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A salt- and dehydration-inducible pea gene, *Cyp15a*, encodes a cell-wall protein with sequence similarity to cysteine proteases

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Abstract

The pea (*Pisum sativum*) gene *Cyp15a* encodes a protein with sequence similarity to cysteine proteases. Expression of *Cyp15a* was investigated during pea seedling development and in response to environmental stress. *Cyp15a* shows increased transcription and elevated mRNA levels in plant tissues that are partially dehydrated or treated with 0.6 M mannitol. *Cyp15a* mRNA levels also increase in seedlings treated with 0.2–0.25 M NaCl or KCl. During development, *Cyp15a* mRNA levels increase within 6 to 12 h in cotyledons and axes during germination and continue to increase for at least 96 h. Illumination of dark-grown seedlings increased *Cyp15a* mRNA abundance in elongating and non-elongating stem tissues. GA and ABA, which modulate the abundance of many seed-localized cysteine proteases, did not significantly modulate *Cyp15a* mRNA levels in stems. The protein encoded by *Cyp15a* contains a typical amino-terminal secretory targeting domain. This domain is followed by a pro-sequence containing ca. 110 amino acids that is found in other cysteine proteases. Polyclonal antibodies, directed against CYP15a, recognized both the larger pro-form and the cleaved mature form of CYP15a on western blots. Immunolocalization assays indicated that both forms of the protein are located in cell walls of stem cortical cells.

Introduction

Plant responses to water limitation are complex and depend on the severity of stress, its duration and the stage of the plant's life cycle affected by the stress [2]. Mild water deficits that reduce cell turgor result in growth inhibition and induction of the plant hormone abscisic acid (ABA) [6, 23]. Elevated abscisic acid levels have been implicated in several plant responses to water limitation including stomatal closure [24], differential inhibition of stem vs. root growth [5], and induction of genes encoding proteins that protect or aid plant adjustment to limiting water conditions [review in 29]. The expression of numerous genes is modulated when plant cell turgor is decreased [2], some but not all, through changes in ABA concentration. For example, genes encoding proteins with homology to cysteine proteases in pea [10] and *Arabidopsis thaliana* [18] are modulated by water deficit but respond minimally to ABA. Other cysteine proteases have been identified that are induced by wounding in tobacco leaves [20] and low temperature in tomato fruit [28].

Cysteine proteases are endopeptidases containing a cysteine residue in their active site. Members

of this superfamily are found in mammals, plants and bacteria. In plants, cysteine proteases and their genes show a variety of expression patterns and responses to plant hormones. For example, aleurain, as well as cysteine proteases EP-A and EP-B, are abundant in germinating barley seeds and may be involved in mobilizing storage reserves in the endosperm [13, 14, 26]. EP-A and EP-B are expressed exclusively in seeds, but aleurain is also expressed in vegetative tissues [17, 26]. Three genes encoding rice cysteine proteinases (oryzains; α, β and γ) have been characterized and, like EP-A and EP-B, they are expressed only in germinating seeds [32]. The level of mRNA corresponding to these proteinases is increased by gibberellic acid (GA) and reduced by ABA [17, 32]. In contrast, P34 is a soybean vacuolar thiol protease whose mRNA is expressed during seed maturation but not during germination or in other tissues [15].

Plant cysteine proteases are located in cell walls (EP-A, EP-B) [17] and vacuoles (aleurain) [12]. Cysteine proteases secreted into cell walls and those destined for vacuoles contain an amino terminal signal sequence of 16 to 25 amino acids that directs the protein to the endoplasmic reticulum [4, 12]. In addition, secreted cysteine proteases, including the pea *Tpp* gene product and rice α and β oryzains, contain an amino terminal pro-sequence of 100 to 130 amino acids [9, 32]. The pro-peptide may maintain the protease in an inactive state and/or facilitate protein folding and stability [30]. In some cases, additional processing events or modifications are required to activate the enzyme [8, 12, 15, 17, 20]. In the case of animal thiol proteases, such as the cathepsins, glycosylation is important for targeting to the lysosome [12, 33]. Aleurain is glycosylated [12] and other cysteine proteinases in plants have either been shown to be glycosylated or to possess glycosylation sites [9, 15]. A role for glycosylation in targeting proteins to vacuoles or the cell wall has not been demonstrated in plants [4]. Efficient targeting of aleurain to vacuoles involves two contiguous protein sequences located in the pro-peptide [13].

In an earlier study, we identified a gene, *Cyp15a*, that showed increased expression when plants were dehydrated until turgor was lost [10]. *Cyp15a* encoded a protein with 41% overall residue identity with cysteine protease 1 from Dictyostelium. Furthermore, amino acids 143 to 190 showed 50–80% sequence identity with other thiol proteases (cathepsin H, papain, aleurain) and the protein contained conserved protease active site residues (Cys-154, His-300) [10]. Based on this analysis, we tentatively identified the protein encoded by *Cyp15a* as a thiol protease. In the present study, we investigated the expression of *Cyp15a* during seedling development and in response to stress. Antibodies against CYP15a showed that the protein is localized in cell walls and that CYP15a is initially synthesized as a prepro-protein.

Materials and methods

Materials

All chemicals were purchased from Sigma Chemical Company, unless otherwise noted. Pea seed (*Pisum sativum* cv. Little Marvel) and Halt fungicide (dimethyl 4,4-0-phenylenebis-(3-thioallophanate), Fertilome) were purchased at a local nursery.

Plant growth

Peas were imbibed and germinated in water containing Halt fungicide (1/2 teaspoon, Halt per liter water) and grown hydroponically in water containing 1 teaspoon Halt per 6 l water. Pea plants were grown at 23 to 25 °C in a controlled environment chamber as described [10]. For germination and early growth studies, pea seeds were imbibed for 12 h with constant agitation. After 12 h, seeds were placed on wet paper towels in the dark throughout the experiment. For other experiments, except where noted, seeds were imbibed overnight, germinated for 3 days in darkness on paper towels then transferred to hydroponics. After 2 additional days, tissue was collected (5 dd). For some experiments, plants

were transferred to continuous white light (incandescent plus fluorescent illumination; 300 μE $m^{-2}s^{-1}$) for 72 h (5 dd + 72 hl). Peas were imbibed overnight, planted into vermiculite and grown for 9 days with 16 h light and 8 h dark to generate protein for western blotting. For northern analysis and protein gels, tissue was frozen in liquid nitrogen at the time of harvest and stored at -80 °C until RNA extraction.

RNA isolation and hybridization

Total nucleic acid was extracted by grinding frozen tissue in liquid nitrogen and homogenizing in 5 to 10 volumes of 0.2 M Tris-HCl pH 8, 0.2 M NaCl, 5 mM EGTA, 5 mM EDTA, 1% SDS, 2.5 mM DTT and 50 μg/ml Proteinase K. The nucleic acids were extracted with phenol/chloroform (1:1), precipitated with 0.3 M sodium acetate (pH 5.2) and 2.5 volumes of EtOH and re-precipitated with 3 M ammonium acetate and 2.5 volumes of EtOH. The nucleic acid was resuspended in water and quantitated by absorbance at 260 nm. Nucleic acid was denatured and run on agarose gels according to Sambrook *et al.* [27], except that gels were 0.8% agarose, 40 mM MOPS (pH 7), 10 mM sodium acetate, 1 mM EDTA. Nucleic acid from gels was blotted onto GeneScreen (DuPont) according to manufacturer's instructions. Nucleic acid was fixed onto membranes by UV crosslinking (UV Stratalinker 1800, Stratagene). Prehybridization, hybridization and washes were done according to protocols in Stratagene BlueScript Exo/Mung DNA Sequencing System Instruction Manual. All manipulations were carried out at 65 °C. Blots were exposed to Kodak-XAR film with an intensifying screen at −80 °C. In some experiments, signals were also quantified by scanning blots with a Betascope 603 Blot Analyzer (Betagen). Antisense RNA probe was made from the full-length *Cyp15a* cDNA clone [10] using T3 polymerase according to Stratagene protocols. Each experiment was repeated one or more times and multiple plants were used for RNA extraction in each experiment.

Antibody generation

Fusion proteins were generated in pGEX-3X vectors (Pharmacia). Portions of the open reading frame from *Cyp15a* were fused in frame to glutathione-*S*-transferase (GST). The fusion proteins used to generate antibody 3444 contained amino acids 1 to 104 of *Cyp15a* and the fusion protein used to generate antibody 3445 contained amino acids 1 to 363 (full-length). Protein was prepared for injection by electrophoresis and electroelution from SDS polyacrylamide gels. *Escherichia coli* containing GST-fusions plasmids were grown and induced with 0.5 M IPTG according to manufacturerer's instruction. After induction, cells were pelleted by centrifugation at 5000 × *g* for 5 min. The pellet was resuspended in SDS loading buffer, vortexed vigorously and frozen at −20 °C until use. Before gel loading, protein was boiled 5 min, briefly sonicated, centrifuged for 5 min and the supernatant was loaded. Fusion protein bands were visualized by Coomassie staining, cut from gels and injected into New Zealand White rabbits in a 1:1 (v/v) solution of Freund's adjuvant. For the initial injection, Freund's complete adjuvant was used and the incomplete adjuvant was used with two successive injections. About 500 μg protein was used for the first 2 injections and 250 μg used for third injection. Injections were given at 4–5 week intervals. Blood samples were taken prior to protein injections (preimmune serum) and 14 days after the final injection (serum). GST antibodies were generated for use as controls. GST was expressed in *E. coli* from the pGEX 3 × vector and antibodies were prepared as described for the fusion proteins.

In vitro *transcription, translation and immunoprecipitation*

Sense RNA was made from the *Cyp15a* full-length clone using T7 polymerase according to Stratagene protocols, except that unlabeled UTP was substituted for radioactive UTP. After the transcription reaction, RNA was precipitated and re-

suspended in water. *In vitro* translation was carried out in the presence of [^{35}S]-methionine using rabbit reticulocyte lysate according to the manufacturer's instructions (Promega). The protein generated was immunoprecipitated as follows: 15 μl of *in vitro* translated protein, 21 μl of water and 4 μl of 20% SDS were mixed and heated to 90 °C for 2 min then 50 μl of immunoprecipitation buffer (100 mM Tris HCl pH 7.5, 600 mM NaCl, 10 mM EDTA, 2% Triton X-100) and 10 μl antibody solution was added and incubated for 45 min at 25 °C with occasional mixing. After this incubation, 50 μl of washed formalin-fixed Staph A cells (Immunoprecipitin, Gibco-BRL) were added, the solution was incubated for 15 min then 1 ml of wash buffer (500 μl immunoprecipitation buffer, 495 μl water, 5 μl 20% SDS) was added, cells were spun briefly in a microfuge, buffer was removed and the pellet was washed 2 more times. After the last wash, cells were resuspended in SDS loading buffer, heated to 90 °C for 3.5 min, centrifuged briefly, and the supernatant was loaded onto an SDS-polyacrylamide gel. Gels were run according to Sambrook *et al.* [27]. After electrophoresis, gels were subjected to fluorography [3], dried onto filter paper and exposed to Kodak XAR film at −80 °C with an intensifying screen.

Western blotting

Soluble protein was obtained from 9-day-old pea shoots, that were wilted in darkness then incubated in a dark humid chamber for 4 h [10]. Wilted shoots (30g) were homogenized in 60 ml of 60 mM Tris-HCl pH 6.8, 30 mM 2-mercaptoethanol and 0.8 mM phenylmethylsulfonyl fluoride (PMSF) using a Polytron (Brinkman). The homogenate was filtered through 2 layers of Miracloth (CalBiochem), the liquid was centrifuged for 15 min at 15000 × *g* and the supernatant was retained. Protein was quantified by Bradford Protein Assay (BioRad). SDS loading buffer was added to protein, followed by sonication, boiling for 3 min, and centrifugation. Protein was separated on 15% SDS-polyacrylamide gels and transferred to Immobilon PVDS membrane (Millipore). Antibody/protein interactions were detected with Enhanced Chemiluminescence reagents according to the manufacturer's instructions (Amersham). The primary antibody was incubated overnight in an *E. coli* lysate to remove *E. coli*-specific antibodies [27]. The secondary antibody used was donkey anti-rabbit IgG-horseradish peroxidase linked whole antibody (Amersham).

Immunocytochemistry

For tissue fixation, plant material was submerged in 4% paraformaldehyde, 0.5% glutaraldehyde in 100 mM sodium cacodylate, 2 mM calcium chloride, pH 7.4 and cut into 1–2 mm^3 blocks. Tissue blocks were placed into fresh fixation buffer and vacuum infiltrated for 10 min and then incubated in this buffer for 2 h at room temperature. Tissue was shaken continuously during all steps of fixation and dehydration. After fixation, buffer was replaced with 1% osmium tetroxide, 100 mM sodium cacodylate and 2 mM calcium chloride. The samples were incubated in osmium tetroxide for 45 min then rinsed with 10% EtOH in water. Tissue was dehydrated for 10 min each in increasing concentrations of EtOH (10%, 25%, 50% and 100% three times). LR White resin (Electron Microscopy Sciences) was infiltrated into tissue in successively increasing concentrations of 50%, 75% and 100% each for 1 h, followed by two 12 h incubations in 100% LR White. The LR White was diluted with 100% EtOH. Tissue was embedded in 100% LR White in aluminum embedding pans and the resin was polymerized at 51 °C for 48 h. Ultra-thin (70 to 90 nm) sections were prepared using an LKB ultratome (Type 4802A) and were affixed to acid-washed nickel grids. Grids were treated with 10% sodium metaperiodate for 10 min, and washed with water prior to blocking and antibody incubations. The secondary antibody used was goat anti-rabbit IgG conjugated to 15 nm gold particles (Amersham). After antibody incubations, grids were post-stained with uranyl acetate for

1 min and Reynold's lead citrate for 1 to 2 min [11]. Transmission electron microscopy was carried out with a Zeiss 10C microscope.

Results

Expression of Cyp15a *during germination and early seedling growth*

Many plant cysteine proteases are expressed in cotyledons and early stages of seedling development and are thought to have a role in mobilizing stored proteins [17, 32]. To assess this possible function for *Cyp15a*, changes in *Cyp15a* mRNA levels were analyzed using RNA blots as a function of time after seed imbibition (Fig. 1). At each time point, except 0 h, the seed coat was removed and the root to shoot axis cut away from the cotyledon. Roots and shoots were separately analyzed in seedlings at 96 h post-imbibition. As shown in Fig. 1, *Cyp15a* mRNA levels were low in dry seeds and increased during the first four days post-imbibition. The increase in mRNA abundance was initially more rapid in the root to shoot axis than in the cotyledons, although by 48 and 96 h the levels were considerably higher in cotyledons compared to roots or shoots.

Cyp15a mRNa levels were also examined in seedlings grown in hydroponics for 5 days in darkness (5 dd) or seedlings grown 5 days in darkness and then transferred to an illuminated chamber for an additional 72 h. At the time of harvest, plants were divided into root tips (bottom 0.5 cm of primary root), non-elongating root sections (all of root, except for bottom 0.5 cm of primary root), non-elongating stem sections (lower 1/2 of the stem for 5 dd plants and the first internode for older plants), zone of stem elongation (upper 1/2 of the stem for younger tissues and all except the first internode for older ones), and leaf bud (apical developing leaves and shoot apex). In the dark-grown material, expression of *Cyp15a* mRNA was highest in non-elongating regions of the root and stem (Fig. 2, top row). The lowest level of expression was in the root tip. After 3 days in the light, expression increased in all tissues (Fig. 2, lower row). Highest expression at this time was in elongating stem sections. Western blots showed that changes in CYP15a

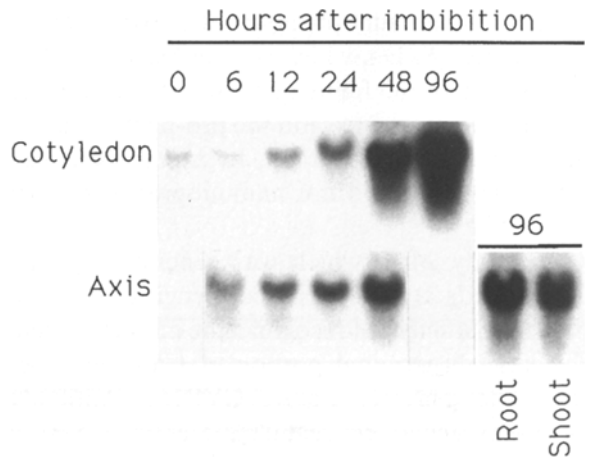

Fig. 1. Cyp15a mRNA abundance in pea during germination and early growth. Total nucleic acid was isolated from pea tissues at various times after imbibition (hours). Total nucleic acid (10 μg/lane) was electrophoresed, blotted and hybridized to the *Cyp15a* probe at 65 °C. A 1.5 kb RNA hybridized to the *Cyp15a* probe as previously reported [10]. X-ray films were exposed for 18 h.

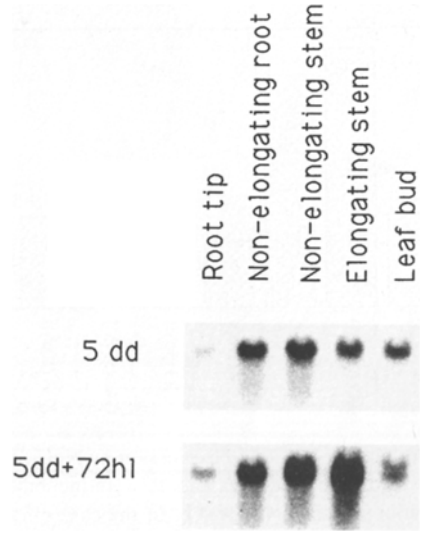

Fig. 2. Cyp15a mRNA levels in pea seedlings grown for 5 days in darkness or 5 days in darkness plus 72 h light. Total nucleic acid extracted from the tissues listed from material grown for 5 dd (top row) or 5 dd + 72 hl (bottom row). Total nucleic acid (10 μg/lane) was electrophoresed, blotted and hybridized to *Cyp15a* probe at 65 °C. X-ray film was exposed for 48 h.

protein levels parallel mRNA abundance (data not shown).

Modulation of Cyp15a *expression by salt, low temperature, GA and ABA*

We previously found that *Cyp15a* mRNA levels increased in leaves and stems when intact seedlings were exposed to 0.6 M mannitol [10]. This result was extended to determine if *Cyp15a* was modulated by inducers known to modulate expression of other plant cysteine protease genes. Plants were grown in hydroponics for 5 days in darkness and then transferred to light for 72 h. For induction studies, shoots were excised just above the cotyledons, placed under water and recut to prevent cavitation. Six shoots were then placed into each solution. After 4 h in light, leaves were removed from stems and stems were harvested into liquid nitrogen. Figure 3 shows the results of one stem uptake experiment. The salts, NaCl and KCl, induced expression of *Cyp15a* more than two-fold. In contrast, low temperature, GA and ABA treatments modulated *Cyp15a* mRNA levels to only a small extent.

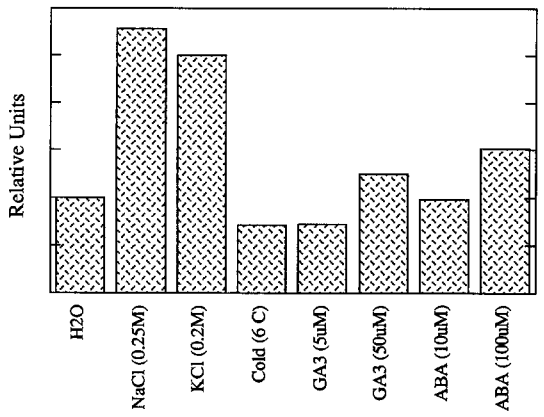

Fig. 3. Modulation of *Cyp15a* expression by NaCl, KCl, GA, ABA and cold treatment. Pea shoots were incubated in different solutions in the light for 4 h. In the case of cold treatment, shoots were placed at 6 °C in a lighted cold room for 4 h. Leaves were removed from shoots and total nucleic acid was extracted from stems. Six plants were used per treatment. Nucleic acid was run on gels, transferred to membrane and hybridized to *Cyp15a* probe. Signal was quantified by scanning the blots with a Betascope 603 Blot Analyzer (Betagen). Relative units were cpm from the blot.

Evidence that the protein encoded by Cyp15a *is synthesized as a prepro-protein*

The deduced amino acid sequence of the protein encoded by *Cyp15a* includes a 16 amino acid N-terminal pre-sequence characteristic of proteins directed to the secretory pathway [4]. In addition, cysteine proteases often contain a pro-sequence between the targeting pre-sequence and the mature protein. As a first step in characterizing these putative domains in CYP15a, antibodies against amino acids 1 to 104 (antibody 3444) and 1 to 363 (antibody 3445) were prepared. Immunoprecipitation and western blotting were utilized to assess the ability of the antibodies to recognize CYP15a. For immunoprecipitation studies, *Cyp15a* cDNA was transcribed *in vitro* and translated in rabbit reticulocyte lysates in the presence of [^{35}S]-methionine. The products of translation are shown in lane 1 of Fig. 4. Aliquots of the translation products were immunoprecipitated with both preimmune and immune serum (Fig. 4, lanes 2–5). For each antibody, the immune serum immunoprecipitated a product of approximately 4 kDa, which is equivalent to the expected size of the full-length CYP15a containing both the pre-sequence and the pro-peptide region (fig. 4, marked at the right, by top arrow). The preimmune sera did not immunoprecipitate any proteins.

Antibody 3444, which was generated against amino acids 1 to 104, should recognize the pro-protein, but may not recognize the mature protein if a large N-terminal portion of CYP15a is removed to generate mature CYP15a. Antibody 3445 was generated against the entire CYP15a (amino acids 1 to 363) and should recognize both the pro and mature forms of the protein. Western blot analysis using antibody 3444 revealed a protein of ca. 38kDa, which we conclude corresponds to the pro-protein (Fig. 4, lane 6, marked by middle arrow). Antibody 3445 recognized this same protein as well as a 30 kDa protein that we

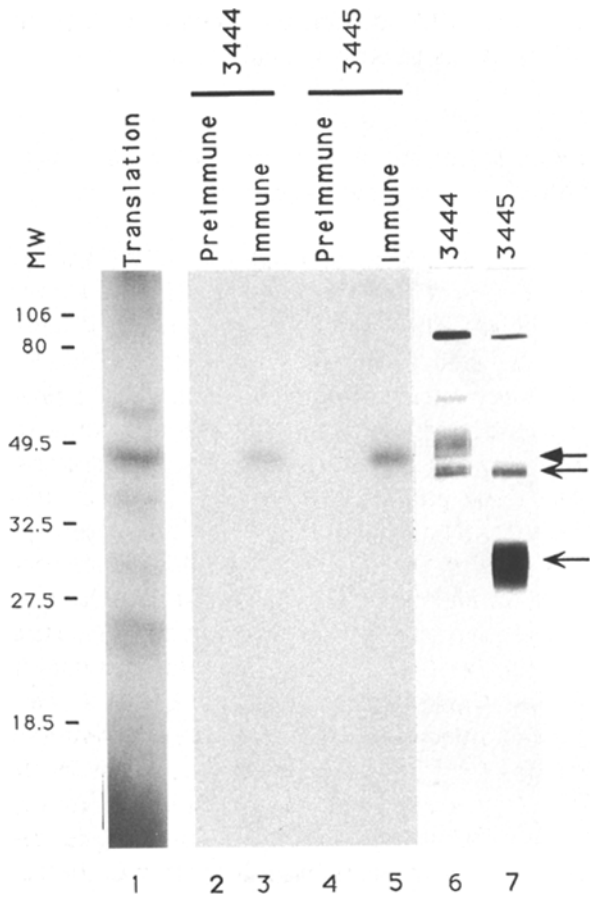

Fig. 4. Immunoprecipitation of CYP15a and western blotting of soluble plant protein. Lane 1: *In vitro* transcription and translation of *Cyp15a* cDNA. Lanes 2–5: immunoprecipitation of translation products with antibodies listed at top of each lane. Lanes 6–7: western blot of soluble protein from pea shoots bound to immune serum 3444 at a dilution of 1:4000 and serum 3445 at a dilution of 1:8000. Molecular weight marker numbers are shown to the left of the figure. The top arrow to the right of the figure identifies the immunoprecipitated products in lanes 1, 3 and 5. The middle arrow points to the pro-form of CYP15a in lanes 6 and 7. The bottom arrow marks the processed mature CYP15a protein in lane 7.

conclude is the mature protein (Fig. 4, lane 7, marked by bottom arrow). The preimmune sera did not recognize these proteins (data not shown). Both antibodies recognize a protein of ca. 95 kDa that could be a dimer or trimer of CYP15a. Antibody 3444 also detected additional proteins that are greater than 40 kDa in size. These proteins may represent different forms of CYP15a or other proteins that the antibody recognizes. A second set of antibodies made with the same fusion proteins as antibodies 3444 and 3445 but prepared in different rabbits, recognized the same pattern of proteins. Analysis of the predicted amino acid sequence of *Cyp15a* reveals numerous potential phosphorylation sites (Mac Vector analysis program). There is also a potential N-linked glycosylation site (NFS) at amino acid #249 to 251 [19]. Protein modification may explain, in part, the diffuse nature of the processed CYP15a.

Immunolocalization and evidence for cell wall localization

Cysteine proteases in plants have been reported in cell walls and vacuoles. In order to determine the location of CYP15a in pea cells, ultra-thin sections of stem tissue were incubated with CYP15a primary antibody and 15 mM gold-conjugated secondary antibody to visualize protein localization at high resolution. A number of different tissues were examined including; elongating and non-elongating stem from 5 dd, 5 dd + 72 hl, 5 dd + 72 hl plus 4 or 24 h treatment with 0.25 M NaCl. In all tissues, protein was immunolabeled in the cell walls of cortical cells using antibody 3445 or antibody 3444 but not with preimmune sera. Typical examples of immunolabelling are shown in Fig. 5. Sections of cortical cells from elongating stem of 5-day-old dark-grown peas were immunodecorated using antibody 3445 (Fig. 5a) and not by the preimmune serum corresponding to that antibody (Fig. 5b). Panels 5c and d show portions of cell walls from non-elongating pea stem tissue from pea plants treated with 0.25 M NaCl for 24 h prior to tissue fixation and incubation with antibody 3444 (Fig. 5c) and preimmune sera 3444 (Fig. 5d). Immunolabelling of CYP15a was not observed in vacuoles and few gold particles were observed in the cytoplasm. As an additional control to check for non-specific antibody interactions, GST antibodies were incubated with stem sections. These antibodies did not detect protein in cell walls, and immunodecorated proteins in the cytoplasm to only a small extent.

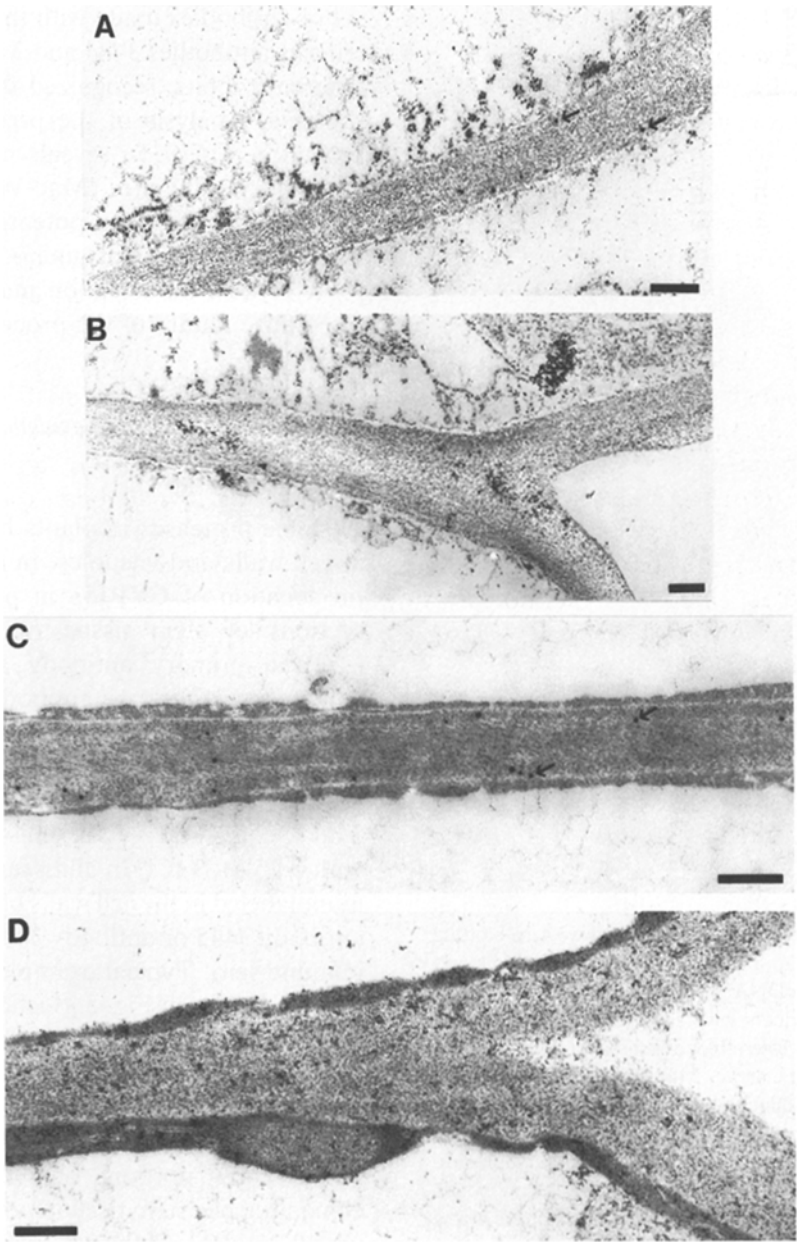

Fig. 5. Immunolocalization of CYP15a in pea stem cortical cell walls. Transmission electron micrographs of stem sections incubated with primary antibody or preimmune serum (1:50 dilution) and 15 nm gold-conjugated secondary antibody (1:100 dilution). Bar = 2.5 μm. Arrows on A and C point to representative labeled CYP15a. A. 5-day-old dark-grown elongating pea stem, incubated with primary antibody 3445. B. Same as A except incubated with preimmune serum 3445. C. 5 dd + 72 hl grown mature pea stem treated with 0.25 M NaCl for 24 h prior to tissue fixation and imbedding, incubated with primary antibody 3444. D, same as C, except incubated with preimmune serum 3444.

Discussion

The protein encoded by *Cyp15a* was identified as a member of the cysteine protease superfamily based on overall sequence similarity and conservation of a number of features including active site residues, cysteines involved in disulfide bond formation and glycine residues for folding [1, 16].

The highest level of overall sequence similarity (68%) is with *Arabidopsis rd19* [18]. *Arabidopsis rd19*, shares many features with *Cyp15a*. The expression of both genes is responsive to salt and drought but not by wounding, GA, heat or cold. These genes may serve a similar function in plants. When only the mature protein sequence of *Cyp15a* is compared to other sequences in the Swiss Protein data bank, the highest levels of sequence similarity after *rd19* are with cathepsin L from rat, mouse and human (ranking of 2, 3 and 5) (Genetics Computer Group, Madison, WI). Cathepsin L proteins are characterized by high endopeptidase activity with native substrates and activity on the synthetic substrate benzyloxycarbonyl-Phe-Arg-7 (4 methyl) coumorylamide [33]. Cathepsin L proteins are thought to be involved in intracellular protein turnover in the normally functioning animal, but may be involved in extracellular degradation in certain disease states [25]. Similarly, *Cyp15a* may play a role in normal plant development and have additional functions in plants subjected to osmotic stress.

Expression of *Cyp15a* during germination and early seedling growth differs from most of the plant cysteine proteases previously described, because expression is high in both cotyledons and in the root to shoot axis. Expression of *Cot44*, which encodes a cysteine protease from *Brassica napus*, is similar but not identical to *Cyp15a* [7]. *Cot44* is expressed at high levels in germinating seedlings and to a much lower extent in leaves, roots and stems of older plants. *Cot44* mRNA was localized to the stem vascular tissue and epidermis and to a small extent to the cortex. The protein encoded by *Cyp15a* was found by immunolocalization to be in cortical cells and was not observed in the vascular tissue or epidermis of pea stems (data not shown). *Cyp15a* can also be distinguished from *Tpp*, another pea gene that encodes a cysteine protease. *Tpp* is expressed in unpollinated senescent pea ovaries, roots, stems and leaves. In contrast to *Cyp15a*, *Tpp* is down regulated by GA [9]. Expression of *Cyp15a* increased when plants were transferred to light. Linthorst *et al.* have also reported on a light-modulated thiol protease in tobacco [20]. Illumination of pea seedlings stimulated the accumulation of *Cyp15a* mRNA the most in the stem zone of cell elongation. Illumination of pea seedlings causes a decrease in stem elongation. Levels of *Cyp15a* mRNA are also increased by water deficit which results in a decrease in stem growth [5, 22].

The sequence of CYP15a revealed the presence of a putative signal or pre-sequence (amino acids 1 to 20) and a pro-sequence (amino acids 20 to 131). Translation of *Cyp15a in vitro*, followed by immunoprecipitation demonstrated that CYP15a, could be synthesized as a 40 kDa precursor. Analysis of CYP15a in whole-cell protein extracts revealed the presence of a 38 kDa protein that was detected by antibodies against the putative CYP15a pro-sequence (amino acids 1 to 104) or the entire CYP15a protein (amino acids 1 to 363). In contrast, only the antibodies against the entire protein detected CYP15a proteins of 30 kDa indicating that the 38 kDa pro-protease is most likely cleaved to release a 30 kDa mature protein. Immunolabelling showed that CYP15a was located in cell walls. CYP15a was not detected in vacuoles. We conclude that CYP15a, like EP-A and EP-B from barley aleurone cells [17], is secreted into cell walls. CYP15a antibodies detected protein in whole cell extracts (Fig. 4, lanes 6 and 7), as well as in the cell walls (Fig. 5). This suggests that the association of CYP15a with the cell wall is not tight. Immunolocalization studies also indicated that the pro-form of CYP15a exists in the cell wall. In some cases, the pro-form of cysteine proteases are not active. An example of this is a cathepsin L from a transformed mouse fibroblast cell line. The pro-form of this protein, called the major excreted protein, is secreted from the cells in an inactive form. It can be activated by lowering the pH, which is also accompanied by a decrease in size of the protein correlating to the removal of the pro-sequence [21]. Acidification of cell walls is involved in cell growth and changes in extracellular pH occur during water deficit [31]. Further studies [30] will be required to determine if CYP15a processing is altered under these conditions and to identify the function of CYP15a.

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