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Ethylene regulates the expression of a cysteine proteinase gene during germination of chickpea (*Cicer arietinum* L.)

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Abstract

Synthetic oligonucleotides corresponding to conserved regions of cysteine proteinases were used as primers in the RT-PCR amplification of a fragment of cDNA corresponding to a region of a cysteine proteinase gene expressed during germination of chickpea (*cac* for *Cicer arietinum* cysteine proteinase). The identity of the PCR-amplified fragment was confirmed by sequencing and the fragment used as a probe to investigate the pattern of *cac* gene expression during germination and its hormonal regulation. The corresponding transcript is undetected in the seed during embryogenesis and before imbibition, being detected 24 h after imbibition. Ablation of the embryonic axis before imbibition results in a dramatic decrease in the amount of transcript detected. Expression of the *cac* transcript in excised cotyledons is restored in the presence of aqueous extracts from embryonic axes and also by incubating the excised cotyledons in 1 mM ethephon. Experiments with various known inhibitors of ethylene action indicate that ethylene activates the expression of *cac* gene in the cotyledons of chickpea during normal germination.

Introduction

The degradation of proteins in germinating seeds is a complex process in which several types of proteolytic enzymes are involved. According to their active site, proteolytic enzymes are classified as aspartic-acid proteinases, serine proteinases, metallo proteinases and cysteine proteinases [31]. Although all four types of proteinases have been purified from seeds [11, 25, 35, 36] it is recognized that during germination, there is a marked increase in the activity of cysteine proteinases, which are responsible for the catabolism of the majority of reserve proteins [24, 28].

In cereals, the hormonal control of transcription of cysteine proteinase encoding genes has been demonstrated for barley aleurain and rice oryzains. Aleurain, as well as oryzain gene transcription, is activated by gibberellic acid and repressed by abscisic acid [23, 24]. In legumes,

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X70375.

synthesis *de novo* of cysteine proteinases has been demonstrated in several instances [5, 20] but the hormonal factors mediating its regulation and their precise mode of action have not been resolved.

The role of ethylene in germination has been intensively studied from a physiological point of view, and this hormone is considered to promote germination via an increase in aerobic respiration and influencing water potential of the seed [12]. The cotyledons of legume seeds have been used to measure binding of ethylene to specific receptors [29], but at the molecular level, there is no evidence of the control of gene expression by ethylene during germination. This is in contrast with the situation in other plant developmental processes, such as fruit ripening, leaf abscission or defence reactions in which expression of a number of genes has been shown to be regulated by ethylene [8, 10, 15, 16]. Here we report the first evidence at the molecular level for a germination-related gene which is regulated by the phytohormone ethylene.

Materials and methods

Plant material

Seeds of *cicer arietinum* L. were imbibed in distilled water and allowed to germinate in the dark on moistened filter paper at 25 °C. Cotyledons were harvested at desired stages of germination and stored at −70 °C prior to use. 50 μM of abscisic acid (ABA) and 1 mM ethephon were added when indicated.

Effects of inhibitors on proteolytic activity

Crude extracts were obtained by homogenizing cotyledons in 0.1 M sodium succinate pH 5.5, 10 mM 2-mercaptoethanol in a Sorvall omnimixer. The homogenates were centrifuged (20000 × *g*, 30 min) and the supernatant dialysed against extraction buffer prior to precipitation with ammonium sulphate. Proteins precipitating in the presence of 30–70% ammonium sulphate were resuspended in extraction buffer and again dialysed. Proteolytic activities in the extracts (0.5 ml per gram of cotyledon) were determined by mixing 400 μl of the extract with 400 μl of Azocasein solution (1% w/v in the extraction buffer). After 1 h incubation at 37 °C, proteins were precipitated with 200 μl 50% TCA (trichloroacetic acid) and pelleted by centrifugation. An aliquot (600 μl) of the clear supernatant was mixed with 600 μl of 2 M NaOH and the absorbance at 430 nm determined. Inhibitors used are: Pepstatin A (1 μg/ml for aspartic acid proteinases), PMSF (phenylmethylsulphonylfluoride, 1 mM) for serine proteinases, iodoacetic acid (1 mM) for cysteine proteinases and EDTA (10 mM) for metallo proteinases, according to the procedure described by Storey and Wagner [31]. Azocasein and inhibitors were obtained from Sigma Chemical Company. The inhibitors were preincubated with the extracts for 30 min before proceeding with the reaction.

First-strand cDNA synthesis

Total RNA was extracted according to the SDS-phenol method [4] with the modifications of Martin and Northcote [17]. Poly(A)$^+$ RNA was purified by affinity chromatography using an oligo-dT cellulose column (Boehringer). cDNA was synthesized using the Moloney-murine leukemia virus reverse transcriptase according to the manufacturer's instruction (Stratagene).

Oligonucleotide synthesis

Degenerate oligonucleotides were synthesized corresponding to conserved amino acid sequences of known cysteine proteinases for use as primers in PCR amplification. The oligonucleotide corresponding to the 5′ region was derived from the amino acid sequence SCWAFS from Ser-24 to Ser-29 in the published sequence of C14, a tomato cold-induced cysteine proteinase [26] and the oligonucleotide corresponding to the

3′ region from the amino acid sequence QPVS from Glu-130 to Ser-133 in the same nucleotide sequence. Both oligonucleotides contained an *Eco* RI site to facilitate further cloning of the PCR-amplified fragments [27]. The resulting oligonucleotides were 5′-GGGAATTCAG(TC)-TG(TC)TGGGCNTT(TC)TC-3′ and 5′-GGG-AATTC(GA)CTNACNGG(TC)TC-3′ respectively.

PCR amplification

PCR reaction was carried out using a Perkin Elmer/Cetus DNA Thermal Cycler apparatus (model 480) and the Perkin Elmer/Cetus kit, according to the manufacturer's protocol. The primers were used at a concentration of 1 μM and the template DNA at a concentration of 1 μg/ml. The final reaction volume was 100 μl. Denaturation, annealing and extension temperatures were 94 °C for 1.5 min, 48 °C for 2 min and 72 °C for 3 min respectively. This cycle was repeated 30 times. cDNA generated from cotyledons 48 h after imbibition was used as well as total DNA from chickpea as a template.

Cloning of the PCR obtained fragment

An aliquot of the PCR reaction was run in a 2% agarose gel to visualize the products. The band of interest was purified from the gel by repeated freezing and thawing in liquid nitrogen in the presence of phenol, followed by ethanol precipitation and restriction digestion using *Eco* RI. The resulting fragments were cloned in pBluescript (Stratagene) using T4 DNA ligase (Amersham).

DNA sequencing

DNA sequencing was performed with the T7 DNA sequencing kit (Pharmacia). The amino acid sequence was deduced using the DNasis program and aligned to other cysteine proteinases using the Clustal program.

Northern analysis

Total RNA was fractionated by gel electrophoresis on denaturing 3% formaldehyde 1.5% agarose gels and blotted onto Hybond membranes (Amersham). RNA transfer buffer was 20 × SSC. The membranes were prehybridized in 0.25 M sodium phosphate pH 7.2, 1 mM EDTA and 7% SDS for 30 min at 65 °C. Hybridization was carried out in the same conditions by adding the labelled denatured probe to fresh buffer and incubating for 16–24 h. The probe consisted of 20 ng of purified PCR product labelled with ^{32}P with the Random Primer labelling kit (Boehringer). After hybridization, the membranes were washed with 40 mM sodium phosphate pH 7.2, 1 mM EDTA and 5% SDS at 65 °C for 30–60 min twice and subsequently with 40 mM sodium phosphate pH 7.2, 1 mM EDTA and 1% SDS at 65 °C for 30–60 min twice, prior to exposure to Kodak-Xomat film at −70 °C.

Gas chromatography

Ethylene biosynthesis from the seeds was quantified by gas chromatography using a Pye Unicam PU 4500 gas chromatograph (Philips). Nitrogen was the carrier gas and the temperature of the column was 110 °C. For ethylene quantification, five seeds collected after 48 h of imbibition were incubated for 5 h in 50 ml plastic tubes.

Results

Effect of inhibitors on proteolytic activity during germination

It has been reported in several instances that proteolytic activities increase in the course of germination [24, 28, 38]. We were interested to determine the relative contribution to the total proteolytic activity of each of the proteinase groups at different stages. Table 1 summarizes the effect of various known proteinase inhibitors on the proteolytic activities of extracts from

Table 1. Effect of proteinase inhibitors on the proteolytic activity of extracts obtained from chickpea cotyledons after 0 and 96 h of imbibition. Data are expressed as the percentage of inhibition at each time. Inhibitors are: phenylmethylsulphonyl fluoride for serine proteinases; pepstatin A for aspartic acid proteinases; iodoacetic acid for cysteine proteinases and EDTA for metallo proteinases. Nomenclature and concentrations used are in accordance with Storey and Wagner [31].

Inhibitor	0 h	96 h
1 mM PMSF	38.3 ± 12.3	36.6 ± 3.4
1 μg/ml Pepstatin A	83.4 ± 5.3	52.3 ± 1.3
1 mM iodoacetic acid	19.3 ± 10.2	42.7 ± 2.7
10 mM EDTA	69.3 ± 5.3	60.4 ± 7.4

chickpea cotyledons at the early (dry seed) and late (96 h after imbibition) phases of germination. Cysteine proteinase activity increases in the course of germination of chickpea, simultaneously, activity due to aspartic acid proteinases decreases during this period of time.

PCR amplification

cDNA obtained from cotyledons harvested 48 h after imbibition was used as a template for PCR amplification. The resulted fragment was 333 bp in length as predicted from previously published sequences of other cysteine proteinases [1, 6, 14, 21, 22, 26, 33, 34].

Nucleotide sequence analysis

Figure 1 shows a comparison with the deduced amino acid sequences for other cysteine proteinases in the region corresponding to the PCR amplified chickpea *cac* clone. Amino acids comprising the active site, hydrophobic cores and cysteines involved in disulphide bridges known to exist in other cysteine proteinases [6, 22, 26] are shown to be conserved in the deduced amino acid sequence for the amplified chickpea *cac* gene, indicating that the gene encodes a cysteine proteinase.

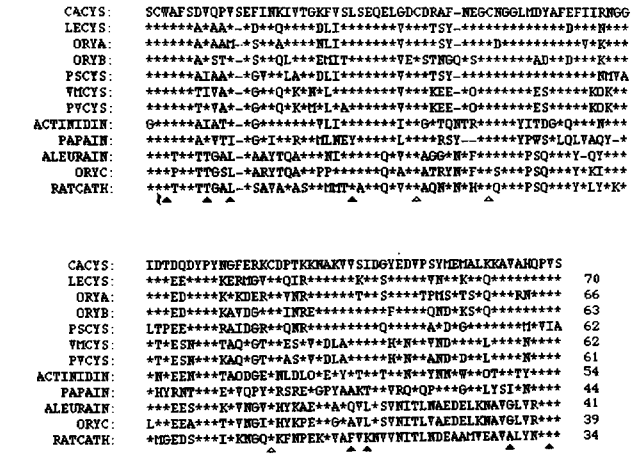

Fig. 1. Comparison of amino acid sequence of the PCR obtained fragment with the corresponding region in other cysteine proteinases. Optimal alignment of sequences was done with CLUSTAL program. Ratcath, cathepsin H [32]; Ory A, B and C, oryzains A, B and C [34]; LECYS, *Lycopersicum esculentum* cysteine proteinase [26];PSCYS, *Pisum sativum* cysteine proteinase [14]; VMCYS, *Vigna mungo* cysteine proteinase [1, 2]; PVCYS, *Phaseolus vulgaris* cysteine proteinase [33]; CPCYS, *Carica papaya* cysteine proteinase (papain) [6]; aleurain [23] and actinidin [22]. Numbers on the right indicate percent of identity of each amino acid sequence with CACYS (*C. arietinum* cysteine proteinase). Wavy line, amino acids in the active site involved in catalysis; open arrowheads, cysteines involved in disulphide bonds in actinidin and papain; solid arrowheads, residues in the actinidin hydrophobic core [6, 22, 26].

Expression of the chickpea cysteine proteinase gene during germination

Expression was determined by northern analysis using total RNA. Results of the northern analysis are presented in Figs. 2, 3 and 4. In Fig. 2, lanes 1, 2 and 3 contain RNA extracted from embryogenic seeds harvested at 15, 30 and 40 days after anthesis. Although proteolysis resulting from the activity of cysteine proteinases is detected in the last of these developmental stages (unpublished), this activity may not be attributable to the enzyme encoded by the *cac* gene as *cac* mRNA is undetected in any of the three phases of embryogenesis tested. The transcript is also undetected in the cotyledons of dry seed (lane 4), accumulating 24 h after imbibition (lane 5). The

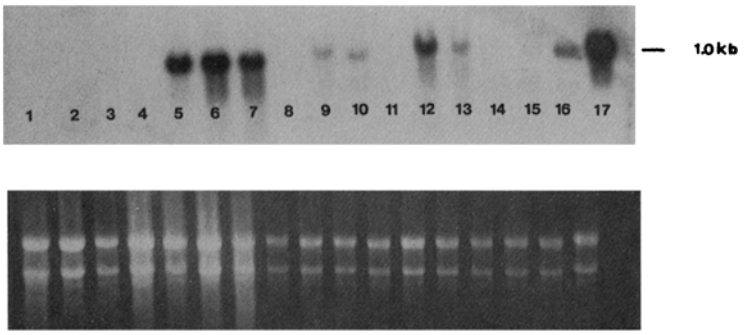

Fig. 2. Top panel: northern analysis showing the cysteine proteinase *cac* gene expression in cotyledons and embryonic axes of *C. arietinum* in different developmental stages and growth conditions. Lanes 1, 2 and 3 contain RNA from three progressive steps in the embryogenic development of *C. arietinum* seeds; lane 4, cotyledons at 0 h of imbibition; lanes 5, 6 and 7, from cotyledons 24, 48 and 96 h after imbibition respectively; lanes 8, 9 and 10 contain RNA obtained from cotyledons imbibed for 24, 48 and 96 h respectively after ablation of the embryonic axis; lanes 11, 12 and 13 contain RNA from cotyledons imbibed for 24, 48 and 96 h respectively after simultaneous ablation of the embryonic axes and removal of the testa; lanes 14, 15 and 16 contain RNA obtained from seeds imbibed for 12, 24 and 48 h respectively in the presence of abscisic acid; lane 17 contains RNA from embryonic axes of seeds imbibed for 48 h. Bottom panel: stained gel showing rRNAa.

amount of transcript reaches a maximum 48 h after imbibition (lane 6) decreasing by 96 h after imbibition (lane 7). Ablation of the embryonic axis before imbibition of the cotyledons causes an obvious reduction in the amount of *cac* mRNA present at 24, 48 and 96 h after imbibition (lanes 8, 9 and 10) indicating that a factor present in the embryonic axis may activate the expression of the chickpea *cac* proteinase gene. Simultaneous removal of the testa and ablation of the embryonic axis before imbibition partially restore *cac* gene expression 48 h and 96 h of imbibition (lanes 12 and 13).

We have investigated *cac* gene expression in cotyledons imbibed in the presence of abscisic acid (ABA), a well known inhibitor of germination. Lanes 14, 15 and 16 contain total RNA from cotyledons imbibed in the presence of 50 μM ABA for 12, 24 and 48 h. From the comparison of lanes 5 and 6 with lanes 15 and 16, ABA appears to inhibit expression of the *cac* gene. At present it is unclear whether this inhibition is the result of direct action of ABA on *cac* gene expression or if it is the indirect result of the general inhibition of germination which ABA is known to manifest [7].

cac gene transcript is strongly expressed in embryonic axes 24 h after imbibition (lane 17), indicating that the proteinase encoded by this gene is not specific for the degradation of reverse proteins in the cotyledons.

To verify the hypothesis that a factor present in the embryonic axis regulates *cac* gene expression in cotyledons, we incubated excised cotyledons in the presence of aqueous extracts obtained from embryonic axes (180 axes per litre). Figure 3 shows a northern analysis containing RNA from cotyledons imbibed for 48 h in different conditions: control seed (lane 1), excised cotyledons (lane 2), excised cotyledons after removal of the testa (lane 3) and excised cotyledons imbibed in the presence of aqueous extracts obtained from embryonic axes of chickpea (dry seed) (lane 4). The result in lane 4 indicates that *cac* gene expression is partially restored to levels observed in the control by the addition of these aqueous embryonic axis extracts. The level of *cac* mRNA accumulation is closer to that in controls when the excised cotyledons are grown in 1 mM ethephon (lane 5), indicating that ethylene may activate *cac* gene expression in chickpea cotyledons.

To investigate the role of ethylene in the induction of *cac* gene expression during normal germination, chickpea seeds were imbibed in the

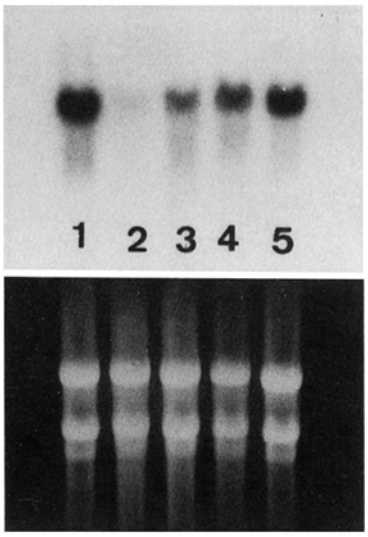

Fig. 3. Top panel: northern analysis of *cac* gene expression in cotyledons of *C. arietinum* imbibed for 48 h in different conditions. 1, control, normal seed, 2, seeds after ablation of the embryonic axis at time 0 h; 3, seeds after simultaneous ablation of the embryonic axis and removal of the testa at time 0 h; 4, seeds imbibed in aqueous solutions containing extracts of embryonic axes (180 axes per litre) after ablation of the embryonic axis at time 0 h; 5, seeds imbibed in the presence of ethephon 1 mM after ablation of the embryonic axis at time 0 h. Bottom panel: stained gel showing rRNAs.

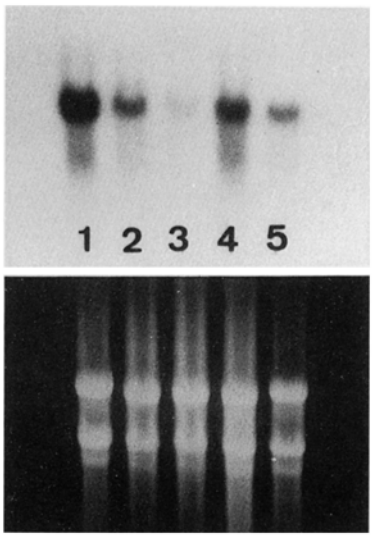

Fig. 4. Top panel: northern analysis of *cac* gene expression in cotyledons of *C. arietinum* imbibed for 48 h in the presence of specific inhibitors of ethylene action. 1, control, normal seed; 2, seeds imbibed in the presence of 2 mM silver thiosulphate; 3, seeds imbibed in 4 mM silver thiosulphate; 4, seeds imbibed in 1 mg/ml norbornadiene; 5, seeds imbibed in 2 mg/ml norbornadiene. Bottom panel: stained gel showing rRNAs.

presence of known inhibitors of ethylene action [9, 30]. Figure 4 shows the result of this experiment. Silver thiosulphate (4 mM) and norbornadiene (2000 ppm), were both effective inhibitors of *cac* mRNA accumulation. This result supports the hypothesis that ethylene is involved in the regulation of *cac* gene expression in germinating chickpea cotyledons. The fact that more than 90% of the seeds germinated in the presence of each inhibitor at the concentrations used, indicates that the effect of the inhibitors is not a general blockade in germination.

Gas chromatographic analysis

Table 2 shows the amount of ethylene released by seeds at 48 h of imbibition as well as by excised cotyledons and excised cotyledons deprived of the testa. The amount of ethylene released by the seeds during normal germination confirms results reported previously [13] being significantly higher than the amount released by the excised cotyledons. No ethylene was detected from excised cotyledons imbibed without testas.

Table 2. Ethylene evolution (nl per seed per hour) from chickpea seeds imbibed during 48 h in different conditions.

Normal seed (embryo + testa + cotyledons)	0.41 ± 0.09
Excised cotyledons (testa + cotyledons)	0.04 ± 0.01
Excised cotyledons minus testa (cotyledons alone)	undetected

Discussion

In cereals, regulatory mechanisms controlling *de novo* synthesis of cysteine proteinases operate at a transcriptional level, gibberellic acid inducing and abscisic acid repressing the expression of cysteine proteinase genes. This has been reported for barley aleurain and rice oryzains [23, 24]. In

legumes, although *de novo* synthesis of cysteine proteinases during germination has been long recognized [3, 5, 24, 28, 37], little is known of the mechanisms controlling their regulation.

The results presented here show that cysteine proteinase activity increases in the course of germination in *Cicer arietinum*. To study the molecular mechanism controlling this rise in activity, we have cloned a fragment of cDNA corresponding to a cysteine proteinase gene expressed during germination. The method could be of interest to characterize at the molecular level families of genes expressed in different tissues or different periods during the plant's life cycle. For example, Granell *et al.* [14] have reported the expression of a cysteine proteinase gene during ovary senescence in *Pisum sativum*, Tanaka *et al.* [33] have purified a cysteine proteinase from *Phaseolus vulgaris* pods and Ogushi *et al.* [21] reported the nucleotide sequence of its corresponding gene. We have detected cysteine proteinase activity during embryogenic development of *C. arietinum*, and present here evidence that the protein responsible may be synthesized from a different mRNA than that corresponding to our PCR-amplified clone (Fig. 2). Thus, several cysteine proteinase genes appear to be expressed differentially during the life cycle of a legume. Our objective is to find out how this activation may occur during germination.

The amount of *cac* mRNA increases from undetectable levels to its maximum value within the first 48 h of imbibition and is drastically reduced by removal of the embryonic axis. It has been known for a long time that removal of the embryonic axis causes a decrease in the total proteolytic and amylolytic activities of the cotyledons, this effect being partially reversed by removal of the testa. These observations apply to a range of different legume genera but their molecular bases are poorly understood [19, 38]. It was suggested that accumulation of amino acids subsequent to excision of the embryonic axis may inhibit proteinase activity or proteinase synthesis [19], however, positive regulatory mechanisms may be responsible for the transcription (or alternatively, increased stability) of these mRNAs in the early stages of germination. In this case, activator molecules produced in the embryonic axis could be responsible for this positive control. The system presented here has been used successfully to test this possibility. By adding aqueous extracts of the embryonic axes to the excised cotyledons, the level of accumulation of mRNA increased to close to its normal value (Fig. 3, lane 4), indicating that a factor present in aqueous extracts from embryonic axes is able to activate *cac* gene expression.

Ethylene evolution has been reported from homogenates obtained from pea epycotils [18] and the data presented here (Table 2) confirm that ethylene is produced by germinating chickpea seeds as was reported before [13]. Our results support the hypothesis that ethylene is active as a signal molecule responsible for an increase in the amount of *cac* mRNA in the cotyledons. First, when specific inhibitors of ethylene action were added to the germinating seeds, *cac* transcript was notably reduced. Second, the embryonic axis is responsible for most of ethylene production in the germinating seed and when the excised cotyledons are imbibed, the decrease in ethylene production reported in Table 2 is accompanied by a notable decrease in the amount of transcript detected (Figs. 2 and 3).

The system reported here has been useful to identify a function for ethylene in the cotyledons of chickpea at the molecular level. This development may contribute to establish the link between the ethylene receptors, reported in the cotyledons of legumes and the molecular events that ethylene binding may trigger during germination.

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