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Biotic and abiotic stress can induce cystatin expression in chestnut

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Abstract A cysteine proteinase inhibitor (cystatin) from chestnut (*Castanea sativa*) seeds, designated CsC, has been previously characterized. Its antifungal, acaricide and inhibitory activities have allowed to involve CsC in defence mechanisms. The CsC transcription levels decreased during seed maturation and increased throughout germination, an opposite behavior to that shown by most phytocystatins. No inhibition of endogenous proteinase activity by purified CsC was found during the seed maturation or germination processes. CsC message accumulation was induced in chestnut leaves after fungal infection, as well as by wounding and jasmonic acid treatment. Induction in roots was also observed by the last two treatments. Furthermore, CsC transcript levels strongly raised, both in roots and leaves, when chestnut plantlets were subjected to cold- and saline-shocks, and also in roots by heat stress. All together, these data suggest that chestnut cystatin is not only involved in defence responses to pests and pathogen invasion, but also in those related to abiotic stress.

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Key words: Chestnut cystatin; Expression pattern; Biotic stress; Abiotic stress; Germination

1. Introduction

Phytocystatins are 12–16 kDa plant proteins with no disulphide bonds, which inhibit cysteine proteinases [1–3]. Several members of this protein family have been characterized in different plant species [4–6] and homology with animal cystatins has been described, although phytocystatins cluster in a specific subgroup of the cystatin superfamily [3]. A defence role of phytocystatins is now widely assumed based on their in vitro inhibition of digestive proteinases from coleopteran pests and nematodes, as well as the enhanced resistance to these phytophagous organisms shown by transgenic plants expressing plant cystatins [6–10]. The induction of these cysteine proteinase inhibitors by wounding and/or methyl jasmonate reported in tomato and soybean [11,12], as well as their accumulation in tomato leaves expressing the prosystemin transgene [13,14], support the involvement of cystatins in plant defence mechanisms.

In seeds, phytocystatin mRNAs show an expression pattern similar to that of major seed storage proteins [4,5]. Likewise, the inhibitor levels decrease at about the same rate as total protein throughout the germination process [15,16]. Both facts, together with the inhibition of endogenous cysteine proteinases by plant cystatins [12,17], have led to the proposal of a role for these inhibitors in the regulation of protein turnover during seed development. Interestingly, the involvement of cystatins as modulators in programmed cell death has been recently reported in soybean [18].

The above data are mostly referred to herbaceous crops, mainly cereals and legumes. By contrast, a very limited effort has been devoted to uncover cystatins from other plant sources. Seeds from temperate forest trees, such as chestnut, seem particularly attractive, because of their high moisture content at shedding and the long periods that they remain in the soil before and during germination. Differences in the regulation of protein turnover and of plant defence components could be expected in such tree seeds with respect to those of crops. In this context, we have recently purified and characterized a cystatin from chestnut (*Castanea sativa*) seeds [19]. Its cDNA clone was also isolated, and the recombinant inhibitor expressed in *Escherichia coli*. Besides affecting commercial cysteine proteinases of plant and animal origin, the chestnut cystatin (CsC) also inhibits digestive proteases from insect pests and mites. Furthermore, it shows antifungal activity, inhibiting the growth of phytopathogenic fungi, such as *Botrytis cinerea* [20], an activity not described previously for phytocystatins.

We report here the expression patterns of CsC during seed maturation and germination, its induction by fungal infection, wounding and methyl jasmonate, and its response to heat, cold and saline stresses.

2. Materials and methods

2.1. Plant material

European chestnut (*C. sativa* Mill.) seeds were harvested in Zarzalejo (Madrid, Spain), frozen in liquid nitrogen, and stored at −80°C. Seeds were collected at three different stages directly from the trees, which corresponded to 90 (M1), 110 (M2) and 140 (MT, mature) days after flowering. Germination was performed in a growth chamber (16 h day/8 h night, 24°C/18°C, 70% RH), and samples were analyzed immediately (G1) and 4 (G2), 8 (G3) and 12 (G4) days after emergence of the radicle.

Treatments (see below) were carried out with plantlets 18–20 cm in height, grown under the same conditions used for germination.

The data presented in each figure are representative results of, at least, two independent experiments.

2.2. Treatments

Infection with the fungal pathogen *Botrytis cinerea* was performed by inoculation with a spore suspension (10 μl; 10^3 spores in 0.01% Tween 20). The suspension was placed on the leaf face, and both inoculated and non-treated leaves harvested 8 h after inoculation. Control plantlets were treated with 0.01% Tween 20.

Plantlets were wounded or treated with a 50 μM jasmonic acid (JA; Apex organics) solution essentially as described in [21]. All leaves were sprayed with JA, and samples were collected 8 h and 24 h after the treatment. In the case of wounding, all leaves of each plantlet were injured twice (24 h interval), and samples were harvested 6 and 10 h after the second injury. Alternatively, systemic induction was tested by wounding central leaves, and then analyzing the upper and lower ones after 8 and 24 h. Besides leaves, stems and roots were also analyzed in the JA treated and wounded plantlets.

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Heat-stress experiments were performed with well-watered plantlets at either 32 or 40°C and 80% RH, and the material was analyzed after 3 h and 8 h. Cold treatments were carried out at 4°C for up to 4 weeks. For salt stress assays, plantlets were watered with 200 μM NaCl, and samples were collected at 4, 10 and 24 h.

2.3. Southern blot analysis

Genomic DNA from chestnut seed was isolated by standard methods, and digested with *Bam*HI, *Sac*I, and *Hin*dIII endonucleases. Restriction fragments were separated by electrophoresis in 0,8% agarose, transferred onto Hybond N membranes (Amersham), and hybridized at 60°C, using a ^{32}P-labelled probe corresponding to the complete coding region of the CsC [19].

2.4. RNA extraction and gel-blot hybridization

Total RNA was extracted from chestnut seeds or plantlets as described previously [22]. RNA was quantified spectrophotometrically, fractionated on 1.2% formaldehyde agarose gels, and transferred to nylon membranes (Hybond N, Amersham) as in [23]. Hybridization was performed overnight at 60°C, using a ^{32}-labelled probe corresponding to the complete coding region of cystatin [19]. After hybridization, membranes were washed twice in 1×SSC (75 mM NaCl, 7.5 mM sodium citrate), 0.5% (w/v) SDS at 37°C for 15 min, and twice in 0.1×SSC, 1% SDS at 60°C for 15 min. Autoradiographs were taken on Kodak X-Omat-S film exposed overnight at −80°C.

2.5. Inhibition and protease activity assays

Flour was obtained from seeds at different maturation and germination stages, and defatted with acetone (1×; 5:1, w/v; 1 h; 4°C). Defatted flour was extracted with PBS buffer (100 mM sodium phosphate pH 7.0, 0.15 M NaCl; 1×; 5:1, w/v; 1 h; 4°C), and the extract dialyzed against 0.1 M ammonium acetate, and freeze-dried. Protein concentration was quantified according to Bradford [24].

Proteinase activity, as well as its inhibition, was assayed as in [19], using BANA (*N*-benzoyl-DL-arginine-β-napthylamide) as substrate. Commercial papain (EC 3.4.22.2, 0.13 μM; Sigma), and purified CsC [19] were used when required.

3. Results and discussion

3.1. A single gene for cystatin is present in chestnut

Southern blot hybridization patterns (Fig. 1) using three different restriction endonucleases were consistent with the presence of one copy of the cystatin gene per chestnut haploid genome.

3.2. Expression of CsC during seed development and germination

Northern blot analysis and inhibition tests were performed in order to evaluate the potential role of CsC on protein turnover throughout seed development and/or germination. The complete coding sequence of a cDNA clone for CsC [19] was used as a hybridization probe to estimate the cystatin mRNA levels. As shown in Fig. 2, a single band of around 700 b was detected at all stages analyzed. However, the CsC message content varied greatly during seed maturation, showing a decrease from M1 to mature stage (MT). In contrast, its level increased, throughout the germination process. The opposite expression patterns have been described for major storage proteins in chestnut seeds [25]. Furthermore, most phytocystatins maintain high levels of their corresponding mRNAs at seed maturity [5,12,26], although exceptions have been detected in rice and carrot [4,27]. The increase of CsC message content during germination is even more divergent with respect to the patterns previously described for plant cystatins, which drastically decrease throughout this process [15,16].

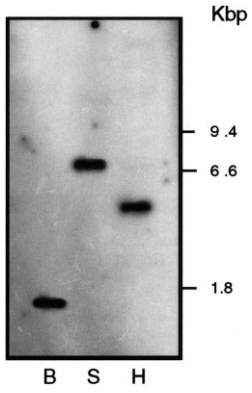

Fig. 1. Southern blot analysis of CsC gene. Genomic DNA samples (10 μg each) from chestnut seed were digested with the restriction endonucleases *Bam*HI (B), *Sac*I (S) or *Hin*dIII (H). The ^{32}P-labelled complete coding region of CsC was used as hybridization probe.

A second line of evidence involving cystatins in the regulation of protein turnover in seeds is based on the susceptibility of endogenous proteinases that degrade seed storage proteins to these inhibitors [12,17]. To test this possibility in chestnut seeds, inhibition assays were carried out using extracts from the different seed maturation and germination stages analyzed by Northern blotting. The results obtained are summarized in Table 1. Tests were performed at pH 6.5, according to previous experiments to determine the pH optima of the different

Table 1
Inhibition of the proteolytic activity in crude extracts from different maturation and germination stages by CsC and other proteinase inhibitors

Seed stage[a]	Inhibition (%)						
	+Cyst[b]			+$CaCl_2$[c]			
	CsC[d]	E-64	Leup	Leup	PMSF	KI	BB
M1	0	10±1	12±2	13±1	35±1	0	0
M2	0	7±1	15±2	15±3	19±0	0	0
MT	0	10±2	26±2	82±1	69±3	7±1	5±1
G1	0	0	18±3	45±2	41±3	0	0
G2	0	0	15±1	58±1	46±1	24±3	0
G3	0	0	10±1	61±1	41±1	0	15±2
G4	0	9±0	33±1	52±1	51±3	0	0

Values are means±S.E.M. ($n=4$).
[a]Seed samples as in Fig. 1. Protein amount was 100 μg/assay.
[b]Assays were performed in 50 mM sodium phosphate pH 6.5, 10 mM cysteine, 10 mM EDTA (cysteine proteinase activity buffer).
[c]Assays were performed in 50 mM Tris–HCl pH 6.5, 20 mM $CaCl_2$ (serine proteinase activity buffer).
[d]Inhibition concentration was 1.5 μM/assay. Leup: leupeptin; PMSF: phenylmethanesulfonyl fluoride; KI: soybean Kunitz inhibitor; BB: soybean Bowman–Birk inhibitor.

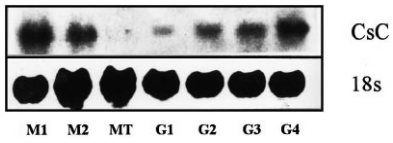

Fig. 2. Expression pattern of CsC mRNA during seed maturation and germination. 20 μg of total RNA were loaded per lane. Seed maturation stages: 90 days (M1) and 110 days (M2) after flowering, and mature seeds (MT). Germination stages: 0 (G1), 4 (G2), 8 (G3) and 12 (G4) days after radicle emergence. Similar RNA loading on this and next figures was verified by hybridization with a barley 18S ribosomal cDNA probe (18S).

seed extracts (maximum protease activity at pH 6–10 in all samples). Two buffers were used for each inhibition assay, containing cysteine or $CaCl_2$, which mainly accounted for cysteine or serine proteinase activity, respectively. The results obtained clearly indicate that the main proteolytic activities at all seed stages analyzed were not affected by CsC. Moreover, the major endogenous proteinases of chestnut seeds detected by the methods used, seem to be non-susceptible to standard inhibitors of cysteine proteinases (E-64), but partially blocked by PMSF and leupeptin, suggesting that enzymes of the serine type are prominent components of the total activity tested.

The results so far described suggest that CsC does not play a relevant role in the control of chestnut seed protein turnover during maturation and germination. However, its involvement in the regulation of non-prominent cysteine proteinases can not be completely discarded.

3.3. *Induction of CsC transcript by fungal infection, wounding and JA*

Different lines of evidence have allowed the involvement of phytocystatins in plant defence mechanisms (see Section 1). The in vitro inhibition of digestive proteinases from insects and mites [19], as well as its antifungal activity [20], suggest a role for CsC in chestnut seed and plantlet protection against pests and pathogens. To further support this potential role, the possible increase of CsC transcript levels induced by fungal infection, and by treatments closely related with pest and pathogen damage (mechanical wounding and JA accumulation), was investigated by RNA gel blot analysis.

Experiments to compare the expression levels of CsC transcript in different organs of chestnut plantlets, as well as in mature seeds, were performed (Fig. 3). No detectable transcript was found in seed, whereas low expression levels were observed in root and stem as compared with that of leaf.

CsC message accumulation was induced in chestnut leaves 8 h after inoculation with *Botrytis cinerea* (Fig. 4). CsC gene induction was observed both in infected and non-inoculated leaves, thus indicating a systemic response against fungal infection. Compared to control leaves, higher levels of CsC transcripts were still evident in leaves of treated plantlets 48 h after inoculation, although basal levels were observed at 24 h. No similar studies using pathogenic fungi have been reported for other phytocystatins.

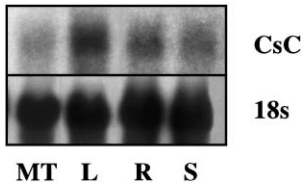

Fig. 3. Expression patterns of CsC transcript in mature seed (MT), leaf (L), root (R) and stem (S) of non-treated chestnut plantlets 18 cm in height. 20 μg of total RNA were loaded per lane.

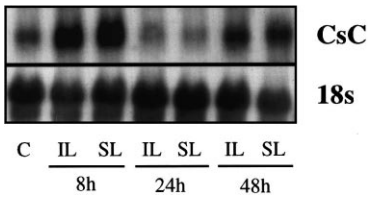

Fig. 4. Induction of CsC transcript accumulation by fungal infection. Leaves were inoculated with *Botrytis cinerea* spores, and samples from infected (IL) and non-infected (SL) leaves were analyzed at 8 h, 24 h and 48 h after inoculation. Leaves from non-treated plantlets were used as control (C). 20 μg of total RNA were loaded per lane.

Wounding also elicited both local and systemic accumulation of CsC transcripts in leaves, roots and stems of chestnut plantlets (Fig. 5). Two different treatments were tested. When all leaves were injured, a strong induction of CsC message was observed in leaves and roots after 10 h, and a weak response was achieved in stems. Alternatively, when only central leaves were wounded, increased levels of CsC transcripts were found in the treated leaves and the lower ones after 8 h, but no induction was detected in the upper leaves, roots or stems. In contrast, strong responses were obtained in the upper leaves but not in the lower ones or in roots and stems after 24 h. These results indicate a sequential systemic response in leaves, with the CsC message being detected first in the lower leaves, and later in the upper ones, with respect to the wounded leaf. This differential response suggests the presence of a systemic signal traveling through the phloem, as well as a bipolar transport, which is in line with results previously reported for other proteinase inhibitors [28,29].

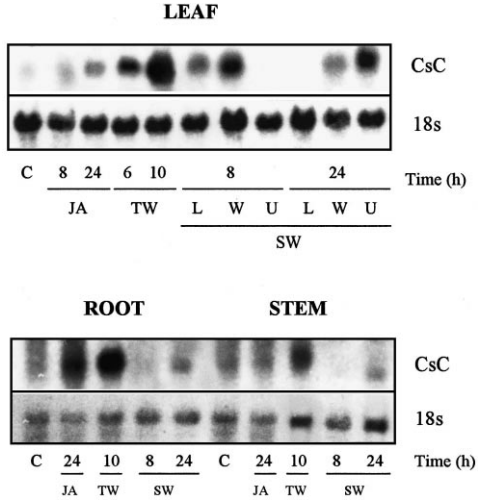

Fig. 5. Induction of CsC transcript accumulation by wounding or treatment with JA. Samples from plantlets treated with 50 μM JA, wounded in all leaves (TW), or only in the central leaves (SW), were analyzed at the times indicated after treatment. 20 μg of total RNA were loaded per lane. C: samples from control plantlets; W: wounded leaves; L: lower, and U: upper leaves.

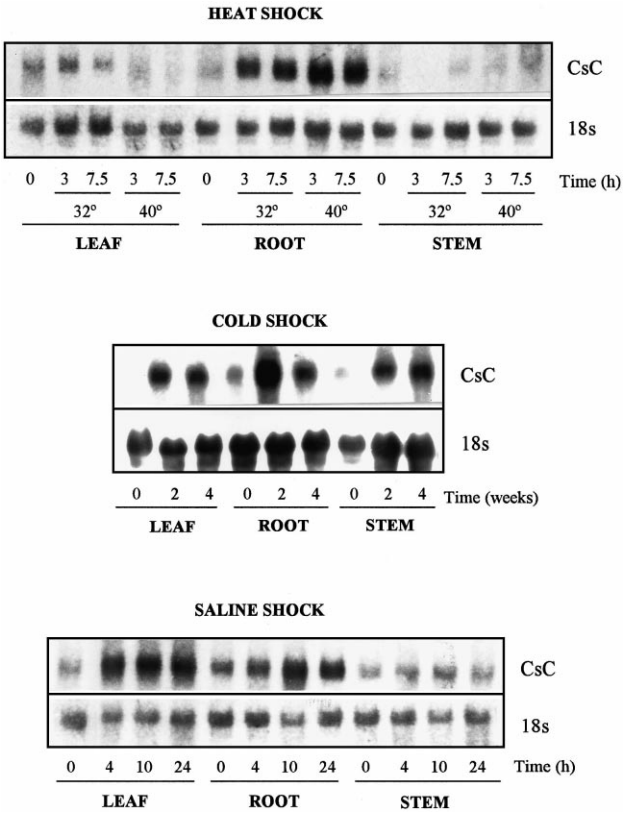

Fig. 6. Induction of CsC transcript accumulation by heat- (32 or 40°C), cold- (4°C), or saline- (200 mM NaCl) shocks. Samples were analyzed at the times indicated after treatment. Patterns of control plantlets at the final time of each treatment were similar to those presented in the figure. 20 μg of total RNA were loaded per lane.

JA is one of the plant hormones involved in signal transduction pathways which mediate the plant response to wounding and biotic stress [30]. Treatment of chestnut plantlets with JA (Fig. 5) induced CsC message accumulation in leaves and roots, but not in stems. Joint consideration of the data presented in Figs. 4 and 5, indicates that the levels of CsC transcript in leaves increase in response to fungal infection, wounding and JA treatment. This behavior is fully in agreement with a role for CsC in chestnut defence mechanisms. Interestingly, JA and wounding also elicit a CsC message accumulation in roots, a chestnut organ particularly susceptible to pathogen attack during the large germination periods of these seeds.

Information on the induction of other phytocystatin transcripts by the treatments used in this work is still very limited. However, soybean and potato cystatins have shown to be induced by wounding and methyl jasmonate [12,31], while tomato cystatin responds to methyl jasmonate but not to mechanical injury [11].

3.4. Induction of CsC transcripts by heat-, cold-, and saline-stress

Phytocystatins have not been directly implicated in abiotic stress responses. However, accumulation of cystatin messages by cold shock has been reported in avocado [32], and by abscisic acid treatment (a plant hormone involved in abiotic stress responses) in potato [31]. On the other hand, cysteine proteinases are induced by different stress conditions, such as senescence, dehydration, and glucose starvation [33–36], although the presence and/or increase of their potential endogenous inhibitors have not been studied in these reports. These data, together with the suspected connections between the responses to biotic and abiotic stress, led us to investigate the effect of heat-, cold- and saline-shocks on the level of CsC transcript in chestnut plantlets. The results obtained are summarized in Fig. 6. All three shocks induced a strong CsC message accumulation in roots, and cold and saline stresses also in leaves. By the contrary, a substantial increase appeared only by cold-shock in stems. No apparent phenotypical effect was observed after the different treatments.

Therefore, both biotic and abiotic stress elicit a CsC message accumulation, suggesting that CsC is probably involved in a response mechanism of chestnut plants which can be shared, at least in part, by the two stress types. Such a general response system, probably mediated by different hormones (abscisic acid or JA) depending on the stimulus, have been recently proposed for herbaceous plants [37].

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