Abundant Accumulation of the Calcium-Binding Molecular Chaperone Calreticulin in Specific Floral Tissues of Arabidopsis thaliana

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Calreticulin (CRT) is a calcium-binding protein in the endoplasmic reticulum (ER) with an established role as a molecular chaperone. An additional function in signal transduction, specifically in calcium distribution, is suggested but not proven. We have analyzed the expression pattern of Arabidopsis thaliana CRTs for a comparison with these proposed roles. Three CRT genes were expressed, with identities of the encoded proteins ranging from 54 to 86%. Protein motifs with established functions found in CRTs of other species were conserved. CRT was found in all of the cells in low amounts, whereas three distinct floral tissues showed abundant expression: secreting nectaries, ovules early in development, and a set of subepidermal cells near the abaxial surface of the anther. Localization in the developing endosperm, which is characterized by high protein synthesis rates, can be reconciled with a specific chaperone function. Equally, nectar production and secretion, a developmental stage marked by abundant ER, may require abundant CRT to accommodate the traffic of secretory proteins through the ER. Localization of CRT in the anthers, which are degenerating at the time of maximum expression of CRT, cannot easily be reconciled with a chaperone function but may indicate a role for CRT in anther maturation or dehiscence.

CRT is a calcium-binding protein that was originally detected in the ER (Ostwald and MacLennan, 1974). Quantitative measurements of the ability of CRT to bind to calcium have led to the identification of three domains, each with distinct dissociation constants for calcium and molar ratios of calcium to protein at maximum binding capacity (Michalak et al., 1992). CRT, together with protein disulfide isomerase, luminal binding protein, endoplasmic, and calnexin (a membrane-anchored relative of CRT), are the major calcium-caging proteins in cells. Although measurements as detailed as those for CRT have not been made for the others, the calcium-binding capacity of CRT may be highest on a molar basis, suggesting that CRT is the major calcium storage in animal cells (Milner et al., 1991).

More recent, functional evidence suggests an important role for CRT in the regulation of cytoplasmic and luminal calcium concentrations. CRT and calcium-ATPase are redistributed during the process of phagocytosis, with CRT becoming concentrated around particles being ingested (Stendahl et al., 1994). This concentration occurs before phagocytosis is complete and also before the measurable increase in the concentration of intracellular calcium occur, indicating that redistribution of the stores precedes calcium release. Thus, relatively low numbers of calcium storage organelles may achieve effective local calcium concentration changes. Camacho and Lechleiter (1995) demonstrated that overexpression of CRT in Xenopus laevis oocytes inhibited the repetitive calcium waves mediated by inositol 1,4,5-triphosphate. Mery et al. (1996) overexpressed CRT in a mouse cell line and measured increases in intracellular calcium storage and decreases in store-operated calcium influx. These results suggest an active role for CRT in calcium storage and distribution.

Other observations, however, suggest that CRT may have cellular roles beyond that of calcium storage and calcium pool regulation. Putative roles include its action in the ER as a chaperone and an action in the cytoplasm as a second messenger in gene regulation, as demonstrated by its interaction with cell-adhesion-related molecules and steroid hormone receptors in the nucleus.

Nigam et al. (1994) selectively retained proteins on columns containing denatured protein for the purification of selectively binding chaperones. After release from the column by coupling to ATP hydrolysis, among others, luminal binding protein, CRT, and members of the thioredoxin superfamily were obtained. For CRT, ATP-induced elution was further stimulated by calcium. Further supporting a chaperone function, transient interactions between CRT and newly synthesized proteins in the ER, including glycosylated hemagglutinin of influenza virus, have been shown (Peterson et al., 1995). Intermediates of hemagglutinin, which bound to luminal CRT (as well as membrane-anchored calnexin), were either not fully disulfide-bonded or incorrectly folded. Also, calnexin and CRT promote correct folding by inhibiting aggregation, preventing premature oxidation and oligomerization, and suppressing degradation of incompletely folded glycopolypeptides (Hebert et al., 1996). Thus, both calnexin and CRT appear to be chaperones that are important to protein maturation within the ER.

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Abbreviations: CRT, calreticulin; EST, expressed sequence tag.
Several results are consistent with a chaperone function during cell proliferation and secretion. Patton et al. (1995) applied growth factors to vascular smooth muscle cells, the primary component cell of the blood vessel wall. Including CRT, several other proteins related to protein synthesis and folding were up-regulated. CRT is also abundant in proliferating skeletal muscle myoblasts and B16 melanoma cells (Michalak et al., 1992). Hensel et al. (1994) observed in pancreatic cells a 2.3-fold increase in CRT following stimulation by cholecystokinin or cerulein, a treatment that stimulates exocrine production.

In contrast, several reports support specific functions for CRT outside of the ER. Interactions with both integrin receptors and steroid hormone receptors have been reported (Rojiani et al., 1991; Leung-Hagesteijn et al., 1994; White et al., 1995). In addition, functional aspects have been demonstrated. White et al. (1995) used anti-CRT antibodies to block laminin-dependent cell spreading. Also, purified CRT successfully competes with cell surface CRT and similarly prevents cell spreading. Finally, CRTs have been shown to inhibit the transcriptional activities of androgen and retinoic acid receptors in vivo and to inhibit neuron differentiation (Burns et al., 1994; Dedhar et al., 1994).

CRT has also been found in plants, although functional analysis lags that done in animal systems. CRT has been purified from leaves (Menegazzi et al., 1993), and Crt cDNAs have been found during searches for proteins associated with pollination (Chen et al., 1994), for proteins containing ER-retention signals (Denecke et al., 1995), for glyclosylated proteins (Benedetti and Turner, 1995), and by immunological screening (Kwiatkowski et al., 1995).

Our study is a paradigmatic use of the possibilities provided by the abundant Arabidopsis ESTs, which allow gene and transcript characterization and a functional analysis of proteins with a minimum of time expenditure. We describe the Arabidopsis Crt genes and provide an immunological characterization of the encoded proteins, including quantitative protein expression data and immunocytochemical localization in floral tissues and specific cell types where expression is particularly high. Three Arabidopsis Crt genes encode similar proteins, which retain the fundamental characteristics of ER-localized proteins. Expression is particularly high in developing ovules and nectaries and in a cluster of cells with an unknown function on the posterior of anther sacs. The results are discussed in terms of the likely roles of CRTs in the cells of these tissues.

**MATERIALS AND METHODS**

**Search for Arabidopsis thaliana in the EST Database**

The tblastn algorithm was used to search the Arabidopsis database of EST (http://www.ncbi.nlm.nih.gov/dbEST/index.html) using as a query the amino acid sequence of a human CRT (accession no. 84739). The Arabidopsis CRT sequences that we found were used in subsequent searches to identify the sequences deposited in the database (http://genome-www.stanford.edu/Arabidopsis/). cDNA clones were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) and sequenced entirely on both strands.

**Growth of Plants**

Ecotype RLD of A. thaliana was grown in a controlled environment chamber (Hoffman, Albany, OR) under constant illumination (100 µE) at 20°C. For heat treatment, plants were transferred to a chamber set at 40°C for 8 h.

**Southern Analysis**

DNA was prepared from leaves by dodecyltrimethylammonium-bromide extraction (Gustinich et al., 1991) and removal of RNA by LiCl precipitation. Fifteen micrograms of DNA, after restriction enzyme digestion, was electrophoresed, blotted, and hybridized to labeled probes by standard procedures (Ausubel et al., 1995). Probes were prepared and labeled by performing standard PCRs using vector forward and reverse primers to amplify inserts and digoxigenin-dUTP (Boehringer Mannheim) as a label. Detection of the digoxigenin-labeled probe was by use of anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (Boehringer Mannheim) and Lumiphos (Boehringer Mannheim) as a substrate.

**Inverse PCR Cloning**

Two oligonucleotides were synthesized, with the sequences 5'-ACACATGGGTGAGCTGGTC-3' (complement of 460-478 of accession no. U27698) and 5'-ACACATTATCCTCCGCC-3' (479-497 of accession no. U27698). Arabidopsis DNA (1 µg) was digested with SacI. After heat inactivation at 70°C, the DNA was diluted to 2 ng/µL and religated with 10 units of T4 DNA ligase (final reaction volume 500 µL). Ligated DNA was ethanol-precipitated. Three hundred nanograms of ligated DNA was used as a template in a standard PCR reaction using the above primers (1 µM each). PCR cycle parameters were 94°C for 1 min, 57°C for 30 s, and 72°C for 4 min.

**Expression of Arabidopsis CRT in Escherichia coli**

The cDNA clone corresponding to accession no. T20724 contains a complete coding region. Two primers were synthesized, which include the sequence near the translation initiation and termination codons (5'-CCCCCATATGGCGAAAATACCC-3' and 5'-CCCCCTAGAGAGCTAGGCGG-3'). The restriction sites NdeI and XhoI were included for cloning into the same sites of the vector pET20b (Novagen). The resulting recombinant protein contains at its C terminus the additional amino acids LEHH-HHHEH, the first two encoded by the XhoI site and the six His residues encoded by vector sequences. The presence of the His tag allowed one-step purification by nickel-column chromatography. Purified protein was used to prepare anti-CRT antibodies (HTI Bio-Products, Ramona, CA).
Protein Analysis

Protein from Arabidopsis tissues was directly extracted in Laemmli’s buffer (Ausubel et al., 1995). Protein concentration was determined using Bradford reagent (Bio-Rad) on a portion of the extract from which excess SDS was removed by precipitation with 100 mM potassium phosphate buffer, pH 6.8. Protein samples were electrophoresed on denaturing polyacrylamide gels and transferred to nitrocellulose using the Trans Blot Semi-dry Transfer Cell (Bio-Rad). Blots were blocked in TBS containing 1% evaporated milk. Primary and secondary antibodies, both diluted 1:5000 in TBS containing 1% Tween 20 and phosphate buffer, pH 6.8. Protein samples were electrophoresed on denaturing polyacrylamide gels and transferred to nitrocellulose using the Trans Blot Semi-dry Transfer Cell (Bio-Rad). Blots were blocked in TBS containing 1% Tween 20 and 1% milk, were incubated sequentially with the blot, each for 30 min, with a thorough washing following each antibody. The secondary antibody was goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (GIBCO-BRL). Enhanced chemoluminescence detection reagents (Amersham) were used to detect the conjugate on BioMax MR film (Kodak).

Immunocytology

Floral tissues were fixed with glutaraldehyde, dehydrated, cleared, and embedded according to the method of Dixon and Klessig (1995). Embedded samples were then sectioned in 10-μm slices, mounted, and immunostained. The secondary antibody was goat anti-rabbit IgG (H+L)-alkaline phosphatase conjugate (GIBCO-BRL). Both primary and secondary antibodies were used at a dilution of 1:1000. For detection of alkaline phosphatase activity, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Boehringer Mannheim) were used as the substrates.

RESULTS

CRT Sequences in the EST Database

*A. thaliana* CRTs were identified by tblastn searches in the EST database (Table I). Of the sequences identified, three emerged as being useful: accession numbers T20724, T22451, and R65185. The first and third are derived from nearly full-length clones containing the translation initiation codon. The second is derived from a partial clone corresponding to accession no. Z26445 (Benedetti and Turner, 1995). They are each derived from distinct genes, and we have assigned the gene names Crt1, Crt2, and Crt3, corresponding to accession numbers T20724, T22451, and R65185, respectively (Table I).

Regular searches of the database EST were conducted using the open reading frame of each cDNA in a tblastn analysis to find additional or novel, CRT-like sequences too divergent to be detected by a hybridization analysis. Four classes of sequences (Table I) have been identified. For each class the longest known sequence (ESTs or GenBank entries) is assigned a prototype. The 5’ end of each sequence relative to the prototype is indicated (Table I). The Crt1 class has six EST members, Crt2 is represented by four EST members, whereas the third class is represented only by the accession used to define Crt3. Class 4 includes two members with similarities to the CRTs, but these are, in fact, calnexins.

DNA Analysis of Crt Genes and Cloning of the Crt2 5’ End

Inaccuracies in the reported single-pass EST sequences are inevitable and, thus, assignments of particular ESTs to gene groups must be controlled by independent techniques. Southern analyses were conducted for Crt1 and Crt2, which have multiple EST members, to determine whether very closely related genes exist. Using the 3’ end of Crt1 as a probe, we detected single bands in DNA digested with BamHI and EcoRI, whereas two bands were detected with HindIII (Fig. 1, left). For Crt2, DNA was digested with EcoRV and SacI, enzymes that cut within the sequence of T22451, and analyzed by hybridization with the insert of this partial cDNA (Fig. 1, right). The single BamHI fragment and the single EcoRI fragment for Crt1 and the pair of SacI fragments for Crt2 suggest that no closely related genes, i.e. within a few percentage points of identity, exist for either gene. The presence of two fragments in the HindIII analysis of Crt1 and three fragments in the EcoRV analysis of Crt2 indicate either a closely related gene for each or the presence of introns in each. In the 5’ end of the Crt2 gene (below) three introns are present. Cloning of the complete Crt1 and Crt2 genes will determine which alternative is correct. At this point, we conclude that all of the ESTs in each group of Table I are derived most likely from a single gene and at most two genes.

In the absence of a full-length cDNA clone corresponding to Crt2, the 5’ end of the gene was obtained by inverse PCR. Oligonucleotides corresponding to the sequence upstream of the SacI site in the sequence of T22451 were synthesized, labeled, and hybridized to a Southern blot (data not shown). The 5’ end of Crt2 was contained on the 3-kb SacI fragment identified in Figure 1, right. Following

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>5' End Relative to Prototype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crt1</td>
<td>T20724</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>T88392</td>
<td>1:150</td>
</tr>
<tr>
<td></td>
<td>T46404</td>
<td>1:432</td>
</tr>
<tr>
<td></td>
<td>Z26445</td>
<td>1:770</td>
</tr>
<tr>
<td></td>
<td>N96508</td>
<td>1:877</td>
</tr>
<tr>
<td></td>
<td>Z26033</td>
<td>1:1143</td>
</tr>
<tr>
<td>Crt2</td>
<td>U27698</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>T45719</td>
<td>1:91</td>
</tr>
<tr>
<td></td>
<td>T22451</td>
<td>1:429</td>
</tr>
<tr>
<td></td>
<td>H36323</td>
<td>1:519</td>
</tr>
<tr>
<td></td>
<td>AA006196</td>
<td>1:693</td>
</tr>
<tr>
<td>Crt3</td>
<td>R65185</td>
<td>1:1</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Z18242</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>T46259</td>
<td>1:660</td>
</tr>
<tr>
<td></td>
<td>T21101</td>
<td>1:866</td>
</tr>
</tbody>
</table>

Table I. Classification of Crt and Crt-like sequences of *A. thaliana*

The three classes of Crts defined here are displayed showing the ESTs found for each family. The first nucleotide of each EST is mapped relative to the nucleotide number within the prototype for each class.
Characteristics of the cloned Crt2 gene fragment

Table II. Characteristics of the cloned Crt2 gene fragment

<table>
<thead>
<tr>
<th>Exon</th>
<th>Accession no.: U66344</th>
<th>Organism: A. thaliana</th>
<th>5' nucleotide reported in accession no. U27698</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>733–817</td>
<td>Exon 1</td>
<td>1419–1526</td>
</tr>
<tr>
<td>2</td>
<td>1621–1813</td>
<td>Exon 3</td>
<td>1911–2038</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Sequences of predicted N-termini (this accession and U27698)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>NSARASA. . . . . . . .</td>
<td></td>
</tr>
<tr>
<td>23.5 kb</td>
<td>EcoRI HindIII EcoRV SacI</td>
<td>0.5 kb</td>
<td>218–223</td>
</tr>
</tbody>
</table>

Figure 1. Southern analysis of Crt1 and Crt2. DNA was digested with the indicated enzymes and probed with the 3' untranslated region of accession number T27024 for Crt1 and the insert corresponding to accession number T22451 for Crt2.

Table III. Comparison of the predicted amino acid sequences of CRT1, CRT2, and CRT3

<table>
<thead>
<tr>
<th>Motif</th>
<th>CRT1</th>
<th>CRT2</th>
<th>CRT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leader</td>
<td>1–21</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>RWVKSDWK</td>
<td>37–45</td>
<td>. . . E</td>
<td>. . . L</td>
</tr>
<tr>
<td>AGEWKHT</td>
<td>50–56</td>
<td>Present</td>
<td>. . . TF</td>
</tr>
<tr>
<td>DCGGGY</td>
<td>107–112</td>
<td>Present</td>
<td>E. . . A</td>
</tr>
<tr>
<td>MFGPDICG</td>
<td>134–141</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>KIKETSAK</td>
<td>209–218</td>
<td>Present</td>
<td>R. . . VKN</td>
</tr>
<tr>
<td>KPEDWD</td>
<td>218–223</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>KPEDWD</td>
<td>253–258</td>
<td>Present</td>
<td>E. . . E</td>
</tr>
<tr>
<td>...</td>
<td>. . .</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

We have deposited the full sequences of the clones corresponding to accession numbers T20724 and R65185 in GenBank under accession numbers U66343 and U66345, respectively. Table III presents the identities and similarities between the three CRT proteins and shows that Crt1 and Crt2 are closely related, whereas Crt3 is more divergent. The cDNA derived from Crt1 encodes a protein with the observed features (Table III) of CRTs from other species (Michalak et al., 1992), including a hydrophobic leader sequence consistent with co-translational insertion into the ER, a Pro- and Lys-rich central domain (P-domain), a C-terminal domain rich in acidic residues, a sequence similar to the consensus for bipartite nuclear localization signals (Dingwall and Laskey, 1991) starting at position 209 (-KKKDPGAKK-), and an -HDEL C-terminal ER retention signal (C terminus). Several other highly conserved amino acid sequences with yet unknown functions (Benedetti and Turner, 1995) are also present in Crt1 and are noted in Table III. These include sequences conserved among diverse organisms, -KPEDWD-, -DCGGGY-, and -MFGPDICG-, and sequences conserved only in plants, -RWVKSDWKK- (position 37) and -AGEWKHT- (position 50). Following the pattern established by calculations of identity and similarity, Crt2 has most of these motifs conserved, whereas in Crt3, several are not perfectly conserved. The substitution of glutamate for aspartate in the second motif listed retains the acidic charge in the Crt2 protein. For Crt3, several substitutions alter type and charge of amino acid, indicating potentially diverse functions for this protein.
Arabidopsis thaliana Calreticulin Protein Family

Protein Analysis

CRT1 was expressed in E. coli using the expression vector pET20b (Novagen). One hour after induction the protein constituted greater than 50% of total protein, and because of the high content of Lys, aspartate, and glutamate (35 mol % total), it remained soluble. Using the purified protein, we prepared antibodies in rabbits. The antibody is able to detect 1 ng of purified protein on a protein blot when using a 1:5000 dilution. Thus, CRT can be detected in plant extracts when as low as 0.01 to 0.001% of total protein.

Sequence conservation between the CRTs is sufficiently high so that a polyclonal antibody raised against one CRT should recognize other CRTs. This point has been tested by analyzing seed extracts of wheat, sorghum, millet, lettuce, tomato, tobacco, Distichlis spicata, and Mesembryanthemum crystallinum. In all species, a signal was detected (results not shown). The strengths of these signals were similar to that of Arabidopsis. Also, the relative mobilities of these signals were centered near the Arabidopsis signals, but, clearly, variations existed. Thus, the antibody raised against Arabidopsis CRT1 can readily recognize a wide variety of CRTs from both monocots and dicots.

Immunological analysis by protein blotting was conducted to determine which tissues contained CRT and to quantify the amount of CRT present. A variety of plant organs was surveyed, with two distinct bands being identified in all tissues: a lower band, which migrates slightly faster than the purified His-tagged CRT1, and an upper band, which migrates distinctly slower. Initially, these bands and the position of the purified His-tagged CRT1 were calibrated with a full-molecular-weight marker set to demonstrate their correct migration. Because the purified His-tagged CRT1 contains the hydrophobic leader sequence and the His tag (an additional 3,364 D), we interpret the lower band as being cleaved, but unmodified, protein. The predicted masses of CRT1, CRT2, and CRT3 after cleavage of the hydrophobic leader sequences are 46,228, 45,294, and 46,869 D, respectively. To calculate the masses of the processed CRTs, we assumed cleavage of the hydrophobic leader sequence immediately before the first Ala or Ser upstream of the first aspartate occurring after a hydrophobic region. The maximum variation is 1,575 D, an amount that would be almost indistinguishable and much less than the distance between the purified His-tagged CRT1 and the upper band. Thus, the upper band is most likely cleaved and posttranslationally modified protein. We do not yet know the ratios of the three CRTs in any of these bands.

The highest concentrations of CRT are found in developing flowers, particularly in the gynoecium (Fig. 2). The ovules of a single stage 17 gynoecium, a stage marked by the fall of organs from green siliques (Bowman, 1994), were dissected from the rest of the gynoecium, and the two tissues were extracted in an equal volume. Expression is not limited to ovules, since distinct bands were detected in both fractions. However, the gynoecium without ovule sample contained approximately 8 times higher total protein concentration, indicating a high level of expression on a protein basis in the ovules. To determine expression in ovules, an extract from the ovules was co-analyzed with purified CRT as a standard (Fig. 3). The signal obtained in the ovule extract is approximately equal to that in the lanes containing 200 and 400 ng of purified CRT, indicating that in ovules, 2 to 4% of total protein is CRT.

In Figure 4 the amount of CRT during seed development was measured. Ten micrograms of protein from stage 17

Figure 2. Western analysis of CRT in the gynoecium. Four siliques were dissected for the ovule and the gynoecium without ovule fractions. Also, four whole siliques were extracted with all extractions in 75 μL. One-fourth of each extract was analyzed. Lane 1, Purified CRT (150 ng); lane 2, ovule protein (7 μg); lane 3, gynoecium without ovule protein (56 μg); and lane 4, total gynoecium protein (63 μg).

Figure 3. Quantification of CRT in ovules. Lane 1, 10 μg of ovule protein; lane 2, 400 ng of purified CRT; lane 3, 200 ng of purified CRT; lane 4, 100 ng of purified CRT; and lane 5, 50 ng of purified CRT.

Figure 4. Quantification of CRT in mature seeds. Lane 1, 10 μg of immature ovule protein; lane 2, 10 μg of mature seed protein; lane 3, 20 μg of mature seed protein; lane 4, 40 μg of mature seed protein; lane 5, 80 μg of mature seed protein.
ovules was compared with various amounts of protein from mature seeds. The signal obtained with 10 μg of ovule protein is approximately equal to the signal from 80 μg of mature seed protein, indicating that the amount of CRT decreases during seed development. The CRT remaining in mature seeds was intact. Only one minor degradation product was observed.

Expression of a human Crt gene is induced by heat shock (Conway et al., 1995). To test whether accumulation of Arabidopsis CRT could be induced by such treatment, plants grown at 20°C were transferred to 40°C for 8 h and allowed to recover overnight at 20°C. Samples were taken prior to the temperature shift, immediately at the end of the heat shock, and again after recovery. The treatments did not result in any increased accumulation (Fig. 5). The apparent decrease in the sample taken immediately after the heat treatment is due to the loading procedure, which was based on equal protein amounts in all lanes: the increased accumulation of heat-shock proteins (one 32-kD protein shown) results in lower amounts of all other proteins being loaded.

Immunocytology

Protein analysis identified flowers as the organ in which CRT is most abundant. Immunocytological analysis thus concentrated on flowers (Fig. 6). The samples shown are from stage 13 flowers (Bowman, 1994). This stage, approximately 12 d following primordium formation, is characterized by opening buds, visible petals, anthesis, and stamens not yet extending above stigma. The locations are depicted by the immunocytological signals and photographs of live tissues.

Three tissues contain particularly high amounts of CRT: the posterior of pollen sacs, developing endosperm in immature ovules, and nectaries. Figure 6A depicts the immunological staining of a single flower showing each of these locations. In B, the bulge of cells on the posterior of the sac is evident. This bulge is present before (left) and after (right) dehiscence of the anther sac. In C, the immunological staining of these cells is indicated. The epidermal layer over the group of cells does not stain, but the signal is located in the cells underlying this bulge. Serial sections were stained (results not shown) and staining was observed only in the center of the pollen sac, in the posterior.

In D to F developing ovules are depicted. In the middle section, cells of the endosperm stain, whereas the embryo does not. In the right section at lower magnification, several ovules are depicted, giving the equivalent of a series of sections. Staining is general throughout the endosperm and ovules and different staining intensities are evident. Without knowing the exact order and timing of fertilization of each embryo, it is difficult to compare the intensity of staining with maturity (F). Nonetheless, the lack of staining in a very early embryo in E and then staining of some embryos in F indicates that CRT is increasing over time.

In G to I nectaries are shown. Several nectaries may be located at the base of the silique and are distributed around the periphery. This group of cells may lie inside of the petal layer or between the sepal and petal. In H a nectary is depicted with staining only in the basal half. In I an entire nectary is stained with an antibody. This is interpreted as the middle nectary being at a slightly earlier stage of development and nectar secretion than the nectary on the right.

DISCUSSION

To systematically analyze the role(s) of CRT in plants, we chose to make use of the benefits provided by the large number of ESTs reported from A. thaliana. These partial sequences facilitate the analysis of gene families and allow systematic and efficient use of resources for functional analyses. ESTs also accelerate the detection of related sequences, which are too distantly related to permit detection by DNA hybridization. This is exemplified by Crt3, which, based on its less than 70% nucleotide identity to Crt1 and Crt2 and low transcript abundance, would have been difficult or impossible to find by hybridization. Based on EST analysis, we were able to characterize the three genes of the CRT family in Arabidopsis to study protein localization and function. Knowledge gained by analysis of Arabidopsis ESTs can later be extended to other species in which CRT may have more specialized functions.

The three Crt genes in Arabidopsis encode proteins that share features of CRTs observed in other species. In particular, all contain a hydrophobic leader sequence and an ER retention signal. We will discuss these features as well as the locations of CRT expression determined here in the context of the three functions with which CRTs have been associated: molecular chaperone, calcium storage/release, and signaling.

Early in seed development high rates of storage protein synthesis are characteristic for developing endosperm. Consistent with the accumulation of CRT observed here in developing ovules, Boston et al. (1996) found in maize that genes encoding CRT, calnexin, Glc-regulated protein 94, and luminal binding protein are induced by treatments that disrupt protein folding. Interactions of these proteins
with other proteins have been demonstrated, although specific targets have not yet been identified. This role of chaperone is confirmed by abundant protein expression in both the ovules and nectaries (Fig. 6, F and I).

The presence of significant amounts of CRT in the mature seeds of Arabidopsis and other species is intriguing. Late-embryogenesis-abundant and certain ABA-regulated proteins (Skriver and Mundy, 1990) accumulate in mature seeds and are found in the cytoplasm. They are highly hydrophilic proteins, similar to CRT, and are proposed to protect the seed during desiccation and dormancy. Potentially, CRT found in mature seeds could have a specific function rather than simply being left over from the storage protein synthesis phase. CRT may be providing a function in the ER that is analogous to the function of late-embryogenesis-abundant and certain ABA-regulated proteins in the cytosol. In mature seeds the two bands observed early in development remain. One may expect a pool of the unglycosylated forms to accumulate when the rate of synthesis of CRT is high, but the persistence of the pool even in dry, mature seeds suggests that the lower, presumably unglycosylated band has a function that is distinct from the function of the glycosylated form.

Secretory cells are characterized by very high rates of protein synthesis. Denecke et al. (1995) demonstrated in germinating tobacco seeds an increase in the concentration of CRT during proliferation of the secretory apparatus. The barley aleurone layer secretes hydrolases mobilizing starch reserves to supply the heterotrophically growing seedling. GA3 stimulates secretion of these hydrolases and treatment with the hormone results in increased amounts of CRT transcripts (Denecke et al., 1995). Also demonstrated by Denecke et al. (1995) was a specific, ATP-dependent interaction with other proteins in vivo under various stress conditions, during which misfolding of proteins readily occurs.

Secretions from the nectary attract pollinators, provide moisture and carbohydrates for germinating pollen, and potentially secrete growth factors that are necessary for floral development (Roshchina and Roshchina, 1993). Little information about nectaries is available from Arabidopsis, but they have been studied in other species. Nectary secretions are primarily composed of sugars and amino acids. The secretory cells are characterized by extensively developed rough ER, smooth ER, and dictyosomes in close association with the plasma membrane (Arumugasamy et

Figure 6. Immunocytochemical analysis of CRT in Arabidopsis floral tissues. A, Cross-section of flower stained with antibody; B, profile of anthers before (left) and after (right) dehiscence showing a bulge on the posterior of the anther sac; C, cross-section of the anther sac stained with antibody; D, ovule; E, cross-section of the ovule and the embryo stained with antibody; F, lower magnification showing cross-sections of several ovules; and G, nectary located at base of gynoecium. Below the nectary is the pedicel and above is a filament; the sepals and petals have been removed. H and I, Cross-sections of two nectaries stained with antibody. Arrowheads in G to I indicate the apex of the nectary. The nectary in H is not yet secreting.
al., 1993; Roshchina and Roshchina, 1993). During secretion nectar first accumulates in the ER and is then secreted by exocytosis. The principal pathway involved in pre-nectar transport from the phloem cells to the nectary cells is the ER (Sawidis, 1991). The basal cells of the nectary, closest to the vasculature supplying carbohydrates, begin synthesis of nectar prior to more apical cells. CRT presence is correlated with this pattern of nectar production, first accumulating in the basal cells (Fig. 6H). CRT may be involved in the synthesis of enzymes that are responsible for the transformation of Suc to other carbohydrates found in nectar or other proteins involved in the exocytosis process. The identification of specific target proteins for CRT interaction in the nectary would assist greatly in understanding nectar secretion.

Although the ability of plant CRTs to bind to calcium has been demonstrated by $^{45}$Ca$^{2+}$ overlay assays (Chen et al., 1994; Opas et al., 1996), no specific storage/release function part of a signal transduction pathway has yet been demonstrated in plants. However, calcium and magnesium concentrations, but neither potassium nor sodium, increase during nectar secretion (Heinrich, 1989). Since nectar secretions are derived from the ER, CRT may have a role in this release of calcium. For other floral processes, gradients of calcium have been described, although the expression of CRT has not yet been related at the subcellular level. Changes in calcium distribution in tissues and cells following pollination have been observed. In petunia a calcium gradient exists, with the level highest near the stigma and decreasing toward the base of the style (Bednarska, 1995). Following pollination, the gradient is reoriented with the highest concentrations measured at the base of the style. Within cells of the transmitting tissue, calcium concentrations are highest at the polar ends of cells. Within pollen tubes that are rejected, calcium concentrations remain high. All of these provide potential systems for analyzing a CRT role in calcium distribution. Napier et al. (1995) found no subdomains of the ER enriched for CRT, although the tissues that were analyzed were not given any specific treatment, which would be proposed to lead to redistribution of CRT prior to calcium release.

Evidence for CRT localization outside of the ER has yet to be reported in plants, and, indeed, the interactions of CRT with integrins and steroid hormone receptors in animal systems pose a conceptual problem: all known CRT genes encode the ER import and retention signals and, thus, no cytosolic isoforms may be expected. Opas et al. (1996) found CRT only in the ER of plant protoplasts, and Napier et al. (1995) detected identical patterns of staining of the ER of maize root tip cells using an anti-CRT antibody and an anti-HDEL antibody. Alternative explanations, as suggested by Dedhar (1994), would involve re-entry into the cell of exported protein, but there is no experimental evidence that this occurs.

To our knowledge, the immunocytochemical staining of CRT in a group of cells directly under the abaxial surface of anthers is the first to mark this cluster of cells. Search of the literature revealed no information concerning the identity or function of this cell cluster, although it is evident in published micrographs (Clark and Meyerowitz, 1994, compare with figure 3B; Bowman, 1994, compare with plate 4.1, A and B). An analysis of these cells may reveal not only information about CRT but basic information about the biology of pollen sacs and the process of dehiscence. It is not known whether protein synthesis rates are high in these specific cells or whether the ER is abundant. High rates of protein synthesis would be surprising because the anther sac as a whole at this stage of floral development is degenerating.

Beginning biochemical studies of CRT in plants and the developmental control and cell specificity of CRT expression reported here indicate that CRTs have more than a "housekeeping" function. The regulation of intracellular localized calcium concentrations is fundamental to many biological processes and, clearly, the evidence points to CRT as a major store. Molecular chaperones are involved in practically all biological processes, whether developmentally regulated or related to the maintenance of homeostasis following environmental disturbance. From an applied standpoint, information about CRT expression is relevant to the understanding of both floral development and seed storage tissue functioning. The study of CRT in plant-specific processes will enable us to extend what has been learned from the analysis of animal systems concerning CRT functions.

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LITERATURE CITED

Arabidopsis thaliana Calreticulin Protein Family


