

## AtCYS1, a cystatin from *Arabidopsis thaliana*, suppresses hypersensitive cell death

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In plants, cysteine protease inhibitors are involved in the regulation of protein turnover and play an important role in resistance against insects and pathogens. *AtCYS1* from *Arabidopsis thaliana* encodes a protein of 102 amino acids that contains the conserved motif of cysteine protease inhibitors belonging to the cystatin superfamily (Gln-Val-Val-Ala-Gly). Recombinant *A. thaliana* cystatin-1 (*AtCYS1*) was expressed in *Escherichia coli* and purified. *AtCYS1* inhibits the catalytic activity of papain ( $K_d = 4.0 \times 10^{-2} \mu\text{M}$ , at pH 7.0 and 25 °C), generally taken as a molecular model of cysteine proteases. The molecular bases for papain inhibition by *AtCYS1* have been analysed taking into account the three-dimensional structure of the papain–stefin B complex. *AtCYS1* is constitutively expressed in roots and in developing siliques

of *A. thaliana*. In leaves, *AtCYS1* is strongly induced by wounding, by challenge with avirulent pathogens and by nitric oxide (NO). The overexpression of *AtCYS1* blocks cell death activated by either avirulent pathogens or by oxidative and nitrosative stress in both *A. thaliana* suspension cultured cells and in transgenic tobacco plants. The suppression of the NO-mediated cell death in plants overexpressing *AtCYS1* provides the evidence that NO is not cytotoxic for the plant, indicating that NO functions as cell death trigger through the stimulation of an active process, in which cysteine proteases and their proteinaceous inhibitors appear to play a crucial role.

**Keywords:** *Arabidopsis thaliana*; cystatin; cysteine protease; hypersensitive response; programmed cell death.

Cysteine protease inhibitors inactivate proteases by trapping them in a(n) (ir)reversible, tight equimolar complex [2]. Plant cystatins, homologous to animal cysteine protease inhibitors [3], have been characterized in several monocots

and dicots, including rice, maize, soybean, Chinese cabbage [4], chestnut, potato and tomato [5–13]. Cystatins show different expression patterns during plant development and defence responses to biotic and abiotic stresses [14]. Moreover, cystatins may play a role in the regulation of protein turnover and plant defence against insect predation and pathogens [13].

Wounding causes extensive changes in the pattern of defence protein synthesis leading to localized resistance at the site of the lesion. The response includes the production of phytoalexin, enhanced lignification and suberization of the cell wall, and systemic induction of protease inhibitors [15,16]. Cystatin accumulation occurs after activation of both long- and short-distance signal cascades, triggered by accumulation of systemin or by cell wall fragments. Many insects such as *Hemiptera* and *Coleoptera* rely on cysteine proteases for the majority of the proteolytic activity responsible for protein digestion in the gut [17]. Remarkably, cystatins have been shown to inhibit the activity of digestive proteases from coleopteran pests *in vitro*, as well as the inhibition of larval development *in vivo*. Thus, cystatins function as 'toxins' by targeting the major proteolytic digestive enzymes of herbivore insects [6,11,18]. Moreover, cysteine proteases play a fundamental role in virus replication; therefore, constitutive expression of a rice cystatin in tobacco induces virus resistance [19].

Recently, a synthetic gene encoding the mature form of a soybean cystatin has been reported to effectively block cell death triggered by either oxidative stress or avirulent

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**Abbreviations:** *AtCYS1*, *Arabidopsis thaliana* cystatin-1; DAB, 3,3'-diaminobenzidine; GST, glutathione *S*-transferase; HR, hypersensitive response; IPTG, isopropyl thio- $\beta$ -D-galactoside; NO, nitric oxide; PCD, programmed cell death; PR, pathogenesis-related; SNP, sodium nitroprusside; Z-Phe-Arg-AMC, *N*- $\alpha$ -benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amido-4-methylcoumarin).

**Enzymes:** glucose oxidase, from *Aspergillus niger* (EC 1.1.3.4); lysozyme, from chicken egg (EC 3.2.1.17); papain, from *Carica papaya* L. (EC 3.4.22.2); thrombin, from bovine plasma (EC 3.4.21.5).

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Note: Full length chicken cystatin numbering is used throughout the text [1].

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pathogens, when transiently expressed in cultured soybean cells [20]. Thus, a role for cysteine proteases can be envisioned in programmed cell death (PCD) by regulatory protein degradation. Note that cysteine proteases have been implicated in the differentiation of *Zinnia elegans* cells into tracheary elements, which involves mesophyll cell death [21]. *SAG12* from *Arabidopsis thaliana* is a senescence-associated gene, which encodes a cysteine protease that is coordinately expressed with hypersensitive cell death [22]. Cystatins may therefore function as modulators of cysteine protease activity during plant growth, development and seed maturation [23].

The activation of PCD appears to play an important role during the hypersensitive disease-resistance response against pathogen attack; however, it is imperative that plants maintain the capacity to regulate this process [20]. Here, we describe the molecular and biochemical characterization of the *A. thaliana* cystatin-1 AtCYS1 that accumulates following wounding and during the hypersensitive response. Moreover, the constitutive expression of *AtCYS1* suppresses PCD triggered by either avirulent pathogens or oxidative and nitrosative stresses in both *A. thaliana* suspension cultures and in transgenic tobacco.

## Materials and methods

### Materials

Papain (from *Carica papaya* L.), bovine thrombin, chicken egg white lysozyme, glucose oxidase (from *Aspergillus niger*), agarose-*p*-aminobenzamidine, agarose-glutathione, *N*- $\alpha$ -benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amido-4-methylcoumarin) (Z-Phe-Arg-AMC), chloral hydrate, 3,3'-diaminobenzidine (DAB), *N*-lauroylsarcosine, L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, dimethylsulfoxide, dithiothreitol, isopropyl thio- $\beta$ -D-galactoside (IPTG), sodium nitroprusside (SNP), methyl jasmonate, kanamycin, ampicillin, rifampicin, Evan's blue, trypan blue, protein molecular markers, Tris and reduced glutathione were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Restriction enzymes were purchased from Promega (Madison, WI, USA). All the other chemicals were purchased from Merck AG (Darmstadt, Germany). All products were of analytical or reagent grade and were used without further purification.

### Southern blot analysis

Genomic DNA was isolated from *A. thaliana* and tobacco leaves as reported previously [24]. In brief, 10  $\mu$ g of genomic DNA was cut with indicated restriction enzymes, fractionated on 0.8% (w/v) agarose gels, transferred to nylon filters and hybridized to a radioactive probe prepared from the complete *AtCYS1* cDNA. Prehybridization and hybridization were performed as described previously [25].

### Northern blot analysis

Total RNA was extracted from 4 week old *A. thaliana* plants at fixed times following indicated treatment. The nitric oxide (NO) donor SNP was prepared and infiltrated into

leaves as described previously [26]. A bacterial suspension containing  $5 \times 10^6$  CFU·mL<sup>-1</sup> of virulent *Pseudomonas syringae* pv. *maculicola* or the isogenic avirulent strain carrying *avrRpm1* was infiltrated into leaves as described [27]. Plants were sprayed with 45.5  $\mu$ M methyl jasmonate prepared in 0.1% (v/v) ethanol. Leaves, stems, roots, flowers and siliques were cut and frozen directly into liquid nitrogen. RNA from *A. thaliana* and tobacco frozen tissues was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) as described by the manufacturer. Then, 5  $\mu$ g of total RNA were separated on 1.5% (w/v) agarose gels containing 6% (v/v) formaldehyde, blotted onto Hybond N<sup>+</sup> membrane (Amersham Biosciences, Little Chalfont, UK) according to manufacturer's instructions, and cross-linked by UV irradiation. For hybridization analysis, the purified *Bam*HI/*Sac*I fragment (500 bp), containing the entire *AtCYS1* coding sequence, was used as probe. The level of PR-1 transcripts in tobacco was determined using the PCR amplification of tobacco Pr1-1a (GenBank accession number X12737) as a probe. Prehybridization and hybridization were performed as described previously [25].

### *E. coli* expression and purification of recombinant AtCYS1

The pGEX 4T-3-AtCYS1 expression vector contains a 380 bp fragment that was obtained by PCR amplification of the *AtCYS1* cDNA using specific primers carrying a *Bam*HI site (forward: GGATCCGCGGATCAACAAGCAGGAACA) and a *Sal*I site (reverse: GTCGACTCACGTGGTCTGAGAGCACAC) for directional cloning (restriction sites underlined). The amplified fragment was subcloned into the pGEM-T vector (Promega), sequenced, cut with *Bam*HI and *Sal*I, and then introduced into the expression vector pGEX4T-3 (Amersham Biosciences) cut with the same restriction enzymes. The resulting construct (pGEX 4T-3-AtCYS1), which expresses AtCYS1 as a fusion protein with the 26 kDa glutathione *S*-transferase (GST), was introduced in *E. coli* JM101 competent cells.

Cultures of JM101 *E. coli* containing the pGEX 4T-3-AtCYS1 construct were grown to saturation at 30 °C in Luria-Bertani broth supplemented with 50  $\mu$ g·mL<sup>-1</sup> ampicillin, diluted 1 : 100 in 500 mL of fresh ampicillin-containing Luria-Bertani broth, and grown until  $D_{600} = 0.6$ . IPTG was then added to a final concentration of 0.5 mM and cells were grown for additional 2.5 h at 30 °C. Cells were collected by centrifugation for 15 min at 3000 *g*, resuspended in 10 mL of ice-cold 10 mM Tris/HCl buffer, pH 8.0 (containing 1.0 mM EDTA and 150 mM NaCl), supplemented with 0.1 mL of freshly prepared 10 mg·mL<sup>-1</sup> lysozyme in water, and incubated for 15 min. Then, 0.1 mL of 1.0 M dithiothreitol and 1.4 mL of 10% (w/v) *N*-lauroylsarcosine were added and the solution sonicated for 1 min. After sonication, the solution was centrifuged at 30 000 *g* for 20 min, and the supernatant was recovered, supplemented with 4 mL of 10% (v/v) Triton X-100 and brought to a final volume of 20 mL with 10 mM Tris/HCl buffer pH 8.0 (containing 1.0 mM EDTA and 150 mM NaCl). Then, the lysate solution was mixed with 1.0 mL bed of agarose/glutathione in NaCl/P<sub>i</sub> (120 mM NaCl, 2.7 mM KCl, 10.0 mM phosphate buffer salts, pH 7.4) and gently shaken for 1 h, at room temperature.

After three NaCl/P<sub>1</sub> washes, the recombinant protein was eluted with the elution buffer (50.0 mM Tris/HCl, 20.0 mM glutathione, pH 9.0), and then digested with 100 NIH units of bovine thrombin per mg of fusion protein, for 4 h at 25 °C. After digestion, bovine thrombin was removed by affinity chromatography on agarose-*p*-aminobenzamidine according to the supplier's specifications, and the purified AtCYS1 was collected. Electrophoresis analysis was performed on 12% (w/v) SDS/PAGE gels according to standard methods [28]. After staining the gels with Coomassie Brilliant Blue, the images were acquired using a Fluor-S Molecular Imager scanner (Bio-Rad, Hercules, CA, USA). The correctness of the amino acid sequence was checked by chemical sequencing.

#### Determination of values of $K_d$ for papain inhibition by recombinant AtCYS1

Values of  $K_d$  for AtCYS1 binding to papain have been determined by the inhibitory effect on papain-catalysed hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC [29–31]. Briefly, active papain (final concentration, 0.1  $\mu$ M) was incubated for 30 min with AtCYS1 (final concentration, 0.02–2.5  $\mu$ M). Z-Phe-Arg-AMC (dissolved in dimethylsulfoxide) was added (final concentration,  $4.0 \times 10^{-5}$  M), and fluorescence (excitation wavelength 380 nm, absorption wavelength 460 nm) was measured over 3 min, at pH 7.0 (0.1 M sodium phosphate buffer) and 25 °C. Prior to each experiment, papain was reductively activated by incubation with  $1.0 \times 10^{-3}$  M dithiothreitol, as already reported [29,30]. The concentration of active papain was determined by active site titration with L-*trans*-epoxysuccinylleucyl-amido(4-guanidino)-butane [29,32].

#### Molecular modelling of recombinant AtCYS1

The molecular model of AtCYS1 was built using the NMR structure of oryzacystatin-I as a template (Protein Data Bank accession number 1EQK) [33]. In detail, an initial search of suitable modelling templates was performed with BLAST [34] on the Protein Data Bank [35]. A multiple sequence alignment between AtCYS1 and other cystatins with known three-dimensional structure was then obtained using the program CLUSTALW [36]. The template structure was selected on the basis of highest sequence homology and the three-dimensional structure of AtCYS1 was built using MODELLER (Release 6), a program that models protein three-dimensional structure by satisfaction of spatial restraints [37]. Model consistency and viability were assessed using the protein structure validation software PROCHECK v.3.5 [38] available online (<http://www.ebi.ac.uk/Thornton/software.html>). The overall average G factor calculated by PROCHECK [38], a measure of how 'normal' the stereochemical properties of the model are, is  $-0.18$ , a value well above the threshold for 'poor' structures (overall average G factor  $< -0.5$ ). The complexes formed by papain with AtCYS1, chicken egg white cystatin, oryzacystatin-I and stefin A were modelled by superimposing the inhibitor's structure (AtCYS1, present study; oryzacystatin-I, PDB accession no.: 1EQK [33]; chicken egg white cystatin, PDB accession no.: 1A67 [39]; and stefin A, PDB accession no.: 1DVC [40]) onto the three-dimensional structure of the stefin B–papain

complex (PDB accession no.: 1STF) [41], using the fit routines of the program SWISS-PDB VIEWER [42].

#### Agrobacterium strain and vector plasmid

The pBI-AtCYS1 vector plasmid (11 kb) contains a 380 bp fragment that was obtained by PCR amplification of the *AtCYS1* cDNA, using specific primers carrying an *Xba*I site (forward: 5'-TCTAGACTCGTGCCGCGAAAATGGCG-3') and a *Sac*I site (reverse: 5'-GAGCTCTCACGTGGTC TGAGAGCACAC-3') for directional cloning (restriction sites underlined). The amplified fragment was subcloned into pGEM-T (Promega), sequenced, cut with *Xba*I and *Sac*I and then introduced into the binary vector pBI121 (Clontech, Palo Alto, CA, USA) under the control of CaMV35S promoter, replacing the *uidA* coding region. The resulting binary plasmid (pBI-AtCYS1) was mobilized in the *Agrobacterium tumefaciens* EHA105 disarmed strain by electroporation at 2500 V of an *A. tumefaciens* culture grown overnight and washed with 10% (v/v) glycerol [43]. Bacterial cultures were grown in the Luria–Bertani medium [44] containing 150 mg·L<sup>-1</sup> each of kanamycin and rifampicin, and diluted in Murashige and Skoog liquid medium to achieve a  $D_{550} = 0.6$  for plant transformation.

#### *Arabidopsis thaliana* transformation

*A. thaliana* ecotype Col-O cell suspensions were grown and transformed as described previously [20]. In brief, cells were coinoculated with  $5 \times 10^8$  *A. tumefaciens* EHA105 cells [45] carrying pBI-AtCYS1 or pBI121 in 24-well culture plates with moderate shaking at 25 °C. After 48 h, the bacteria were removed by extensive washing over Miracloth (Calbiochem, San Diego, CA, USA) and resuspended in the original volume of fresh medium. An aliquot of cells transformed with pBI121 was used for estimation of the transformation efficiency [20]. Physiological experiments were then performed in 12-well tissue culture plates (1 mL per well). *P. syringae* pv. *maculicola* carrying the *avrRpm1* avirulence gene was kindly provided by R. Innes (Indiana University, IN, USA) and was grown as already described [46]. Except where otherwise noted, reagents were added to *A. thaliana* cells simultaneously, with bacteria at the indicated final concentrations. The NO-donor SNP was dissolved in water and used within 2 h.

#### Cell death in *Arabidopsis thaliana* suspension cultured cells

Cell death was assayed 24 h after the indicated treatments by incubating *A. thaliana* suspension cultured cells for 15 min with 0.05% (w/v) Evan's blue. Unbound dye was removed by extensive washing. The dye bound to dead cells was solubilized in 50% (v/v) methanol, 1% SDS for 30 min at 50 °C and quantified by  $A_{600}$  [47].

#### Tobacco transformation

Leaf discs of tobacco (*Nicotiana tabacum* L. cv. *Xanthi*) were transformed according to the literature [48]. Transgenic plants were transferred into pots and hardened in a greenhouse. The stable integration and expression of the

transgene in the regenerated plants and their progenies was verified using PCR, Southern and Northern blot analysis.

### Cell death and oxidative burst in tobacco plants

*Pseudomonas syringae* pv. *phaseolicola* (NPS3121) was provided by K. Shirasu (John Innes Centre, Norwich, UK). Bacteria were grown as described previously [46]. H<sub>2</sub>O<sub>2</sub> production by the oxidative burst was visualized *in situ* with DAB staining as described [49]. Leaf discs were collected 1 h and 5 h after bacterial infiltration and immersed overnight with the DAB solution, then destained and fixed with a solution of 3 : 1 ethanol/glycerol. Cell death was visualized with trypan blue staining [50]. Leaf discs were collected 8 h and 12 h after infiltration of leaves with the NO-donor SNP or bacteria, respectively. Leaf discs were immersed in a boiling solution composed of 10 mL lactic acid, 20 mL 50% (v/v) glycerol, 0.02 g trypan blue and 10 mL phenol for 2 min. The trypan blue solution was decanted and the leaves were destained with 10 mL 70% (w/v) chloral hydrate.

### Papain inhibition by tobacco protein extracts

Leaves (120 mg) from untransformed and selected transgenic tobacco plantlets were homogenized in 500 µL of 10 mM Tris (pH 8.0) and centrifuged at 4 °C for 20 min at 13 000 *g*. Then, 1 mL of 80% (v/v) ammonium sulphate solution was added to the supernatant. Samples were incubated for 1 h in ice and centrifuged at 4 °C for 20 min at 13 000 *g*. Subsequently, the supernatant was discarded and the pellet resuspended in 100 µL of 10 mM Tris (pH 8.0). Samples were diluted to a standard protein concentration (400 µg·mL<sup>-1</sup>) determined according to the literature [51]. Cysteine protease inhibition was assayed as follows. Plant protein extracts (50 µL) were mixed with 10 µL of papain solution (2.0 mg·mL<sup>-1</sup> in 50 mM phosphate buffer, pH 6.8, containing 4.0 mM cysteine) and preincubated at 37 °C for 15 min to allow inhibitor binding to the protease. Next, 100 µL of azoalbumin solution (10 mg·mL<sup>-1</sup> in 50 mM phosphate buffer, pH 6.8) were added and the samples were incubated at 37 °C for 30 min. The reaction was stopped by the addition of 480 µL of 10% (v/v) trichloroacetic acid solution. Samples were kept on ice for 15 min and then centrifuged for 3 min at 8000 *g*. Aliquots corresponding to 500 µL of supernatant were collected and mixed with 100 µL of 3.3 M NaOH to allow staining of the undigested substrate. Papain activity was determined by measuring the hydrolysis of azoalbumin at 440 nm in the absence and presence of tobacco protein extracts. For each tobacco line, three independent protein extracts were analysed. All assays were repeated at least twice.

## Results

### Isolation and molecular characterization of AtCYS1 cDNA

A search of the GenBank EST section (<http://www.ncbi.nlm.nih.gov/dbEST>) for novel cystatins revealed an *A. thaliana* cDNA encoding a polypeptide with a high degree of homology to known cysteine protease inhibitors.

The clone (GenBank accession number ATTS2919) was requested from the *Arabidopsis* Biological Resource Center (The Ohio State University, Columbus, OH, USA). It was then entirely sequenced and used to probe an *A. thaliana* genomic library. A 1.4 kb fragment containing the hybridizing region was subcloned and sequenced. An open reading frame of 306 nucleotides was identified, coding for an 11 kDa polypeptide with high homology to known plant cystatins (Fig. 1A) [3].

### Expression and purification of recombinant AtCYS1

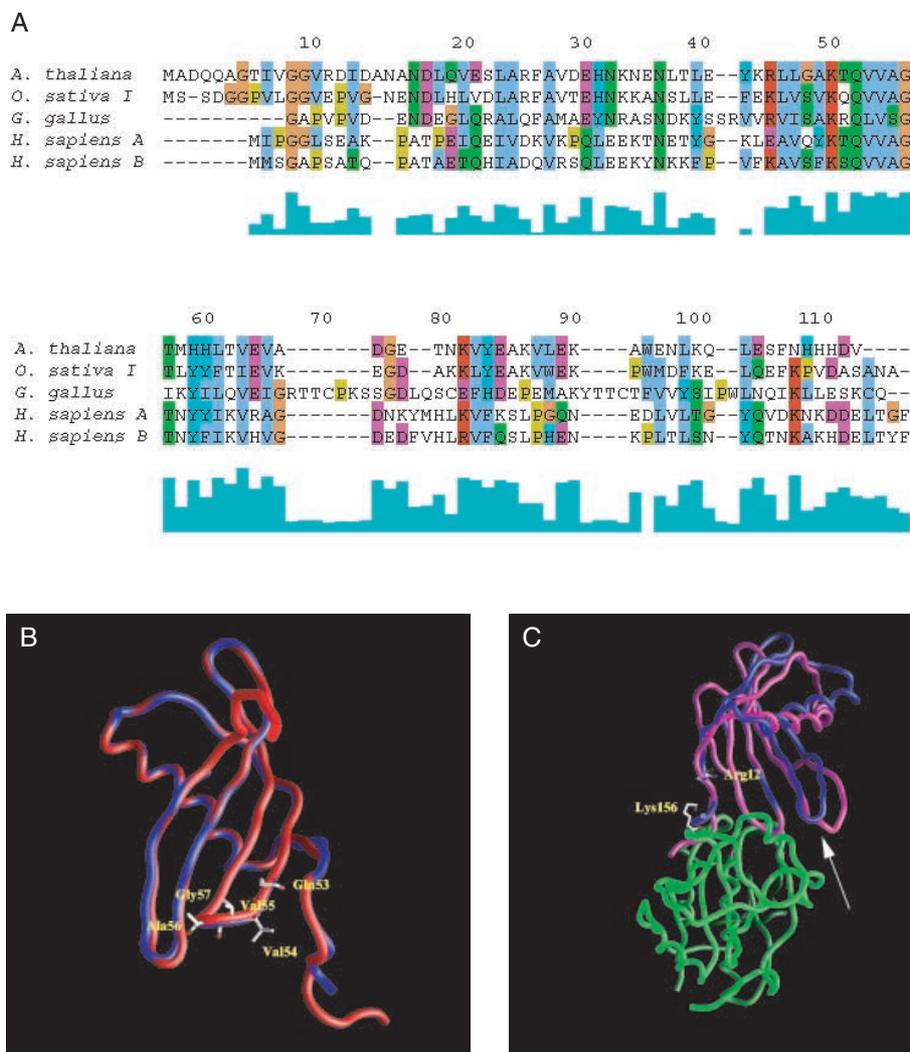
*E. coli* JM101 cells were transformed with the construct pGEX 4T-3-AtCYS1, which directs the synthesis of 39 kDa GST-AtCYS1 fusion protein under the control of the IPTG-inducible Lac promoter. Subcellular localization experiments showed that the overwhelming majority of the induced protein precipitated as insoluble inclusion bodies (data not shown). By inducing pGEX 4T-3-AtCYS1 expression at 30 °C in the presence of 0.5 mM IPTG, however, the solubility of the fusion protein increased dramatically, and as much as 3.6 mg·L<sup>-1</sup> of soluble fusion protein could be recovered, after centrifugation, from the induced supernatant. The fusion protein was purified by affinity chromatography on agarose/glutathione, and the native 13 kDa AtCYS1 was cleaved from the fusion protein by adding bovine thrombin. The thrombin was removed by affinity chromatography, and the 13 kDa native AtCYS1 was recovered. The purity was higher than 95% as judged by SDS/PAGE and chemical sequencing (data not shown).

### Molecular basis for papain inhibition by recombinant AtCYS1

AtCYS1 binding to papain follows a simple equilibrium, the value of the Hill coefficient (*n*) always being equal to 1.00 ± 0.03. The *K*<sub>d</sub> value for AtCYS1 binding to papain is (0.4 ± 0.1) × 10<sup>-2</sup> µM (pH 7.0 and 25 °C; Table 1).

A BLAST search of the Protein Data Bank [35] reveals a high sequence homology between AtCYS1 and oryzacystatin-I [33]. In detail, 70% sequence homology is found between the 88 residues forming the core region of oryzacystatin-I and the corresponding 89 residues of AtCYS1 (Fig. 1A). It must be noted that both the N-terminal 10 residues and C-terminal seven residues display a high flexibility in the NMR structure of oryzacystatin-I [33]. For this reason, the core region of oryzacystatin-I (residues 6–93) was used as a template to model the corresponding region of AtCYS1. Analysis of the molecular model of AtCYS1 shows that all amino acid substitutions, including the single insertion of Ala15A, are easily accommodated in the structure without any gross backbone rearrangement (Fig. 1B). In particular, all the residues forming the hydrophobic core of oryzacystatin-I are conserved or conservatively substituted in AtCYS1. Moreover, all charge substitutions occur on the protein surface and the position and length of secondary structure elements (five β-strands and one α-helix) are conserved in AtCYS1.

As shown in Table 1, values of *K*<sub>d</sub> for binding of plant and animal cystatins to papain span 10<sup>-1</sup>–10<sup>-8</sup> µM (Table 1), reflecting differences in enzyme–inhibitor recognition. To provide a rationale for the striking difference in



**Fig. 1. Amino acid sequence alignment of selected cystatins and schematic representation of the molecular models of AtCYS1 and AtCYS1–papain complex.** (A) Sequence alignment of Atcys1 (*A. thaliana*), oryzacystatin I (*O. sativa* I), chicken egg white cystatin (*G. gallus*, Gly9-truncated form [1]), human stefin A (*H. sapiens* A) and human stefin B (*H. sapiens* B). Residues are coloured according to their chemical properties. The green boxes under the alignment indicate the degree of sequence consensus. Note the high conservation of the Gln53-Val54-Val55-Ala56-Gly57 sequence forming the first hairpin loop of cystatins. The alignment was obtained using the program CLUSTALW [36]. (B) Molecular model of AtCYS1 (blue) superimposed onto the three-dimensional structure of oryzacystatin I (red) used as a template for modelling. (C) AtCYS1 model (blue) superimposed onto the three-dimensional structure of the complex formed by papain (green) and stefin B (magenta). The arrow indicates the different conformation of the AtCYS1 second hairpin loop, as compared to stefin B. AtCYS1 is rotated by approximately 180 degrees with respect to (B). The figure was made using GRASP [69]. For details, see text.

**Table 1. Dissociation equilibrium constants ( $K_d$ ) for the interaction between selected cystatins and papain.**

Cystatin	$K_d$ ( $\mu\text{M}$ )	pH and temperature	Reference
AtCYS1	$4.0 \times 10^{-2}$	pH 7.0 and 25 °C	Present study
Oryzacystatin I	$3.0 \times 10^{-2}$	pH 6.5 and 37 °C	[71]
Chicken egg white cystatin	$6.0 \times 10^{-8}$	pH 7.4 and 25 °C	[72]
Human stefin A	$1.8 \times 10^{-7}$	pH 7.4 and 25 °C	[73]
Human stefin B	$4.9 \times 10^{-8}$	pH 7.4 and 25 °C	[74]

affinity observed between binding of AtCYS1, oryzacystatin-I, chicken egg white cystatin, and human stefins A and B to papain (Table 1), molecular models of the complexes of

these inhibitors with papain have been built based on the three-dimensional structure of stefin B in complex with papain [41].

Analysis of the five protease-inhibitor complexes confirm a common mode of cystatin interaction with papain, whereby the inhibitors bind to this protease through a 'tripartite wedge' [41] formed by the N-terminal 'trunk', which occupies the unprimed subsites of the enzyme, the first hairpin loop, containing the highly conserved Gln-Val-Val-Ala-Gly sequence [41], and the second hairpin loop. However, while the first hairpin loop is highly conserved both in sequence and geometry in all the inhibitors analysed, contributions from the trunk and the second hairpin loop vary among the different inhibitors.

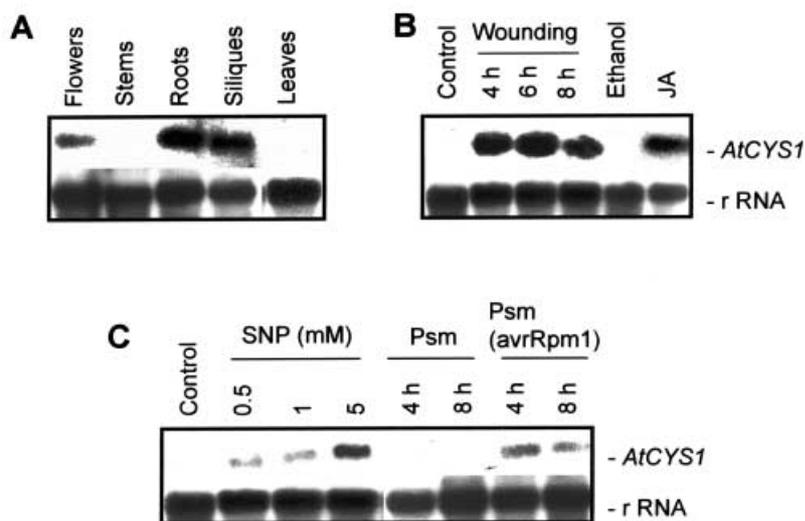
The interaction between papain and stefin B has been demonstrated to rely on tight hydrophobic contacts established mainly by the N-terminal trunk and the first hairpin loop of stefin B [41], with minor contributions coming from the second hairpin loop [52]. The same interactions are basically conserved in the modelled stefin A-papain complex. In chicken egg white cystatin, contribution of the N-terminal trunk to the interaction with papain is greatly reduced due to deletion of the first three residues found in stefins A and B. This effect may be counterbalanced by the interactions observed in the modelled complex between inhibitor Asp15 and Asp18, and papain Lys139 and Lys156. In the papain-AtCYS1 and papain-oryzacystatin-I complexes, steric hindrance is observed between the second hairpin loop and the papain region including Trp177. In addition, in the papain-AtCYS1 complex the inhibitor Arg12 residue faces papain Lys156, resulting in an unfavourable electrostatic interaction (Fig. 1C). This is not observed in the papain-oryzacystatin-I complex in which inhibitor Glu12 may interact with protease Lys156.

### Distribution and expression pattern of AtCYS1

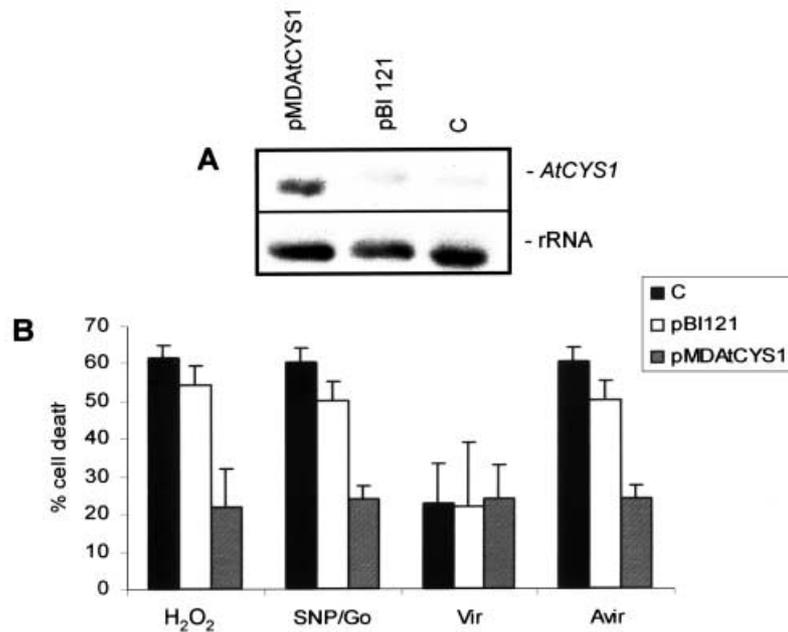
DNA from *A. thaliana* seedlings was probed with the AtCYS1 cDNA clone. A single band was detected in genomic DNA cut with *SalI* and *EcoRI*, whereas DNA cut with *HindIII* and *BamHI* revealed two and three bands, respectively (data not shown). This would indicate that AtCYS1 belongs to a small gene family having two very closely related sequences. A BLAST search with the AtCYS1 gene of the *Arabidopsis* genomic sequences from the *Arabidopsis* Genome Initiative project (<http://www.arabidopsis.org/blast>) revealed 100% identity with the locus At5g12140 and 96% identity with a 50 bp region of locus At3g12490 which encodes another putative cysteine protease inhibitor. The search of the *Arabidopsis thaliana* Gene Index ([http://www.tigr.org/tdb/tgi/agi/searching/name\\_search.html](http://www.tigr.org/tdb/tgi/agi/searching/name_search.html)) revealed four other putative cysteine protease inhibitors, as suggested by the presence of the cystatin sequence Gln-Val-Val-Ala-Gly [41]. However, these genes, At2g31980, At2g40880, At4g16500 and At5g05110, share no significant homology with AtCYS1 either at amino acid or DNA level.

Northern blot analysis was used to investigate the expression pattern of AtCYS1. A single transcript of 0.5 kb was readily detected in roots and in developing siliques. The transcript was less abundant in flowers and absent in intact leaves and stems (Fig. 2A). After wounding or treatment with jasmonic acid, transcription of AtCYS1 was induced 4–8 h later, similar to other cysteine protease inhibitors (Fig. 2B).

Infection of *A. thaliana* ecotype Col-O, which contains the *RPM1* resistance gene, with *Pseudomonas syringae* pv. *maculicola* carrying the corresponding *avrRpm1* avirulence



**Fig. 2.** Molecular analysis of AtCYS1 from *Arabidopsis thaliana*. (A) Organ-specific expression of the AtCYS1 gene. Northern-blot analyses were performed using total RNA, with RNA loading checked by reprobing the same filter with an 18S rRNA probe [70]. (B) Effect of wounding and jasmonic acid on AtCYS1 transcript accumulation. Wounded leaves were analysed at the indicated time points. Control leaves infiltrated with water, control leaves infiltrated with 0.1% (v/v) ethanol and leaves infiltrated with 45.5  $\mu\text{M}$  jasmonic acid in 0.1% (v/v) ethanol (JA) were analysed 10 h after treatment. (C) Effect of NO and *Pseudomonas syringae* pv. *maculicola* on AtCYS1 transcript accumulation. Control leaves infiltrated with water and leaves infiltrated with the indicated concentrations of the NO-donor SNP were analysed 2 h after treatment. Leaves infiltrated with  $10^6$  CFU·mL<sup>-1</sup> of virulent or avirulent *P. syringae* pv. *maculicola* were analysed 4 and 8 h after treatment. For details, see text.



**Fig. 3.** Effect of ectopic expression of *AtCYS1* in *Arabidopsis* suspension cultures. (A) Accumulation of *AtCYS1* mRNA transcript. The letter C indicates untransformed control cells. RNA loading was checked by reprobing the same filter with an 18S rRNA probe [70]. (B) Cell death triggered by different stimuli. Cells were treated with 5.0 mM H<sub>2</sub>O<sub>2</sub> (H<sub>2</sub>O<sub>2</sub>), with a solution consisting of 0.5 mM SNP, 5.0 U glucose oxidase, and 10 mM glucose (SNP/Go), with  $5 \times 10^7$  CFU *Pseudomonas syringae* pv. *maculicola* (Vir), and with  $5 \times 10^7$  CFU *Pseudomonas syringae* pv. *maculicola* carrying *avrRpm1* (Avir). Cell death was assayed 20 h after treatment with H<sub>2</sub>O<sub>2</sub> or SNP/Go and 28 h after bacteria inoculation. The experiment was repeated three times. For details, see text.

gene induces a localized hypersensitive response (HR), in which lesion development is accompanied by restriction of bacterial growth and spread [46]. A significant accumulation of *AtCYS1* transcripts occurred 4 h after infection of *A. thaliana* leaves with the avirulent *P. syringae* pv. *maculicola* *avrRpm1*, but not with virulent bacteria (Fig. 2C). This rapid gene induction occurred well before the appearance of HR cell death, which becomes evident only after 12 h (data not shown). The NO-donor SNP induces hypersensitive cell death within 12 h when infiltrated in leaves exposed to sunlight in order to maintain a sufficient level of reactive oxygen intermediates (unpublished observation). Consistent with the involvement of this cystatin gene in the HR, transcription of *AtCYS1* was also induced within 2 h by infiltration of *A. thaliana* leaves with increasing concentrations of SNP (Fig. 2C).

#### Ectopic expression of *AtCYS1* in *Arabidopsis thaliana* cell suspensions suppresses cell death triggered by different stimuli

*A. thaliana* cells were transformed with *A. tumefaciens* EHA105 carrying either *AtCYS1* or *GUS* (control), both under control of the CaMV35S promoter. Transformation with *GUS* allowed an estimation of the transformation efficiency, which was at least 70–80% (data not shown). Because the experiment was completed within 4 days following inoculation, we did not analyse whether expression of the transgene was transient or stable. After transformation, the majority of agrobacteria were removed by extensive washing and the *A. thaliana* cells were in part

analysed for transgene activity (Fig. 3A) and in part challenged with PCD-inducing oxidative stress. The overexpression of *AtCYS1* effectively blocked cell death induced by several stimuli (Fig. 3B). In many systems, oxidative stress was found to be a potent inducer of PCD [53]. Treatment of suspension cultured cells with 5.0 mM H<sub>2</sub>O<sub>2</sub> triggered the cell death program in untransformed as well as in *GUS*-transformed *A. thaliana*, but did not alter cell viability in the *AtCYS1*-transformed line, indicating suppression of PCD by the transgene (Fig. 3B).

It has been shown previously that during HR, NO cooperates with H<sub>2</sub>O<sub>2</sub> to induce cell death from otherwise sublethal amounts of H<sub>2</sub>O<sub>2</sub> [26]. In suspension cultured cells, the addition of NO alone is not sufficient to activate the cell death program because the level of endogenously generated reactive oxygen intermediates is too low [26]. To produce a lasting oxidative burst, suspension cultured cells were treated with 5.0 U of glucose oxidase and 10 mM glucose [54]. This level of stress did not result in a significant amount of cell death. However, supplementation of cultures with the NO-donor SNP (0.5 mM) greatly augmented the degree of cell death in both untransformed and *GUS*-transformed cultures. However, cells transformed with the *AtCYS1* gene were resistant to this treatment (Fig. 3B).

The ability of *AtCYS1* to suppress HR-associated cell death was further characterized by challenging the transformed cells with avirulent pathogens. Infection of *A. thaliana* cell suspensions with  $5 \times 10^7$  CFU *P. syringae* pv. *maculicola* carrying the *avrRpm1* avirulence gene induced HR cell death in both untransformed and *GUS*-transformed lines. In the *AtCYS1*-transformed line, similar

infections failed to cause significant cell death, supporting the involvement of cysteine proteases in hypersensitive cell death (Fig. 3B).

### Inhibition of hypersensitive cell death by AtCYS1 expression in tobacco plants

To study the possible functions of *AtCYS1* in whole plants, transgenic tobacco plants carrying this protease inhibitor gene under the control of the constitutive CaMV35S promoter were regenerated. The expression pattern of *AtCYS1* in different putative transformed plant lines was verified by Northern blot analysis. A single signal of approximately 0.5 kb in most of the putative transgenic plantlets was observed (data not shown). No signal was detected in control plants.

Protein extracts from selected *AtCYS1* transgenic tobacco lines were examined for inhibition of cysteine protease activity. In particular, extracts from tobacco transgenic lines N21, N11 and N30 showed 24.9%, 77.5% and 83.7% inhibition of papain activity, respectively. No inhibitory effects on papain action were observed using control tobacco plants.

Two lines possessing moderate and high levels of inhibitory protease activity were compared to wild type control plants after infiltration of leaves with either SNP or HR-inducing nonhost bacteria. Cell death is triggered by a fine balance of reactive oxygen intermediates and NO, in which the former channel NO through the cell death pathway [55]. In the absence of pathogens, this can be mimicked by exogenous NO in plants exposed to high light (M. Delledonne, unpublished data). SNP (1.0 mM) was infiltrated into wild type and transgenic plants and the appearance of necrotic lesions in leaves was followed over time. Clear lesions appeared as early as 12 h after treatment. A dramatically reduced necrosis occurred in tobacco leaves of the transgenic *AtCYS1* plants infiltrated with the NO-donor (Fig. 4A).

To test the effect of *AtCYS1* on biotically induced HR, tobacco leaves were inoculated with  $10^7$  CFU·mL<sup>-1</sup> *Pseudomonas syringae* pv. *phaseolicola* NPS3121, which elicits the HR [56]. Necrotic lesions typical of the HR response appeared on leaves of control plants 24 h after treatment, while lesions were dramatically reduced on leaves of *AtCYS1* transgenic plants even after 72 h (Fig. 4A).

Generation of H<sub>2</sub>O<sub>2</sub> is a major component of HR. To test whether *AtCYS1* inhibited the HR through mediating the oxidative burst, the production of H<sub>2</sub>O<sub>2</sub> in infected plants was measured. DAB polymerizes on contact with H<sub>2</sub>O<sub>2</sub> in a peroxidase-dependent reaction, enabling H<sub>2</sub>O<sub>2</sub> visualization *in situ* as a reddish-brown precipitate [49]. A strong, brown precipitate was observed in control plants following infection with the incompatible *P. syringae* NPS3121 (Fig. 4B). The same analyses carried out with transgenic plants from the N21 and N30 lines produced very similar results, indicating that the reactive oxygen intermediates signal transduction pathway activated by pathogen recognition was fully functional. Trypan blue staining performed on other leaves from the same experiment confirmed that hypersensitive cell death was strongly reduced in transgenic plants (Fig. 4C). Thus, the

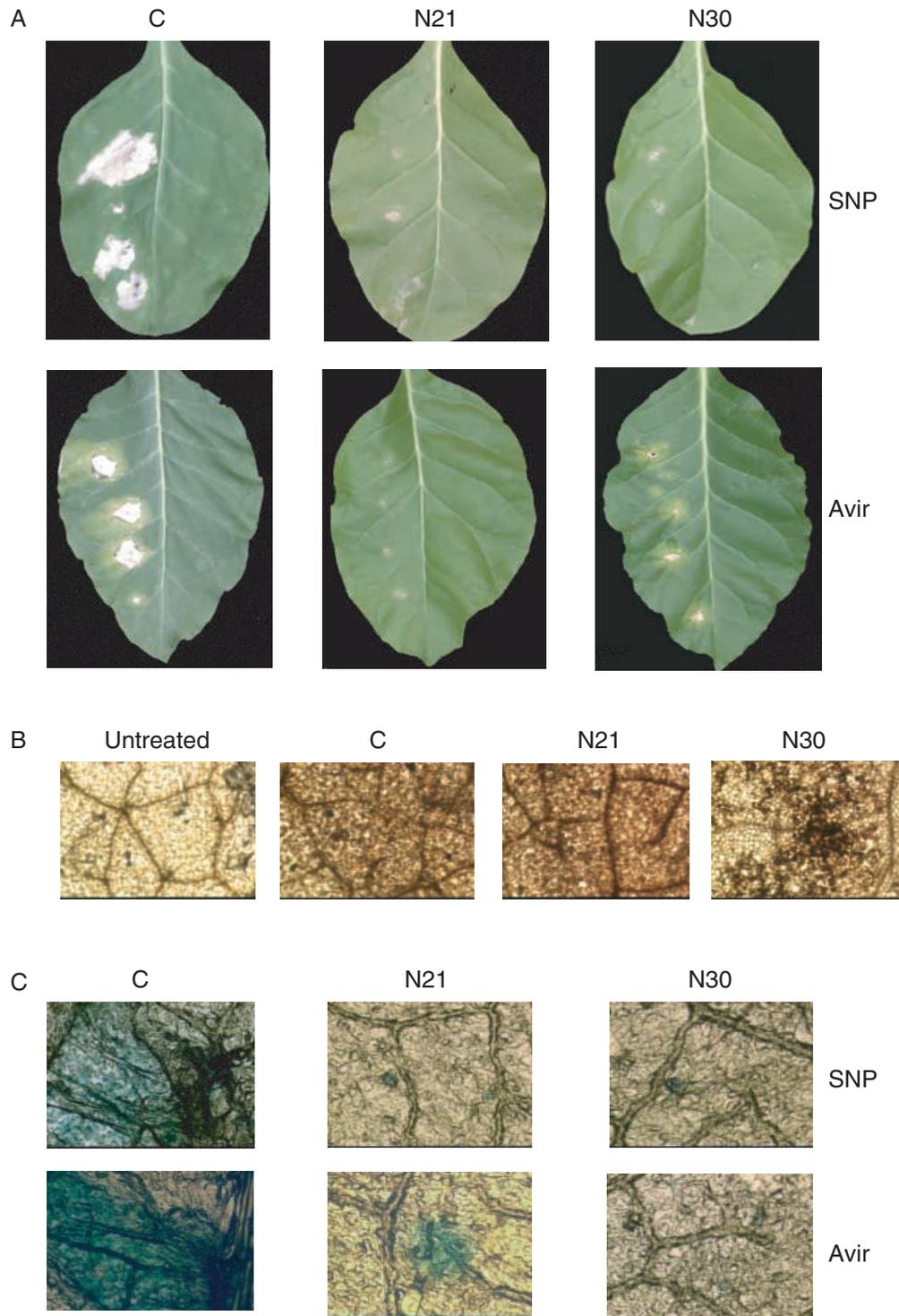
expression of *AtCYS1* blocks hypersensitive cell death without affecting the initial response of the host cells to the incompatible pathogen.

### Discussion

Proteases and related inhibitors have several physiological roles [2]. Among others, plant cysteine proteases are involved in seed germination, and cystatins participate in the control of endogenous protease activity [2,57]. The *AtCYS1* transcript was particularly abundant in developing siliques. This suggests that it modulates the activity of cysteine proteases during seed maturation, when proteins must be accumulated for storage and then hydrolysed for amino acid assimilation during germination [58]. Protease inhibitors also play an important role in various defence mechanisms that offer protection against many kinds of biotic agents such as bacteria, fungi, nematodes and insects. The expression of *AtCYS1* occurring after wounding or exogenous application of jasmonic acid (present study), together with the high degree of resistance to *Chrysomela populi* larvae conferred in transgenic poplar plants, strongly supports the proposed function of this cystatin in resistance against herbivorous insects [25]. However, *AtCYS1* was also induced by treatment with either NO-donor or avirulent pathogens, both of which led to hypersensitive cell death. The overexpression of *AtCYS1* blocked cell death triggered by either avirulent pathogens or oxidative and nitrosative stresses in *Arabidopsis* cell suspensions, supporting the hypothesis that plant cells employ cysteine protease inhibitors to modulate the cell death program. The accumulation of *AtCYS1* in the tissue surrounding the infected cells would avoid the death of those adjacent, uninfected cells, which perceive the cell death triggering signals produced by cells undergoing HR. Thus, such a mechanism would utilize the available resources to avoid unregulated spreading of the suicide response in uninfected areas [20].

This dual role of *AtCYS1*, as both a killer of herbivorous insects and a blocker of HR cell death, is somehow in contradiction with the often observed inverse relationship between systemic plant resistance to microorganisms and herbivorous insects [59]. However, evidence of the trade-off between resistance to pathogens and herbivorous insects is equivocal and the relationship between them seems to depend upon the particular species of plant, herbivorous insect, and pathogen involved [14].

Several lines of evidence suggest that death of host cells during the HR results from the activation of a suicide process by modulating the levels of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and NO [55,60]. The molecular mechanism of this interplay is not clearly understood. *In vitro* studies have suggested that a reaction between gaseous NO and H<sub>2</sub>O<sub>2</sub> produces singlet oxygen or hydroxyl radicals [61]. Alternatively, the toxicity of NO/H<sub>2</sub>O<sub>2</sub> may be due to the production of a potent oxidant derivative of NO/H<sub>2</sub>O<sub>2</sub>, formed via trace metals [62]. Much of the oxidative damage in animals is mediated via iron through the Fenton reaction [60]. However, the observed inhibition of NO-mediated cell death in *A. thaliana* cell suspensions and transgenic tobacco plants overexpressing *AtCYS1* strongly argued in favour of a function as a cell death trigger, through the stimulation of an active



**Fig. 4. Suppression of HR lesions in *AtCYS1* tobacco plants.** Leaves from control (C) or *AtCYS1* transgenic lines (N21 and N30) were infiltrated with 1.0 mM SNP or with  $10^7$  CFU mL<sup>-1</sup> *Pseudomonas syringae* pv. *phaseolicola* (Avir). (A) Pictures of representative leaves were taken 48 h or 72 h after treatment with SNP or avirulent pathogens, respectively. (B) Leaf discs from control and *AtCYS1* tobacco plants were immersed in DAB solution 1 h after inoculation with the avirulent pathogen and stained 24 h later. (C) Leaf discs from control and *AtCYS1* tobacco plants were stained with trypan blue 8 h after infiltration with SNP or 24 h after infiltration with *Pseudomonas syringae* pv. *phaseolicola*. For details, see text.

process in which cysteine proteases appear to play a crucial role. At the same time, these data allow us to rule out a general cytotoxic effect of NO.

Recent evidence points to the mitochondrion as an essential organelle in the regulation of cellular responses to stress [63]. Both H<sub>2</sub>O<sub>2</sub> and NO cause the release of cytochrome *c* from mitochondria, triggering the cell death program [54,64]. Thus, NO released during pathogen attack (especially at elevated SA concentrations) may trigger a reactive oxygen intermediate-amplifying cascade leading to cell death [54]. It has been demonstrated recently in both maize and *A. thaliana* cells that the release of cytochrome *c* from mitochondria induced by D-mannose results in DNA laddering [65]. Furthermore, NO-donors reduced the survival of suspension cultured cells of *Citrus sinensis* by a mechanism consistent with a mitochondrial-dependent apoptotic process [66]. In animals, apoptosis relies on a cascade of enzymes that are mostly cysteine proteases that specifically cleave after Asp residues (caspases). Caspases are both negatively and positively regulated by proapoptotic (Bax) and antiapoptotic (Bcl) factors, respectively. Bax favours the release of cytochrome *c* and of an apoptosis-inducing factor from the mitochondrial intermembranal space. This apoptosis-inducing factor moves directly to the nucleus where it induces chromatin condensation and nuclear fragmentation. Cytochrome *c* activates a caspase-signaling cascade that selectively cleaves vital substrates in the cell, including the nuclease responsible for DNA fragmentation [63,66]. Although caspases have not been found in the genomic and EST plant databases, several lines of evidence demonstrate that cysteine proteases are involved in plant PCD [20,67]. In oat, activation of cell death by victorin has been shown to be mediated by cysteine proteases sensitive to E-64 and calpeptin [68]. Moreover, the overexpression of cystatin in soybean cell cultures was shown to suppress cell death induced by oxidative stress and avirulent pathogens [20]. Therefore, a regulatory role for cysteine proteases can be envisioned in the processing of cell death-promoting proteins into an active form and in the degradation of regulatory proteins that prevent PCD. Thus, cysteine proteases that interact with cystatin may be good candidates for activities that execute the plant cell death.

## Conclusions

Cystatins can be regarded as powerful tools for biotechnological applications. AtCYS1 finely regulates biotic and abiotic induction of PCD in plants, and was found to confer increased resistance to herbivorous insects when expressed in transgenic poplar plants [25]. Thus, the AtCYS1 expression may provide an efficient mechanism for improving plants resistance to either biotic or abiotic stress. However, any biotechnological application of AtCYS1 requires the knowledge of the natural target(s) of its inhibitory activity. In this respect, the expression and the biochemical characterization of AtCYS1 represent a fundamental step towards the identification of the target *A. thaliana* cysteine protease(s).

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