A multicomponent, elicitor-inducible cystatin complex in tomato, *Solanum lycopersicum*

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Summary

- We assessed the ability of the fungal elicitor arachidonic acid to induce cystatin genes in tomato (*Solanum lycopersicum*), using a cDNA expression library from arachidonate-treated leaves.
- The cDNAs of two novel cystatins were isolated, coding for an approx. 11-kDa protein, *Sl*CYS10; and for a 23.6-kDa protein, *Sl*CYS9, bearing an N-terminal signal peptide and a long, 11.5-kDa extension at the C terminus. Both genes were

induced by arachidonate but not by methyl jasmonate, an inducer of the 88-kDa eight-unit cystatin, multicystatin, accumulated in the cytosol of leaf cells upon herbivory.

- A truncated form of *Sl*CYS9, t*Sl*CYS9, was produced by deletion of the C-terminal extension to assess the influence of this structural element on the cystatin moiety. As shown by kinetic and stability assays with recombinant variants expressed in *Escherichia coli*, deleting the extension influenced both the overall stability and inhibitory potency of *Sl*CYS9 against cysteine proteases of herbivorous organisms.
- These findings provide evidence for a multicomponent elicitor-inducible cystatin complex in tomato, including at least 10 cystatin units produced via two metabolic routes.

Introduction

Protease inhibitors of the cystatin protein superfamily regulate proteolysis in various biological processes (Turk *et al*., 1997; Arai *et al*., 2002). Cystatins form a tight, reversible complex with cysteine proteases, acting as pseudosubstrates to enter the active site cleft of target enzymes and cause inhibition. Several roles have been attributed to cystatins in plants, including the control of endogenous cysteine proteases in physiological and developmental processes as diverse as organogenesis, seed development and maturation, storage protein turnover and programmed cell death (Kumar *et al*., 1999; Kuroda *et al*., 2001; Arai *et al*., 2002; Corre-Menguy *et al*., 2002; Belenghi *et al*., 2003; Rojo *et al*., 2004; Martinez *et al*., 2005a). Plant cystatins would also help plants to cope with abiotic stresses such as drought or cold temperatures, and inhibit the (exogenous) proteases of herbivorous organisms during herbivory or pathogenic infection (Pernas *et al*., 2000; Gaddour *et al*., 2001; Arai *et al*., 2002; Van der Vyver *et al*., 2003; Diop *et al*., 2004; Martinez *et al*., 2005b; Massonneau *et al*., 2005; Christova *et al*., 2006). Several lines of evidence suggest a significant role for cystatins in plant defense, including their inhibitory potency against the digestive cysteine proteases of herbivorous arthropods and parasitic nematodes (Zhao *et al*., 1996; Visal-Shah *et al*., 2001; Arai *et al*., 2002), their detrimental effects against pathogenic fungi (Pernas *et al*., 1999; Soares-Costa *et al*., 2002; Martinez *et al*., 2003, 2005b; Yang & Yeh, 2005; Christova *et al*., 2006), and the enhanced resistance of cystatin-expressing transgenic plants against herbivorous insects and pathogens (Guttierrez-Campos *et al*., 1999; Arai & Abe, 2000; Urwin *et al*., 2003; Outchkourov *et al*., 2004).

The induction of cystatin-encoding genes in leaves challenged with methyl jasmonate (MeJa), wounding or insect herbivory also support a protective role for plant cystatins (Bolter, 1993; Botella *et al*., 1996; Jacinto *et al*., 1998; Pernas *et al*., 2000; Wu & Haard, 2000; Belenghi *et al*., 2003; Bouchard *et al*., 2003). Current models for the stress-induced expression of protease inhibitors in plants point to the key role of α-linolenic acid, which

is released from cell membranes upon wounding, then metabolized via the octadecanoid signaling pathway to give jasmonic acid, an inducer of defense-related genes (Farmer & Ryan, 1992; Koiwa *et al*., 1997; Gatehouse, 2002). In Solanaceae, several protease inhibitors, including the serine-type inhibitors, proteinase inhibitors I (Pin-I) and II (Pin-II); the Kunitz inhibitor cathepsin D inhibitor; the inhibitor of metalloproteases, metallo-carboxypeptidase inhibitor; and the eight-unit cysteine-type inhibitor, multicystatin are induced in leaves by wounding, insect herbivory, systemin, jasmonate, MeJa and/or jasmonate analogues or precursors including α-linolenate (Farmer & Ryan, 1992; Hansen & Hannapel, 1992; Hildmann *et al*., 1992; Bolter, 1993; Werner *et al*., 1993; Jacinto *et al*., 1998; Gleddie & Michaud, 2000; Wu & Haard, 2000; Moura & Ryan, 2001; Bouchard *et al*., 2003; Diez-Diaz *et al*., 2004). To document further the role of cystatins as an active player in the plant's defensive machinery, we assessed the ability of the fungal elicitor arachidonic acid to induce the expression of cystatin-encoding genes in tomato (*Solanum lycopersicum*).

Arachidonate, released from germinating spores of the late blight fungus *Phytophthora infestans* and related oomycetes during plant infection (Ricker & Bostock, 1992), is a potent inducer of systemic resistance to pathogens in plants (Bostock *et al*., 1981, 1986; Cohen *et al*., 1991; Coquoz *et al*., 1995; Fidantsef *et al*., 1999). In Solanaceae, this polyunsaturated fatty acid elicits programmed cell death and systemic defense responses via an α-linolenate/jasmonate-independent route presumably involving salicylic acid (Coquoz *et al*., 1995; Yu *et al*., 1997; Knight *et al*., 2001). Genes encoding a circadian rhythm-regulated protein of unknown function, DEA1, and specific forms of 3-hydroxy-3-methylglutaryl coenzyme A reductases and family 1 pathogenesis-related (PR) proteins were shown to be induced by arachidonate while remaining uninduced by jasmonate or wounding (Bostock *et al*., 1992; Choi *et al*., 1992, 1994; Fidantsef & Bostock, 1998; Fidantsef *et al*., 1999; Rivard *et al*., 2004; Weyman *et al*., 2006). Here we describe the differential inducing effects of arachidonate and jasmonate on cystatin-encoding genes, and provide evidence for the occurrence of a multicomponent, elicitor-inducible cystatin complex in tomato leaves.

Materials and Methods

Proteases and inhibitors

Trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane (E-64), papain (from papaya latex, EC 3.4.22.2), phenylmethylsulfonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA) and pepstatin A were purchased from Sigma (Oakville, ON, Canada). LdP30, a digestive cystatin-sensitive protease from the coleopteran insect Colorado potato beetle (*Leptinotarsa decemlineata* Say), was purified by affinity chromatography from third-instar larvae reared on potato plants, using oryzacystatin as an affinity ligand (Visal-Shah *et al*., 2001). The secreted cysteine proteases Mhp1 and Mip1, from the root-parasitic nematodes *Meloidogyne hapla* and *Meloidogyne incognita*, were prepared from preparasitic J_2 larvae as described earlier (Michaud *et al*., 1996).

Plant material

Eight-wk-old glasshouse-grown tomato plants (*Solanum lycopersicum*) cv. Vendor were sprayed with 40 or 400 μm MeJa or arachidonate (Sigma) in 0.125% (v/v) Triton X-100. Control plants were treated with 0.125% (v/v) Triton X-100. After treatment, the plants were kept in different areas of the glasshouse to prevent cross-contamination between treatments. Leaves were harvested 0, 4, 8, 12, 16, 20 or 24 h after treatment, immediately frozen in liquid nitrogen, and stored at −80°C until use.

cDNA library construction and screening

A cDNA expression library was constructed with the ZAP Express cloning vector system (Stratagene, La Jolla, CA, USA) according to the supplier's instructions, using leaves harvested 16 h after treatment with 400 μm arachidonate as source of mRNA (see above). The library was screened with polyclonal antibodies raised in rabbits against purified potato multicystatin, according to Sambrook *et al*. (1989). After three rounds of purification, positive cDNAs were excised from the pBK-CMV phagemid vector. The plasmids were isolated using the Qiaprep Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada), and sequenced in both directions.

Phylogenetic reconstruction

Evolutionary relationships among tomato cystatins were assessed by reconstructing an unrooted phylogenetic tree with the DNA sequences of 26 plant cystatins (Table 1), including those isolated from the tomato leaf cDNA library. Cystatin gene sequences were first aligned using the multalin program (Corpet, 1988). An unrooted phylogenetic tree was then inferred from the alignments by the neighbor-joining distance method of Saitou & Nei (1987) using the Phylogenetic Inference Package (phylip) ver. 3.6, after generating a sequence similarity matrix based on Kimura's two-parameter model (Kimura, 1983).

Northern blot analysis

Total RNA (10 μg), isolated from control and treated leaves according to Logemann *et al*. (1987) was resolved into 1.2% (w/v) formaldehyde–agarose gels and blotted onto nitrocellulose membranes. The membranes were hybridized for 20 h with appropriate ^{32}P-labelle DNA probes and washed under stringent conditions. The filters were subject to autoradiography for 24 h at −80°C, using intensifying screens.

Heterologous expression in *Escherichia coli*

DNA sequences for the mature form of *Sl*CYS9 (with no peptide signal), the mature form of *Sl*CYS9 with no C-terminal extension (t*Sl*CYS9, for truncated *Sl*CYS9), and the eighth domain of tomato multicystatin (*Sl*CYS8, formerly LeCYS8; Kiggundu *et al*., 2006) were amplified using the following primers including *Bam*HI and *Eco*RI cleavage sequences:

5'-AAG GAT CCG CGA ACA GGG AAA ATC AGG AGG ATT CTG C-3'/5'-AGA ATT CTA GTT GTC AGG CTC CAT ACG ATT CAA GTG-3' for *Sl*CYS9; 5'-AAG GAT CCG CGA ACA GGG AAA ATC AGG AGG ATT CTG C-3'/5'AAG AAT TCT AGG TAG GAA CGT CTT CAA CAT GCT TGA A-3' for t*Sl*CYS9; and 5'-AAG GAT CCC AAA TCC TGG GGG CAT TAC CAA TGT TCC AT-3'/5'-AAG AAT TCA TTT CAC TTA GTG GCA TCA CCA ACA AGC TTG AAC TC-3' for *Sl*CYS8. After digestion with *Bam*HI and *Eco*RI, the PCR amplicons were inserted into the protein expression vector pGEX-3X (Amersham Biosciences, Baie d'Urfé, QC, Canada), in frame with the glutathione *S*-transferase (GST)-encoding gene. This vector was introduced into *E. coli* strain Y1091 by electroporation, and used to produce the cystatins as described earlier for other plant cystatins (Michaud *et al*., 1994). The GST affinity partner was removed from cystatins by cleavage with human factor X_a (Novagen, San Diego, CA, USA) according to the supplier's instructions. Purity of the preparations was confirmed by 12% SDS–PAGE. Protein concentrations were determined according to Bradford (1976), with bovine serum albumin as a standard.

Estimation of $K_{i(app)}$ values

The inhibitory activities of *Sl*CYS8, *Sl*CYS9 and t*Sl*CYS9 were assayed by estimating apparent dissociation constants ($K_{i(app)}$ values) for the complexes formed between these proteins and different cysteine proteases. $K_{i(app)}$ values for papain and Ldp30 were determined by the monitoring of hydrolysis progress curves, according to Salvesen & Nagase (1989). Both enzymes were assayed in 50 mm Tris–HCl pH 6.0 with Z-Phe-Arg-para-nitroanilide (Bachem, Torrance, CA, USA) as a substrate. Proteolysis was allowed to proceed at 37°C in reduced conditions (5 mm l-cysteine), after adding a minimal volume of 50 mm Tris–HCl pH 8.0 (ctrl) or of either cystatin dissolved in the same buffer. Activity levels were monitored every 30 s over 10 min at 405 nm, using a Spectronic 1000 Plus spectrophotometer (Milton Roy, Rochester, NY, USA). Approximate $K_{i(app)}$ values for Mhp1 and Mip1 were inferred by mildly denaturing gelatin/SDS–PAGE as described earlier (Michaud *et al*., 1996). Both enzymes were incubated with recombinant cystatins (5 pmole cystatin μl^{-1} nematode extract) for 10 min at 37°C before electrophoresis.

Cystatin stability assay

Cystatin stability in the presence of nontarget (insensitive) proteases was assessed by challenging *Sl*CYS8, *Sl*CYS9 and t*Sl*CYS9 with a third-instar midgut extract from the herbivorous insect Colorado potato beetle (Michaud *et al*., 1995). The purified cystatins were incubated for various periods with the insect extract (1 μg insect protein $pmole^{-1}$ cystatin). Proteolysis was stopped by adding SDS–PAGE sample buffer and incubating the whole mixture for 5 min at 100°C. Degradation of the cystatins was monitored on immunoblots after detection with antipotato multicystatin polyclonal antibodies. To identify proteases responsible for cystatin degradation, the insect extracts were preincubated for 30 min with either 100 μm E-64, 1 mm PMSF, 100 μm pepstatin A or 10 mm EDTA, before incubation with the cystatins (Michaud *et al*., 1995).

Results

The tomato genome encodes (at least) three evolutionarily distinct cystatins

A cDNA expression library was prepared from tomato leaves treated with 400 μm arachidonate as source material. Two screens of 30000 plaque-forming units yielded several clones expressing proteins recognized by antipotato multicystatin polyclonal antibodies. Sequencing and homology searches showed these clones, also retrieved from a cDNA library prepared from γ-linolenic acid-treated leaves (not shown), to encode three different cystatin-like polypeptides. Some clones included an open reading frame for a cystatin of 235 residues referred to as *Sl*CYS9 (GenBank accession no. AF198388), with a predicted signal peptide of 22 amino acids and a long, 103-aa extension at the C terminus (Fig. 1). Other clones encoded a 98-residue cystatin with no C-terminal extension, referred to as *Sl*CYS10 (accession no. AF198389). The last clones encoded polypeptides showing high homology with each of the eight inhibitory domains of potato multicystatin. Sequence alignments, cross-reactions with antimulticystatin antibodies and Northern blot analysis (see below) strongly suggest that these clones, including the entire eighth inhibitory domain *Sl*CYS8 (accession no. AF198390), encode parts of the MeJa-induced 88-kDa multidomain cystatin, multicystatin (Bolter, 1993).

Alignment of the three novel sequences with the model inhibitor oryzacystatin-I (OC-I, or *Os*CYS1 in this study; Abe *et al*., 1987), and with the eighth cystatin unit of potato multicystatin (PMC-8, or *St*CYS8; Waldron *et al*., 1993) revealed significant identity between all these cystatins, at least for the regions homologous to the 12-kDa cystatin, N-terminal moiety of *Sl*CYS9 (Fig. 2a). The sequence of *Sl*CYS9 corresponding to residues G_{39}–T_{132} displayed 67, 56, 52 and 51% identity with the corresponding sequences of *Sl*CYS10, *Os*CYS1, *Sl*CYS8 and *St*CYS8, respectively. The new polypeptides included the typical inhibitory motifs of cystatins, namely a –GG– motif in the N-terminal trunk, the central signature inhibitory motif –QxVxG– (where x is any amino acid) of the first inhibitory loop, and a W residue charateristic of the second inhibitory loop in the C-terminal region, approx. 30 aa distal from the central inhibitory motif. *Sl*CYS9 differed from the other cystatins by including a long, 11.5-kDa extension at the C terminus (Fig. 2a), similar to the extension of cystatins from other plants isolated in recent years (Fig. 2b).

Evolutionary relationships among tomato cystatins and cystatins from other species were visualized by inferring an unrooted phylogenetic tree for the cDNA sequences of 26 plant cystatins (Table 1), using the neighbor-joining distance method of Saitou & Nei, 1987 (Fig. 3). As expected, *Sl*CYS8 formed a clade with the fifth cystatin unit of tomato multicystatin (*Sl*CYS5) and the eight units of potato multicystatin (*St*CYS1–*St*CYS8), while *Sl*CYS9 formed a clade with cystatins from different plant families bearing the 11.5-kDa C-terminal extension. *Sl*CYS10, with no C-terminal extension, grouped with Solanaceae multicystatins, but also showed significant homology with cystatins of other

clades, suggesting the occurrence of at least three evolutionary distinct cystatin-encoding genes in the tomato genome.

_Sl_CYS8, _Sl_CYS9 and _Sl_CYS10 are differentially induced by MeJa and arachidonate

The inducing effects of MeJa and arachidonate on expression of the three cystatin genes in tomato leaves were investigated by Northern blotting. A probe prepared with the cDNA sequence of *Sl*CYS8 hybridized with an mRNA species approx. 2.5 kb in size (not shown), strongly suggesting that this cDNA was indeed encoding the C-terminal part of tomato multicystatin, homologous to the eighth inhibitory domain of potato multicystatin, *St*CYS8 (Waldron *et al*., 1993). As shown in Fig. 4(b,c), *Sl*CYS8 was strongly induced by MeJa, but weakly induced by arachidonate. By contrast, *Sl*CYS9 and *Sl*CYS10 transcripts were present at a basal level in nontreated leaves, not induced further by MeJa, but strongly induced by arachidonate (Fig. 4b,c) and other unsaturated fatty acids including linoleic acid and γ-linolenic acid (not shown). As a control, the blots were probed with labelled cDNAs encoding the wound-induced serine-type inhibitor, Pin-II, and the PR-1 protein, protein P4 (Fig. 4a). In agreement with previous reports (Fidantsef *et al*., 1999; Rivard *et al*., 2004), the gene for Pin-II was induced by MeJa but not by arachidonate, while the gene for protein P4 was induced by arachidonate but not by MeJa, suggesting that the genes coding for *Sl*CYS9, *Sl*CYS10 and protein P4 were responding in a similar way to the fungal elicitor, presumably via an α-linolenate/jasmonate-independent pathway (Fidantsef *et al*., 1999). Overall, these observations suggest that MeJa and arachidonate induce the accumulation of distinct cystatins in tomato leaves, via either jasmonate-dependent or -independent pathways.

The protease inhibitory profile of _Sl_CYS9 is influenced by its C-terminal extension

To determine whether structural elements such as the C-terminal extension of *Sl*CYS9 could influence the overall inhibitory profile of the inducible complement of cystatins in tomato, the activity of *Sl*CYS9 against cysteine proteases was compared with the activity of *Sl*CYS8, and with the activity of a truncated form, t*Sl*CYS9, generated by removing 103 amino acids at the C terminus of the native inhibitor (arrow, Fig. 2a). The recombinant cystatins were produced in *E. coli* (with no signal peptide) and assayed for their respective inhibitory potency against papain and herbivorous pest cysteine proteases. The three cystatins were expressed in and purified from *E. coli* using the GST gene fusion system (Michaud *et al*., 1994), cleaved from the GST moiety (Fig. 5a), then assayed against papain, the herbivorous insect digestive protease Ldp30 and the major extracellular cysteine proteases of two root parasitic nematodes, Mhp1 and Mip1 (Visal-Shah *et al*., 2001). As shown in Table 2, the native form of *Sl*CYS9 showed weak activity against papain and Ldp30, giving $K_{i(app)}$ values in the micromolar range. By contrast, the truncated inhibitor t*Sl*CYS9, structurally related to the model rice cystatin *Os*CYS1 (Fig. 5b), showed $K_{i(app)}$ values in the nanomolar range for the same two enzymes, similar to *Sl*CYS8. The same inhibitory pattern was observed for the nematode protease Mhp1, with estimated $K_{i(app)}$ values in the nanomolar range for *Sl*CYS8 and t*Sl*CYS9, compared with a $K_{i(app)}$ value in the micromolar range for *Sl*CYS9. Noteworthily, cysteine proteases of the closely related nematodes *M. incognita* (Mip1; Table 2) and *M. javanica* (Mjp1;

Michaud *et al*., 1996, not shown), were efficiently inhibited by *Sl*CYS8 but not by *Sl*CYS9 or t*Sl*CYS9, pointing out a differential impact of the C-terminal extension on cystatin inhibitory activity, depending on the target protease assessed.

The C-terminal extension of *Sl*CYS9 also influences its overall structure

Stability assays were carried out with *Sl*CYS8, *Sl*CYS9 and t*Sl*CYS9 to assess the impact of the C-terminal extension on the overall structure of *Sl*CYS9. To this end, the inhibitors were challenged with a larval midgut extract of the Colorado potato beetle, which contains digestive proteases from several mechanistic classes either sensitive or insensitive to plant cystatins (Michaud *et al*., 1995; Novillo *et al*., 1997). As seen in Fig. 6, a significant fraction of *Sl*CYS8 and t*Sl*CYS9 was hydrolyzed by the insect-insensitive proteases after incubation for 30 min under the conditions of the assay, the hydrolytic process being almost complete after 60 min (middle and lower panels). By contrast, *Sl*CYS9 showed a very rapid degradation rate, being completely digested within a few seconds after adding the insect extract (Fig. 6, upper panel), with no degradation intermediate detectable on gel. Pre-incubation of the insect extract with the cysteine protease inhibitor E-64 prevented degradation of all three cystatins. By contrast, preincubation with PMSF (a serine-type inhibitor) or pepstatin A (an aspartate-type inhibitor) had only a partial and transient stabilizing effect, indicating that cystatin-insensitive cysteine proteases in the extracts – presumably cathepsin B-like enzymes (Michaud *et al*., 1995) – were responsible for cleaving the recombinant cystatins.

Discussion

The main goal of this study was to compare the ability of arachidonate and (methyl) jasmonate to induce the expression of cystatin genes in tomato leaves. Several studies described the differential induction of defense-related genes by these two elicitors in Solanaceae, using as models a number of proteins including the serine-type inhibitors Pin-I and Pin-II, the PR-1 protein P4, and different forms of the metabolic effectors lipoxygenases and 3-hydroxy-3-methylglutaryl coenzyme A reductases (Choi *et al*., 1992, 1994; Fidantsef & Bostock, 1998; Fidantsef *et al*., 1999; Rivard *et al*., 2004). As a complement, we observed here that protease inhibitor- (cystatin-)encoding genes in tomato may respond not only to jasmonate, but also to the fungal elicitor arachidonate, presumably via a jasmonate-independent pathway.

The biological significance for the arachidonate-induced expression of cystatin genes in tomato remains to be clarified, but a protective role against invading pathogens appears plausible. Arachidonate is a potent elicitor of systemic defense responses in Solanaceae (Coquoz *et al*., 1995; Fidantsef *et al*., 1999; Weyman *et al*., 2006), notably triggering the expression of antimicrobial PR proteins in leaves (Fidantsef & Bostock, 1998; Fidantsef *et al*., 1999). Little information is available about the structural and functional

characteristics of fungal proteases (St Leger *et al*., 1997; ten Have *et al*., 2004), but the involvement of secreted proteases – including cysteine proteases – during plant tissue infection by *P. infestans* and other pathogenic fungi is well documented (Ball *et al*., 1991; Murphy & Walton, 1996; Paris & Lamattina, 1999; Poussereau *et al*., 2001; ten Have *et al*., 2004). Strong antifungal effects have also been observed recently *in vitro* for two 23-kDa, C-tailed cystatins structurally related to *Sl*CYS9 (Martinez *et al*., 2005b; Christova *et al*., 2006), again suggesting an antimicrobial role for this arachidonate-induced protein.

Despite these unsolved questions about the roles of *Sl*CYS9 (and *Sl*CYS10) *in planta*, our data clearly suggest the existence of a dynamic, elicitor-inducible 'cystatin complex' in tomato, consisting of at least 10 cystatin inhibitory units, *Sl*CYS1–*Sl*CYS10. These cystatins are induced in leaves in response to various stress signals including wounding, systemin and jasmonate (Bolter, 1993; Jacinto *et al*., 1998; Wu & Haard, 2000; this study), the fungal elicitor arachidonate (this study), and the bacterial phytotoxin coronatine (Gleddie & Michaud, 2000), a structural analogue of MeJa (Palmer & Bender, 1995). The occurrence of cystatin genes with distinct specificities and modes of induction in the tomato genome suggests the ability of this plant to synthesize cystatin forms active against a variety of (exogenous) cysteine proteases. From a functional viewpoint, the inducible cystatin complex in tomato would thus show plasticity at both the expression and protease inhibitory levels, making it effective and readily functional under a range of biotic stress conditions.

Structural elements such as the C-terminal extension or the N-terminal signal peptide for cellular secretion on *Sl*CYS9 might also contribute to this functional plasticity. In contrast to *Sl*CYS8, which forms insoluble crystals in the cytosol of tomato leaf cells after synthesis (Gleddie & Michaud, 2000), *Sl*CYS9 bears an N-terminal signal peptide that presumably directs its movement through the cell secretory pathway. No additional sorting signal for the vacuole or the endoplasmic reticulum could be detected in *Sl*CYS9 submitted to the WoLF PSORT Prediction database for plant sorting signals (http://psort.hgc.jp), which suggests an extracellular destination for this protein. N-terminal signal peptides for cellular secretion have been described recently for a number of stress- and developmentally regulated cystatins (Womack *et al*., 2000; Corre-Menguy *et al*., 2002; Rassam & Laing, 2004; Martinez *et al*., 2005b, 2005c; Massonneau *et al*., 2005). *In vivo*, such signals would allow the plant to accumulate cystatins in vacuoles upon wounding or insect herbivory, or to direct their secretion into the extracellular milieu upon fungal or bacterial attack. Whereas the final destination of *Sl*CYS9 in tomato leaf cells still needs to be confirmed empirically, the apparently distinct intracellular targeting of *Sl*CYS8 and *Sl*CYS9 in MeJa- and arachidonate-treated leaves clearly contributes to the overall picture of a dynamic, multifunctional inducible cystatin complex in tomato.

The C-terminal extension of *Sl*CYS9 might also have a certain influence *in vivo*. In contrast to an earlier study reporting a negligible impact for the C-terminal extension of a related cystatin from soybean seeds (Misaka *et al*., 1996), the C-terminal extension of *Sl*CYS9 was shown here to influence strongly both the protease inhibitory potency and

the tertiary structure of the protein. The differential stability of *Sl*CYS9 and t*Sl*CYS9 challenged with insect nontarget proteases indicated the occurrence of distinct sites for proteolytic cleavage at the surface of the two cystatin variants, which suggests that the influence of the C-terminal extension on *Sl*CYS9 inhibitory activity was due, at least in part, to a general effect on the overall structure of the inhibitor moiety. At this point, our inhibitory data suggest a repressive, anticystatin effect for this structural element, but the existence of target proteases strongly inhibited by *Sl*CYS9 *in vivo*, or a proteolytic deletion of the extension following secretion of the protein in the apoplast, cannot be ruled out. Similar extensions at the C terminus of plant cystatins have been described from sources as diverse as soybean seeds, field mustard flower buds, taro corms, strawberry fruits, wheat crowns and senescent leaves of sweet potato (Lim *et al*., 1996; Misaka *et al*., 1996; To *et al*., 1999; Martinez *et al*., 2005b; Yang & Yeh, 2005; Christova *et al*., 2006), but no clear function could be attributed to this ubiquitous structural element based on sequence homology searches in gene databases. After detecting the presence of amino acid strings possibly related to sequence motifs conserved among functional (inhibitory) cystatins, Martinez *et al*. (2005b, 2005c) recently suggested this extension to be a degenerated cystatin sequence resulting from an ancestral gene-duplication event followed by subsequent diverging evolution. Work is under way to assess this idea, also taking into account the reported occurrence in plants of protease inhibitors bearing inhibitor-independent antifeedant or antimicrobial functions (Maskos *et al*., 1996; Joshi *et al*., 1999).

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Figures and Tables

Fig. 1 Nucleotide and deduced amino acid sequences of the arachidonate-induced cystatin *Sl*CYS9 (GenBank accession no. AF198388). Residue T_{132}, in bold, corresponds to the C-terminal residue of the cystatin moiety (Fig. 2a). The amino acid sequence for the inhibitor's (predicted) signal peptide is in bold/italic. The stop codon is marked by an asterisk.

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attcaccacaacag  ATG AGA GTG ATT CGA AGT AGA GCA ATA CTG ATA GTG CTT TTT     56
                 M   R   V   I   R   S   R   A   I   L   I   V   L   F     14

CTG GTT TCT GCG TTT GGG TTA AGC GAA CAG GGA AAA TCA GGA GGA TTC TGC AGT    110
 L   V   S   A   F   G   L   S   E   Q   G   K   S   G   G   F   C   S     32

GAA GAG ATG GCT ACT CTT GGT GGA GTT CAT GAT TCT CAT GGT TCC TCG CAG AAC    164
 E   E   M   A   T   L   G   G   V   H   D   S   H   G   S   S   Q   N     50

AGT GAC GAG ATC CAT AGC CTT GCT AAA TTT GCC GTA GAT GAG CAT AAT AAG AAG    218
 S   D   E   I   H   S   L   A   K   F   A   V   D   E   H   N   K   K     68

GAG AAT GCA ATG ATT GAA TTG GCC AGA GTA GTG AAG GCG CAA GAA CAA ACT GTT    272
 E   N   A   M   I   E   L   A   R   V   V   K   A   Q   E   Q   T   V     86

GCA GGT AAA CTG CAC CAC CTC ACT CTT GAG GTC ATG GAT GCT GGA AAA AAG AAA    326
 A   G   K   L   H   H   L   T   L   E   V   M   D   A   G   K   K   K    104

CTC TAT GAG GCT AAG GTC TGG GTC AAA CCA TGG TTG AAT TTT AAG GAA CTT CAA    380
 L   Y   E   A   K   V   W   V   K   P   W   L   N   F   K   E   L   Q    122

GAG TTC AAG CAT GTT GAA GAC GTT CCT ACC TTT ACT TCT TCA GAT CTA GGA GTT    434
 E   F   K   H   V   E   D   V   P   T   F   T   S   S   D   L   G   V    140

AAG CAA GTA GAG CAG AAC AGT GGA TTG AAA TCA GTG CCT GTG CAT GAT CCT GTT    488
 K   Q   V   E   Q   N   S   G   L   K   S   V   P   V   H   D   P   V    158

GTT GAA GAA GCT GCA GAG CAT GCA ATA AAG ACC ATC CAG CAG AGA TCC AAC TCT    542
 V   E   E   A   A   E   H   A   I   K   T   I   Q   Q   R   S   N   S    176

ATA CAT CCA TAT AAA CTA CAA GAG ATT GTT CAT GCT AAT GCT GAG ATG GCT GAT    596
 I   H   P   Y   K   L   Q   E   I   V   H   A   N   A   E   M   A   D    194

GAT TCT ACA AAG CTT CAT TTG GTC ATC AAA ACC AGC AGG GGA GGG AAG GAA GAG    650
 D   S   T   K   L   H   L   V   I   K   T   S   R   G   G   K   E   E    212

AAG TTC AAA GTT CAA GTG CAG CAC AAT AAT GAA GGT GCG TTC CAC TTG AAT CGT    704
 K   F   K   V   Q   V   Q   H   N   N   E   G   A   F   H   L   N   R    230

ATG GAG CCT GAC AAC TAA gtttgggagatcctacgcctctttagatttctttagttcatctatgg    769
 M   E   P   D   N   *                                                    236

agctatggatctgtttcaagtataataagcatgtaaccagcacaatatttttactacttgcttttgttcat    840

ctgaagtttgtcttcatctagtggattactctgatccaccttaggttgagggcatctttgtcttgtgtcac    911

agttgtaatgtttcaagtattctgaacataactactcggtataaagtaaaaaaaaaaaaaaaaaa         976
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Fig. 2 Alignment of the deduced amino acid sequence of *Sl*CYS9 (GenBank accession no. AF198388) with the sequences of *Sl*CYS10 (AF198389), the eighth inhibitory domain of tomato multicystatin, *Sl*CYS8 (AF198390), and other plant cystatins. (a) Alignment of *Sl*CYS8, *Sl*CYS9 and *Sl*CYS10 with *Os*CYS1 (P09229) and the eighth inhibitory domain of potato multicystatin, *St*CYS8 (P37842). The predicted signal peptide of *Sl*CYS9 is underlined. Key residues for cystatin activity are in bold. The arrow on the threonine residue (T132) marks the C-terminal end of the truncated version of *Sl*CYS9. (b) Alignment of the C-terminal extension of cystatins from tomato (*Sl*CYS9; this paper), sweet potato (*Ib*CYS1; AF117334), soybean (*Gm*CYS1; D31700), strawberry (*Fa*CYS1; AJ845186), apple (*Md*CYS1; AY173139), castor bean (*Rc*CYS1; Z49697) and field mustard (BcCYS1; S65071). Identity (id) and similarity (sim) percentages between each extension and the extension of *Sl*CYS9 are given on the right.

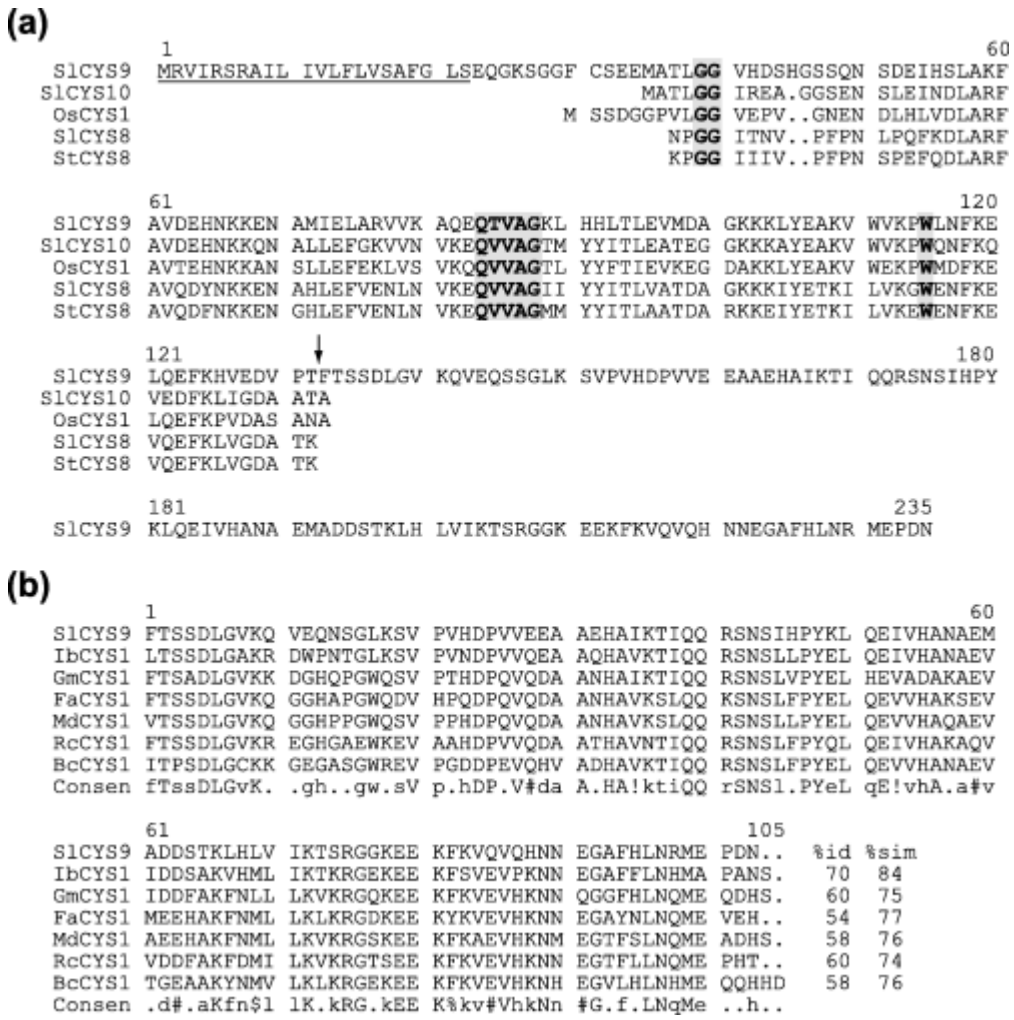

Fig. 3 Neighbor-joined phylogenetic tree for 26 cystatins from different plant taxa (Table 1). The unrooted tree was reconstructed using a distance matrix generated by Kimura's two-parameter model (Kimura, 1983). The analysis grouped the different cystatins into three clades (boxes), correlated with the taxon of origin (Solanaceae and Poaceae (monocot) cystatins) or the presence of a C-terminal extension (23-kDa cystatins). The tomato cystatins *Sl*CYS8, *Sl*CYS9 and *Sl*CYS10 are in bold.

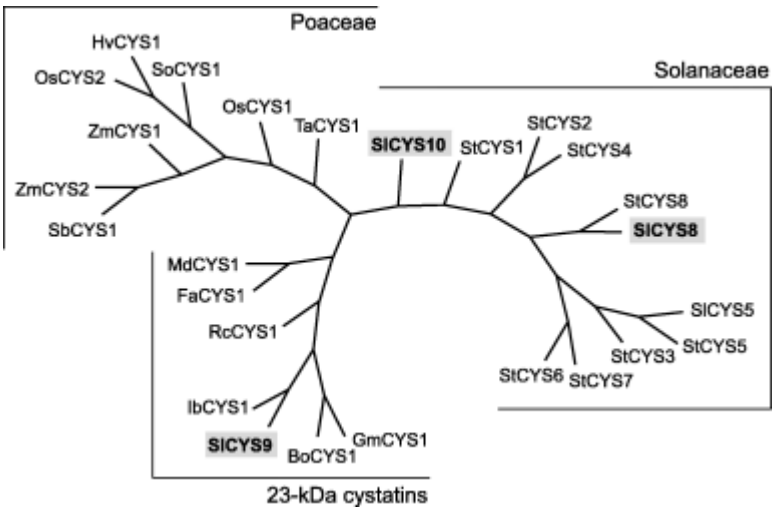

Fig. 4 Northern blot analysis for the induction of Pin-II-, protein P4- and cystatin-encoding genes by methyl jasmonate (MeJa) and arachidonic acid (AA) in tomato leaves. (a) mRNA transcripts of the model proteins Pin-II and protein P4 16 h after treatment with 40 or 400 μM MeJa or AA. (b) mRNA transcripts of *Sl*CYS8, *Sl*CYS9 and *Sl*CYS10 16 h after treatment with 400 μM MeJa or AA. (c) Relative accumulation of *Sl*CYS8, *Sl*CYS9 and *Sl*CYS10 mRNA transcripts 0–24 h after induction with 400 μM MeJa or AA. Data are presented as relative values, compared with transcript signals 20 h post-treatment for each gene considered (value 1.0). Membranes were hybridized with ^{32}P-labelled cDNA probes encoding either Pin-II, protein P4, *Sl*CYS8, *Sl*CYS9 or *Sl*CYS10. Equal loading of RNA in each well was controlled by ethidium bromide fluorescence of total RNA fixed onto the membrane. In (c), relative amounts of mRNA transcripts on nitrocellulose membranes were estimated by densitometry using a Microtek ScanMaker II scanner (Microtek Laboratory, Torrance, CA, USA) and the image analysis software NIH IMAGE 1.6 (National Institutes of Health, Bethesda, MD, USA). CTL, control plants sprayed with 0.125% (v/v) Triton X-100.

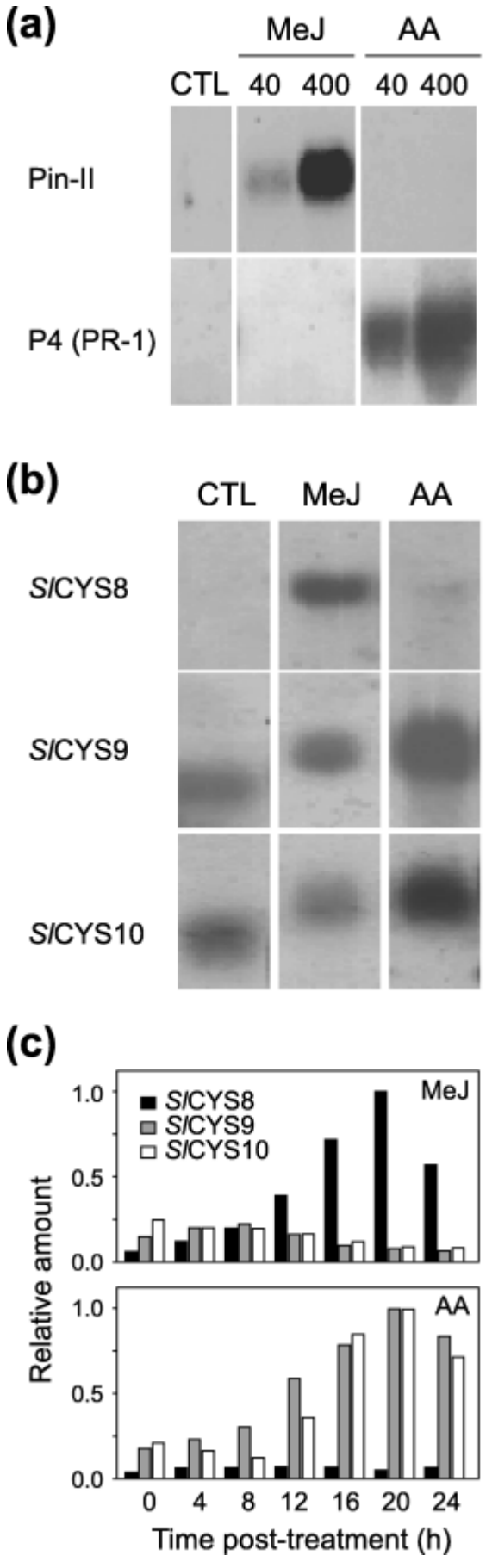
(a)
MeJ
AA
CTL
40
400
40
400
Pin-II
P4 (PR-1)
(b)
CTL
MeJ
AA
SlCYS8
SlCYS9
SlCYS10
(c)
MeJ
SlCYS8
SlCYS9
SlCYS10
AA
Relative amount
1.0
0.5
0.0
0
4
8
12
16
20
24
Time post-treatment (h)

Fig. 5 Heterologous expression of *Sl*CYS8, *Sl*CYS9 and t*Sl*CYS9 in *Escherichia coli* and structural model for t*Sl*CYS9. (a) Recombinant cystatins expressed using the glutathione *S*-transferase (GST) gene fusion system. The inhibitors were expressed (with no signal peptide) attached to GST, purified with reduced glutathione-embedded agarose beads, and cleaved from the GST affinity partner with human factor X_a. Purity of the preparations was confirmed here by 12% (w/v) SDS–PAGE followed by Coomassie Brilliant Blue staining. Values on the left refer to commercial molecular weight markers. (b) Tertiary structure of t*Sl*CYS9, as inferred from structural coordinates of the model cystatin from rice, *Os*CYS1 (OC-I: 1EQK in Protein Data Bank; Nagata *et al.*, 2000). The structure was constructed using MODELLER ver. 6.2 (Sanchez & Sali, 2000) and tested for energy distribution and stereochemical quality using the in-built *Energy* command of MODELLER and the PROCHECK program (Laskowski *et al.*, 1993), respectively. t*Sl*CYS9 was visualized using SWISS-PDB software (Guex & Peitsch, 1997). The resulting model shows the typical structural elements of plant cystatins, including four antiparallel β sheets linked to an α-helix in the N-terminal region, two hairpin inhibitory loops entering the active site of target enzymes, and a protruding N-terminal trunk with the conserved –GG– motif presumably interacting with amino acid side chains of the target enzyme during the inhibitory process (Turk *et al.*, 1997).

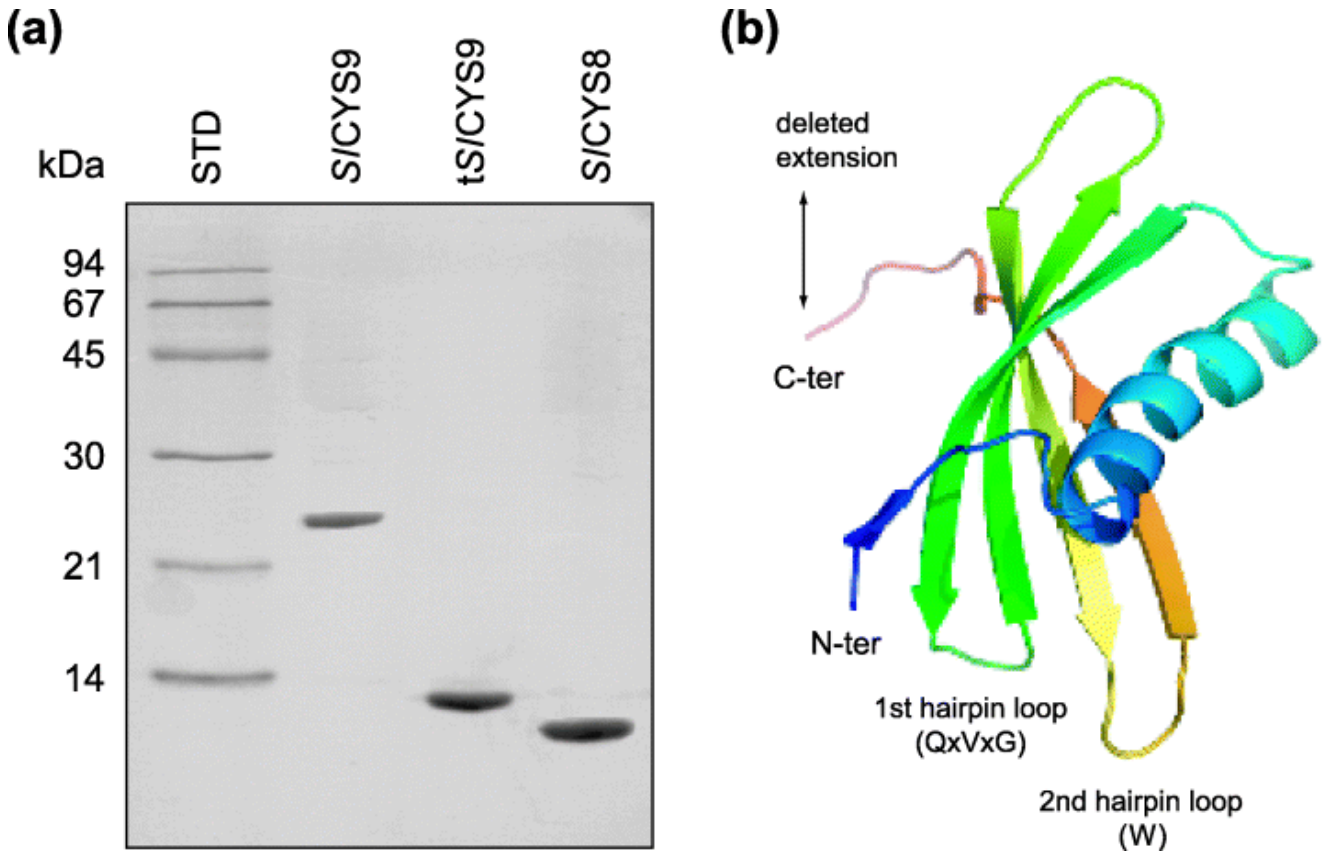

Fig. 6 Degradation of *Sl*CYS8, *Sl*CYS9 and t*Sl*CYS9 by digestive proteases of the Colorado potato beetle. The insect extract was preincubated with water (CTL, control), the low-molecular-weight cysteine protease inhibitor E-64, the serine protease inhibitor PMSF, the aspartate protease inhibitor pepstatin A (PEP), or the metalloprotease inhibitor EDTA before incubation with cystatins. Proteolysis was stopped after various periods by the addition of SDS–PAGE sample buffer, and cystatins remaining in the mixtures were visualized on immunoblots using antipotato multicystatin polyclonal antibodies. Purified *Sl*CYS9 was also loaded alone as a control, as it was readily degraded in the presence of the insect proteases.

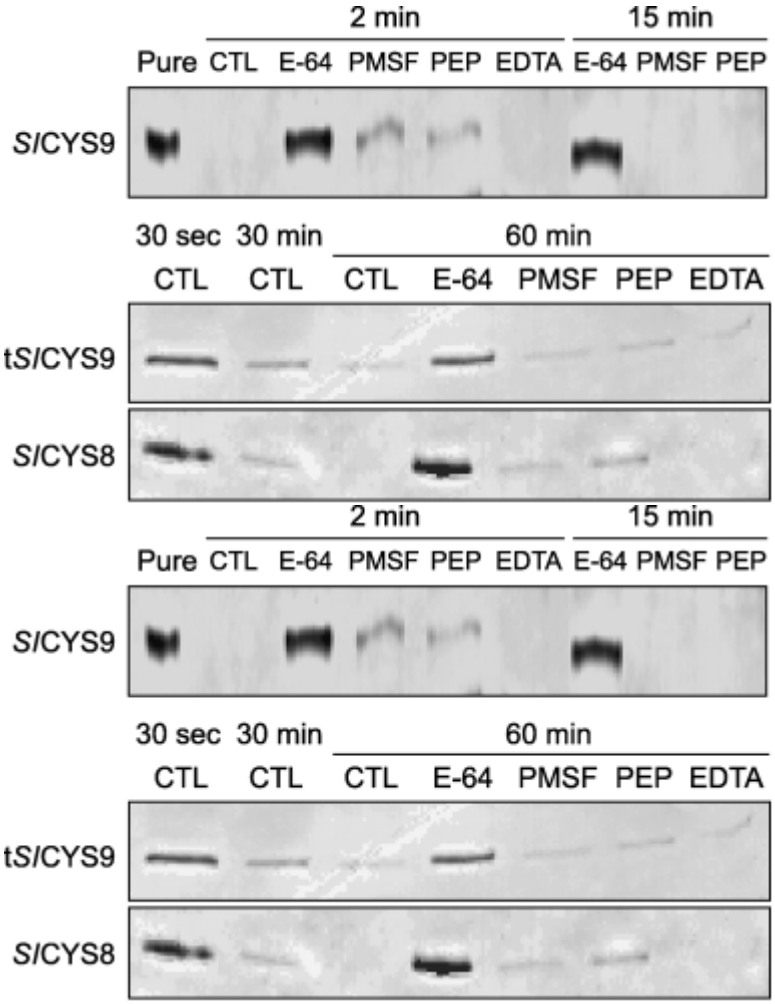

Table 1 Cystatin-encoding sequences used for the phylogenetic reconstruction

Cystatin	Species	Accession number*
*Bc*CYS1	*Brassica campestris*	S65071
*Cl*CYS1	*Coix lacryma*-jobi	AB037156
*Gm*CYS1	*Glycine max*	D31700
*Hv*CYS1	*Hordeum vulgare*	Y12068
*Ib*CYS1	*Ipomoea batatas*	AF117334
*Md*CYS1	*Malus* × *domestica*	AAO19652
*Os*CYS1	*Oryza sativa*	J03469
*Os*CYS2	*O. sativa*	J05595
*Rc*CYS1	*Ricinus communis*	Z49697
*Sb*CYS1	*Sorghum bicolor*	X87168
*Sl*CYS5	*Solanum lycopersicum* †	U153466 ‡
*Sl*CYS8	*S. lycopersicum*	AF198390
*Sl*CYS9	*S. lycopersicum*	AF198388
*Sl*CYS10	*S. lycopersicum*	AF198389
*So*CYS1	*Saccharum officinarum*	AY119689
*St*CYS1	*Solanum tuberosum*	L16450
*St*CYS2	*S. tuberosum*	L16450
*St*CYS3	*S. tuberosum*	L16450
*St*CYS4	*S. tuberosum*	L16450
*St*CYS5	*S. tuberosum*	L16450
*St*CYS6	*S. tuberosum*	L16450
*St*CYS7	*S. tuberosum*	L16450
*St*CYS8	*S. tuberosum*	L16450
*Ta*CYS1	*Triticum aestivum*	AB038392
*Zm*CYS1	*Zea mays*	D10622
*Zm*CYS2	*Z. mays*	D38130
*Data from the NCBI/GenBank database.		

Cystatin	Species	Accession number*
†Tomato: formerly *Lycopersicon esculentum*.		
‡Sequence retreived from the Solanaceae Genomics Network database (http://www.sgn.cornell.edu).		

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Table 2 Inhibition of papain and herbivorous pest cysteine proteases by *Sl*CYS8, *Sl*CYS9 and t*Sl*CYS9 (see Materials and Methods)

Inhibitor	$K_{i(app)}$ (nM)			
	Papain	Ldp30	Mhp1	Mip1
*Sl*CYS8	2.4	7.1	<10	<10
*Sl*CYS9	635	545	>100	>100
t*Sl*CYS9	11.1	11.6	<10	>100

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