGINAL ARTICLE

Zeng-Fu Xu · Whei-Lan Teng · Mee-Len Chye

Inhibition of endogenous trypsin- and chymotrypsin-like activities in transgenic lettuce expressing heterogeneous proteinase inhibitor SaPIN2a

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Abstract SaPIN2a, a proteinase inhibitor II from American black nightshade (*Solanum americanum* Mill.) is highly expressed in the phloem and could be involved in regulating proteolysis in the sieve elements. To further investigate the physiological role of SaPIN2a, we have produced transgenic lettuce (*Lactuca sativa* L.) expressing SaPIN2a from the *CaMV35S* promoter by *Agrobacterium*-mediated transformation. Stable integration of the *SaPIN2a* cDNA and its inheritance in transgenic lines were confirmed by Southern blot analysis and segregation analysis of the R_1 progeny. *SaPIN2a* mRNA was detected in both the R_0 and R_1 transformants on northern blot analysis but the SaPIN2a protein was not detected on western blot analysis using anti-peptide antibodies against SaPIN2a. Despite an absence of significant inhibitory activity against bovine trypsin and chymotrypsin in extracts of transgenic lettuce, the endogenous trypsin-like activity in each transgenic line was almost completely inhibited, and the endogenous chymotrypsin-like activity moderately inhibited. Our finding that heterogeneously expressed SaPIN2a in transgenic lettuce inhibits plant endogenous protease activity further indicates that SaPIN2a regulates proteolysis, and could be potentially exploited for the protection of foreign protein production in transgenic plants.

Keywords Chymotrypsin · *Lactuca* · Protease · Proteolysis · *Solanum* · Trypsin

Abbreviations *CaMV* cauliflower mosaic virus · *cDNA* complementary DNA · *NOS* nopaline synthase · *PAGE* polyacrylamide gel electrophoresis · *PI* proteinase inhibitor · *SaPIN2a Solanum americanum* proteinase inhibitor IIa · *SDS* sodium dodecyl sulphate · *T-DNA* transferred DNA

Z. Xu · W. Teng · M. Chye (✉)
Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong, China
E-mail: mlchye@hkucc.hku.hk
Fax: +852-28583477

Present address: Z. Xu
Key Laboratory of Gene Engineering of the Ministry of Education, Zhongshan (Sun Yat-sen) University, Guangzhou 510275, China

Introduction

It has been proposed that plant proteinase inhibitors (PIs) which act on animal or microbial proteases play a role in the inhibition of proteolytic enzymes from pests or pathogens (Ryan 1981, 1989; Brzin and Kidric 1995). These conclusions arise 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. However, none of these proteases is likely to be the true physiological target of these plant PIs (Laskowski and Kato 1980; Brzin and Kidric 1995).

Reports on the developmental regulation and tissue-specific accumulation of plant PIs (Rosahl et al. 1986; Sanchez-Serrano et al. 1986; Margossian et al. 1988; Hendriks et al. 1991; Pena-Cortes et al. 1991; Lorberth et al. 1992) do suggest they have endogenous functions. A soybean cysteine proteinase inhibitor has been designated a novel role in modulating programmed cell death (Solomon et al. 1999). Another, from cucumber leaves, which does not significantly inhibit commercial proteases of animal or microbial origin, inhibits cucumber glutamyl endopeptidase, suggesting regulation of its activity (Yamauchi et al. 2001).

Recently, we have shown that a *Solanum americanum* proteinase inhibitor II, SaPIN2a, is highly expressed in phloem (Xu et al. 2001). The localization of *SaPIN2a* mRNA and protein to the companion cells and sieve elements suggests a role in the regulation of proteolysis in phloem development/function. To further investigate, we have expressed SaPIN2a in transgenic lettuce and, here, we show that its heterogeneous expression results in the inhibition of plant endogenous protease activity.

Lettuce was chosen for expression of SaPIN2a because it neither possesses detectable trypsin inhibitory activity in its leaves nor responds to any treatments by accumulating inhibitors (Walker-Simmons and Ryan 1977).

Materials and methods

Plant material and growth conditions

Seeds of lettuce (*Lactuca sativa* L. cv. Great Lakes No.118) were obtained from Northrup King Co., Minneapolis, MN, USA. Plant tissue cultures were maintained and propagated in vitro in a growth chamber at 22–24 °C under a 12 h light/12 h dark regime. Plants in soil were grown under natural conditions in a greenhouse.

Generation of transgenic lettuce plants expressing SaPIN2a

The *Agrobacterium tumefaciens*-mediated transformation vector pSa7 was constructed by replacing the *β*-glucuronidase (*GUS*) gene (Jefferson et al. 1987) in pBI121 (Clontech) with the *SaPIN2a* cDNA (Xu et al. 2001). To confirm the absence of any spurious ATG codon between the transcription start site (Jefferson et al. 1987) and the SaPIN2a start codon, this region was sequenced with a primer (5′-CAATCCCACTATCCTTCGCAAGACC-3′) located within the cauliflower mosaic virus 35S (*CaMV35S*) promoter. The binary vector pSa7 was introduced into *A. tumefaciens* LBA4404 by direct transformation (Holsters et al. 1978). Lettuce was transformed according to Curtis et al. (1994) with modifications: petunia nurse cell cultures were omitted from the callus-inducing medium and kanamycin sulphate (100 μg/ml) was added to the media for callus induction, shoot regeneration and rooting.

Segregation analysis of the progeny (R_1) plants of transgenic lettuce

The segregation ratios of kanamycin-resistant (Km^R) to kanamycin-sensitive (Km^S) plants in the progeny (R_1) of self-fertilized primary (R_0) transgenic lettuce were determined by germinating surface-sterilized seeds from each R_0 line on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing kanamycin sulphate (100 μg/ml). After incubation for 2–3 weeks in a tissue-culture chamber (22–24 °C, 12 h light/12 h dark), the seedlings were scored for kanamycin resistance. The segregation ratios were assessed by Chi-square analysis.

Southern blot analysis

Twenty μg DNA, isolated (Dellaporta et al. 1983) from lettuce leaves, was digested with restriction endonucleases, separated by electrophoresis in a 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× standard saline citrate (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5× Denhardt's, 1% SDS, 50 μg/ml denatured, sonicated salmon sperm DNA at 42 °C for 4 h. Random-primed ^{32}P-labelled *SaPIN2a* cDNA was added for hybridization overnight at 42 °C. The blot was washed in 0.1× SSC, 0.1% SDS at room temperature.

Northern blot analysis

Total RNA was extracted (Nagy et al. 1988) from nightshade (*Solanum americanum* Mill.) plants, wild-type lettuce or transgenic lettuce and analyzed by northern blot analysis as previously described (Xu et al. 2001). The *SaPIN2a* cDNA was used in the generation of a random-primed ^{32}P-labelled probe.

Western blot analysis

Total plant protein was extracted according to the procedure of Wu et al. (1997). Protein concentration was determined following Bradford (1976). Total protein was separated by 4–20% gradient SDS–PAGE (Gallagher 1995) for western blot analysis (Sambrook et al. 1989) using polyclonal antibodies raised in rabbit against a synthetic peptide (GESDPRNPKDC) corresponding to amino acids 77–87 of SaPIN2a (Xu et al. 2001). The Amplified Alkaline Phosphatase Immun-Blot Assay Kit (Bio-Rad) was used in detection of cross-reacting bands.

Assay of trypsin and chymotrypsin inhibitory activities

Total plant proteins, extracted with 50 mM Tris (pH 8.1), 20 mM $CaCl_2$, were used in spectrophotometric assays of trypsin or chymotrypsin inhibitory activity as described by Kollipara and Hymowitz (1992).

In the trypsin inhibitory-activity assay, 150 μl of leaf extract was pre-incubated for 3 min at room temperature (RT) in a quartz cuvette (10 mm path length, 3.5 ml capacity) with 100 μl of bovine trypsin (10 μg/ml in 1 mM HCl; Calbiochem Cat. No. 6502) and assay buffer [46 mM Tris–HCl (pH 8.1), 11.5 mM $CaCl_2$], to give a final volume of 1.5 ml. The reaction was initiated by the addition of 1.5 ml of substrate [2 mM *p*-toluenesulphonyl-L-arginine methyl ester (TAME; Sigma Cat. No. T4626) in assay buffer] to the pre-incubation mixture. Recording of absorbance at 247 nm (A_{247}) was immediately initiated. The spectrophotometer was set to auto-zero just before the start of recording and absorbance was measured at 30-s intervals for 3 min. In the standard reaction, 100 μl of bovine trypsin (10 μg/ml in 1 mM HCl), in the absence of leaf extract, was pre-incubated with 1.4 ml of assay buffer.

In the chymotrypsin inhibitory-activity assay, 50 μl of leaf extract was pre-incubated for 3 min at RT with 100 μl of bovine α-chymotrypsin (20 μg/ml in 1 mM HCl; Calbiochem Cat. No. 230832) and assay buffer [0.1 M Tris–HCl (pH 7.8), 0.1 M $CaCl_2$], to give a final volume of 1.5 ml. The reaction was initiated by the addition of 1.5 ml of substrate [1 mM N-benzoyl-L-tyrosine ethyl ester (BTEE; Sigma Cat. No. B6125) in 50% (w/w) methanol] to the pre-incubation mixture. Absorbance at 256 nm (A_{256}) was similarly monitored as described above. In the standard reaction, 100 μl of chymotrypsin (20 μg/ml in 1 mM HCl), in the absence of leaf extract, was pre-incubated with 1.4 ml of assay buffer.

Assay of endogenous trypsin- and chymotrypsin-like activities

Endogenous trypsin- and chymotrypsin-like activities in leaves of wild-type and transgenic R_1 lettuce plants were determined using the same procedures as in the trypsin and chymotrypsin inhibitory-activity assays, with the omission of bovine trypsin or chymotrypsin from the reaction.

Results

Southern blot analysis of transgenic lettuce plants

Lettuce was transformed using an *A. tumefaciens* LBA4404 derivative harboring plasmid pSa7 (Fig. 1a), the binary vector expressing the *SaPIN2a* cDNA from the *CaMV35S* promoter. Southern blot analyses were used to confirm the integration of the *SaPIN2a* cDNA

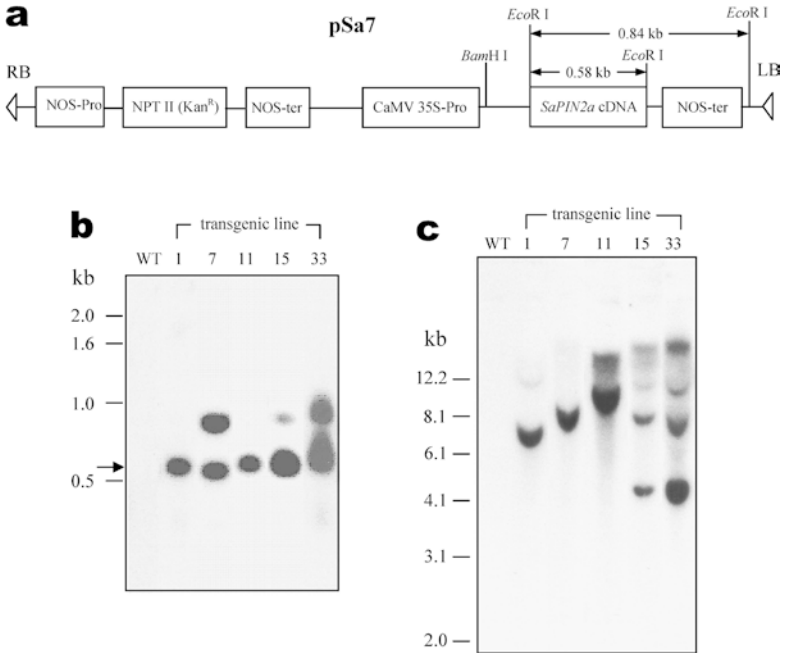

Fig. 1a–c The binary vector pSa7 used in lettuce (*Lactuca sativa*) transformation and Southern blot analysis of R_0 transgenic plants. **a** Structure of T-DNA region of pSa7. *RB*, right border of the transferred-DNA (T-DNA); *NOS-Pro*, nopaline synthase (*NOS*) promoter; *NPT II*, neomycin phosphotransferase II gene encoding resistance to kanamycin (Kan^R); *NOS-ter*, *NOS* terminator; *CaMV35S-Pro*, promoter of cauliflower mosaic virus (CaMV) *35S* RNA; *SaPIN2a*, *Solanum americanum* proteinase inhibitor IIa; *LB*, left border of the T-DNA. **b, c** Twenty μg of genomic DNA was digested with *Eco*RI (**b**) or *Bam*HI (**c**) and fractionated on a 0.8% agarose gel. The DNA was blotted and probed to ^{32}P-labelled random-primed

in the lettuce genome and to estimate the copy number of *SaPIN2a* cDNA in the various transgenic lines.

Results from Southern blot analysis using *Eco*RI-digested DNA of transgenic and regenerated (R_0) lettuce lines, TL1, 7, 11, 15 and 33, show the presence of an expected 0.58-kb *Eco*RI band (indicated by an arrow in Fig. 1b), suggesting integration of the *SaPIN2a* cDNA in the lettuce genome. This band was lacking in wild-type lettuce (Fig. 1b, lane WT). Additional strong hybridization bands in TL7 and 33 may be due to incomplete *Eco*RI digestion of genomic DNA or the presence of rearranged copies of the *SaPIN2a* cDNA in these lines.

Since only one *Bam*HI site is present between the *35S* promoter and the *SaPIN2a* cDNA within the transferred DNA (T-DNA) region on pSa7 (Fig. 1a), the number of bands hybridizing to ^{32}P-labelled *SaPIN2a* cDNA on Southern blot analysis of *Bam*HI-digested DNA should give a good estimate of the transgene copy number. Different hybridization patterns seen with *Bam*HI-digested DNA (Fig. 1c) implied that the various transgenic lines resulted from independent transformation events. Also, the number of strongly hybridizing bands ranged from one (TL1, 7, 11) to three (TL33), corresponding to single or multiple copies of transgene in these lines. Single copies of *SaPIN2a* cDNA in TL1 and 11, and multiple copies in TL33, were further confirmed by segregation analysis of their progenies (Table 1).

Expression of SaPIN2a mRNA in transgenic lettuce

The transcription of *SaPIN2a* in R_0 and R_1 transgenic lettuce lines was examined by northern blot analysis. Total RNA from leaves of R_0 transgenic plants, identified by Southern blot analysis (Fig. 1), was hybridized to ^{32}P-labelled *SaPIN2a* cDNA. As shown in Fig. 2a, *SaPIN2a* mRNA was detected in the transgenic lines TL1, 7, 11, 15 and 33, but not in wild-type lettuce. The *SaPIN2a* transcript in transgenic lettuce (0.93 kb, indicated by an arrow in Fig. 2a) was slightly larger than the endogenous transcript of 0.67 kb in *S. americanum* (indicated by an arrowhead in Fig. 2a, lane Sa), due to the additional 0.26 kb from the nopaline synthase (*NOS*) terminator (Fig. 1a). We observed a more complex mRNA expression pattern in the R_1 transgenic lines, with the presence of a shorter transcript below the expected 0.93-kb band (Fig. 2b). It is worth noting that the shorter transcript found in leaves of some R_0 plants (Fig. 2a) became more prominent in leaves of R_1 plants (Fig. 2b).

Non-detection of SaPIN2a protein in transgenic lettuce by western blot analysis

Total leaf proteins from R_0 transgenic lettuce, expressing *SaPIN2a* mRNA on northern blot analysis (Fig. 2a), were

Table 1 Segregation of the NPT-II gene (kanamycin resistance) in the progeny of transgenic lettuce (*Lactuca sativa*) plants

Parent transgenic line (R_0)	Progeny (R_1) plants[a]		Expected ratio[b]	Chi-square	Probability
	Kan^R	Kan^S	(Kan^R:Kan^S)		
TL1	67	21	3:1	0.061	1.000
TL11	30	10	3:1	0.000	1.000
TL33	176	3	255:1	7.587	0.667
			63:1	0.015	1.000

[a] Kan^R = kanamycin-resistant; Kan^S = kanamycin-sensitive
[b] Ratio 3:1 is for a single functional locus, 255:1 for four functional loci and 63:1 for three functional loci

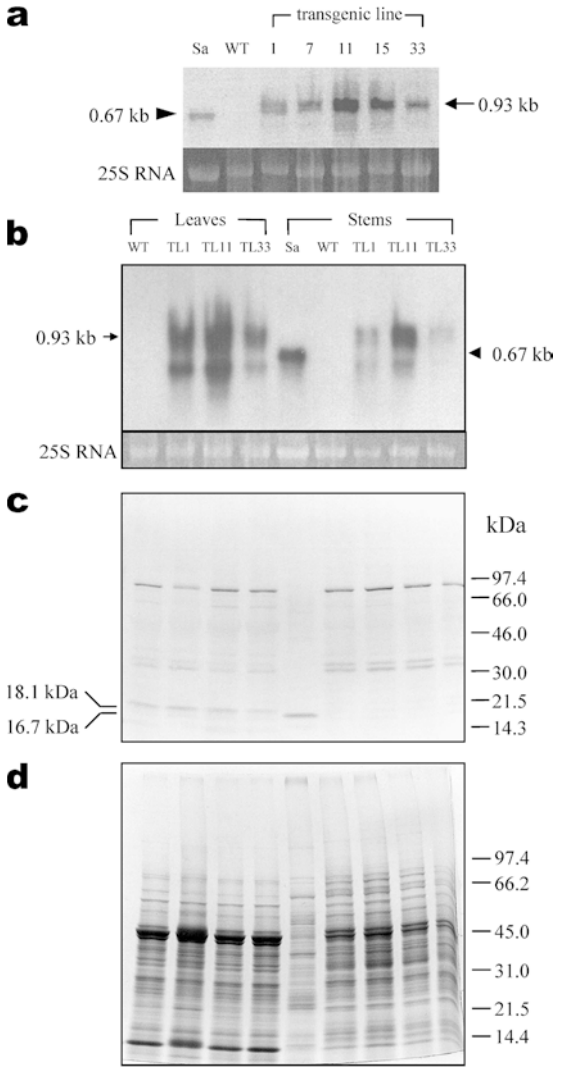

Fig. 2a–d Northern blot and western blot analyses on transgenic lettuce plants. For northern blot analysis (**a, b**), the blots were probed with random-primed ^{32}P-labelled *SaPIN2a* cDNA. The hybridization band corresponding to the expected *SaPIN2a* transcript is indicated by an *arrowhead* (0.67-kb mRNA in *S. americanum* stems) or an *arrow* (0.93-kb mRNA in transgenic lettuce). The 25S ribosomal RNA (rRNA) bands stained with ethidium bromide are shown (*bottom panel*) to indicate the amount of total RNA loaded per lane. **a** Northern blot analysis of RNA from R_0 transgenic lettuce lines. Total RNA (20 μg) was isolated from leaves of wild-type (*WT*) or transgenic lettuce and from *S. americanum* (*Sa*) stems as a positive control. **b–d** Total RNA (20 μg) and total protein (20 μg) were isolated from identical leaves and stems of 38-day R_1 transgenic (*TL1*, *TL11*, *TL33*) and wild-type (*WT*) lettuce plants. Total RNA (20 μg) and total protein (14 μg) isolated from *S. americanum* stems (*Sa*) were included as positive controls. **b** Northern blot analysis of RNA from R_1 transgenic lettuce plants. **c** Western blot analysis of protein from R_1 transgenic lettuce plants using SaPIN2a-specific antibodies. Cross-reacting bands in lettuce leaves (18.1 kDa) and *S. americanum* stems (16.7 kDa) are indicated. **d** Coomassie blue stain of total protein from transgenic and wild-type plants separated on a 4–20% gradient SDS–PAGE gel to demonstrate amounts of total protein loaded in **c**

used in western blot analysis with affinity-purified SaPIN2a-specific antibodies. However, SaPIN2a could not be detected in the leaf proteins from these R_0 transgenic lines (data not shown). Western blot analysis was also carried out using total proteins from R_1 plants from three self-pollinated R_0 lines (TL1, 11 and 33). Figure 2c shows the results of western blot analysis on these R_1 plants, using tissue samples identical to those used in the northern blot shown above (Fig. 2b). Despite very high levels of *SaPIN2a* mRNA in leaves of these transgenic lines (Fig. 2b), no protein band corresponding to SaPIN2a in *S. americanum* stem (Fig. 2c, lane Sa) was detected in transgenic lettuce leaf proteins (Fig. 2c). Some non-specific cross-reacting bands were observed and one (18.1 kDa), present in lettuce leaves but not stems, is very close to the size of native SaPIN2a (16.7 kDa; Fig. 2c). Since native SaPIN2a in *S. americanum* accumulates in stem (Xu et al. 2001), we were interested to investigate whether the cellular transport of SaPIN2a may have accounted for its apparent absence in the transgenic leaves. Hence, northern blot and western bolt analyses were carried out using stems from these transgenic lettuce plants. Although *SaPIN2a* mRNA was expressed in their stems (Fig. 2b) at lower amounts than leaves, SaPIN2a protein remained undetected on western blots with SaPIN2a-specific antibodies (Fig. 2c).

Assay of trypsin and chymotrypsin inhibitory activities

Despite verification of the *SaPIN2a* sequence in pSa7 by DNA sequence analysis and confirmation of the integration of its cDNA by Southern blot analysis (Fig. 1b, c) and of its mRNA expression by northern blot analysis (Fig. 2a, b), the SaPIN2a protein was not observed on western blot analysis. The relatively low sensitivity of western blot analysis may have resulted in the non-detection of SaPIN2a in these transgenic lines. Hence, to further examine for the presence of SaPIN2a, we used proteinase inhibitory-activity assays, which we hoped would prove more sensitive than western blot analysis.

Crude leaf extracts from R_1 transgenic and wild-type plants were tested for inhibitory activity against bovine trypsin and chymotrypsin. Results of trypsin inhibitory-activity assays (Fig. 3a) showed no significant inhibitory activity against bovine trypsin in transgenic lettuce. Leaf extracts from all three transgenic lines analyzed (TL1, 11, 33) showed trypsin activity similar to that of the standard reaction. Surprisingly, leaf extracts of wild-type plants had much higher trypsin activity than the standard reaction containing bovine trypsin (Fig. 3a). In the case of assay of chymotrypsin inhibitory activity (Fig. 3b), reactions of leaf extracts from both transgenic and wild-type plants showed higher chymotrypsin activity than the standard; each transgenic line showed slightly decreased activity compared to wild-type. These results of the inhibitory-activity assays suggest that these transgenic lettuce leaves may possess considerable endogenous trypsin- and chymotrypsin-like activities.

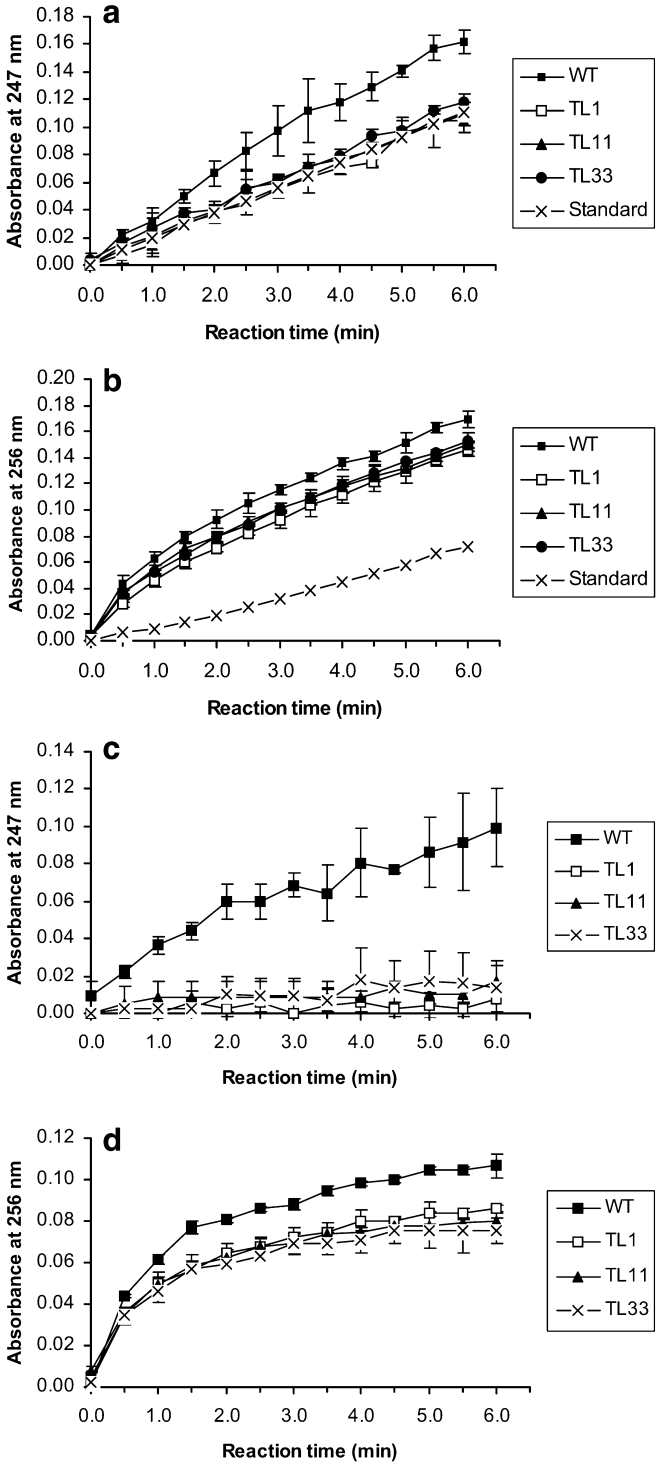

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Fig. 3a–d Assays of trypsin- and chymotrypsin inhibitory activities and trypsin- and chymotrypsin-like activities on transgenic lettuce leaf extracts. Leaf extracts were prepared from 54-day-old wild-type (*WT*) or transgenic (*TL1*, *TL11*, *TL33*) R_1 plants. Each value represents the mean ± SE of three replicates. **a** Trypsin inhibitory-activity assay. One μg of bovine trypsin (Calbiochem) was incubated with 150 μl of assay buffer (standard) or leaf extracts and the residual trypsin activity was determined by measuring the increase of absorbance at 247 nm during substrate hydrolysis. **b** Chymotrypsin inhibitory-activity assay. Two μg of bovine α-chymotrypsin (Calbiochem) was incubated with 50 μl of assay buffer (standard) or leaf extract and the residual chymotrypsin activity was determined by measuring the increase of absorbance at 256 nm during substrate hydrolysis. **c** Trypsin-like activity assay. Leaf extract (150 μl) was directly incubated with substrate in the absence of bovine trypsin. Trypsin-like activity was determined by measuring the increase in absorbance at 247 nm during substrate hydrolysis. **d** Chymotrypsin-like activity assay. Leaf extract (50 μl) was directly incubated with substrate in the absence of bovine α-chymotrypsin. Chymotrypsin-like activity was determined by measuring the increase in absorbance at 256 nm during substrate hydrolysis

Assay of endogenous trypsin- and chymotrypsin-like activities

The endogenous trypsin- and chymotrypsin-like activities in leaves of wild-type and transgenic R_1 lettuce plants were determined using the same procedures as in the assays of proteinase inhibitory activity, with the omission of bovine trypsin or chymotrypsin from the reaction. Results of these experiments are shown in Fig. 3c, d. Both endogenous trypsin- and chymotrypsin-like activities were detected in leaf extracts from wild-type lettuce. However, the assay results from transgenic leaves were unexpected and interesting. The endogenous trypsin-like activities in all three transgenic plants were almost completely inhibited (Fig. 3c), while the endogenous chymotrypsin-like activities in these lines decreased moderately (Fig. 3d). The inhibition of endogenous trypsin- and chymotrypsin-like activities in transgenic lettuce leaves suggests that SaPIN2a protein accumulated at amounts that could inhibit endogenous trypsin- and chymotrypsin-like activities, despite being undetected on western blot analysis.

Discussion

We have demonstrated that the heterogeneous expression of a plant PI, SaPIN2a, in transgenic lettuce inhibits endogenous trypsin- and chymotrypsin-like activities. The significant inhibition of trypsin-like activity and the moderate inhibition of chymotrypsin-like activity in transgenic lettuce, resulting from the expression of SaPIN2a, could not be due to a mutation caused by the T-DNA insertion because all three independent lines (TL1, 11, 33) tested showed similar inhibition. This finding further supports our previous hypothesis that SaPIN2a has an endogenous role in regulating the activity of endogenous proteases in the phloem (Xu et al. 2001). Since it has been shown that the yield and quality of antibodies produced in transgenic plants are significantly affected by endogenous proteolytic degradation (Stevens et al. 2000), heterogeneous expression of PIs could be exploited in the protection of foreign protein production in transgenic plants. The identification of the target endogenous proteases for SaPIN2a would be our future goal. So far, only a trypsin-like enzyme and its endogenous inhibitor have been identified in lettuce seeds (Shain

and Mayer 1965, 1968). In barley, an endogenous protease, a carboxypeptidase, has been shown to hydrolyse a number of ester substrates of trypsin and chymotrypsin (Mikola and Pietila 1972).

The difference in the levels of *SaPIN2a* mRNA accumulation in leaves and stems of transgenic lettuce plants (Fig. 2b) was unexpected. The *CaMV35S* promoter is generally regarded as a strong constitutive promoter and directs high-level transcription in nearly all plant organs (Nagy et al. 1985; Odell et al. 1985). Our observations, taken together with those of others (Jefferson et al. 1987; Benfey et al. 1989; Williamson et al. 1989; Yang and Christou 1990; Sunilkumar et al. 2002), suggest that the *CaMV35S* promoter need not be constitutive.

It has been reported that the expression of transgenes in lettuce is not as efficient as in other transgenic plants (McCabe et al. 1999; Ryder 1999). The mechanism of low transgene expression in lettuce is not well understood and may be associated with DNA methylation (McCabe et al. 1999). Observations on the lack of foreign protein accumulation despite over-expression of its corresponding mRNA have also been reported in transgenic petunia (Jones et al. 1985), tobacco (Jones et al. 1985; Florack et al. 1994), tomato (Seymour et al. 1993), cauliflower (Passelegue and Kerlan 1996) and potato (Gatehouse et al. 1997). In this study, we detected shorter transcripts other than that expected of the *SaPIN2a* mRNA using the *NOS* terminator in transgenic lettuce. This suggests that the mRNA may not have undergone proper processing and could have been further degraded. Inadvertently, this may be a contributing factor to the failure in the detection of SaPIN2a protein in transgenic lettuce on western blot analysis, despite high accumulation of the *SaPIN2a* mRNA.

Whether SaPIN2a possesses the putative inhibitory activity towards bovine trypsin and chymotrypsin, based on its sequence homology to known PIN2 proteins, is still unclear. One possibility is that the quantity of SaPIN2a protein in leaves of transgenic lettuce is insufficient, as indicated by western blot analysis, for assay of in vitro inhibitory activity using bovine trypsin or chymotrypsin. Nonetheless, this amount of SaPIN2a is sufficient to inhibit endogenous trypsin-like activity in lettuce leaves. An alternative explanation is that SaPIN2a could be specific to certain plant endogenous proteases but not to bovine trypsin and chymotrypsin. Protease inhibitors from soybean (Birk et al. 1963) and wheat (Applebaum and Konijn 1966) that inhibited larval gut proteolysis of *Tribolium castaneum* were inactive towards mammalian trypsin and chymotrypsin. The maize proteinase inhibitor (MPI), belonging to the potato proteinase inhibitor I (PIN1) family (Cordero et al. 1994), effectively inhibited midgut chymotrypsin of *Spodoptera littoralis* larvae, but only weakly inhibited bovine chymotrypsin, unlike most PIN1 members which are potent inhibitors of mammalian chymotrypsin (Tamayo et al. 2000). To unequivocally establish the inhibitory activities of SaPIN2a, purified SaPIN2a protein from *S. americanum* stems should be employed for further in vitro activity assays.

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