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Identification and Characterization of cDNA Clones Encoding Plant Calreticulin in Barley

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Two cDNA clones (CRH1 and CRH2) homologous to animal calreticulin, a major calcium storage protein in the lumen of the endoplasmic reticulum, were isolated from an ovary cDNA library of barley through differential screening. The two clones differ in the 3′ untranslated region and the 5′ region that encodes a putative N-terminal signal sequence. CRH1 was mapped to the minus arm of chromosome 1. CRH2 was mapped to the minus arm of chromosome 2. The deduced amino acid sequences share 50 to 55% identity with animal calreticulins and exhibit the same three-zone characteristic. Recombinant protein stained blue with Stains-all and bound $^{45}Ca^{2+}$ when transferred to nitrocellulose membranes. A native protein of ~55 kD was identified in ovary extract. Elevated gene expression was observed in ovaries 1 day after pollination and during early embryogenesis. CRH1 was expressed at a higher level than CRH2. These studies demonstrate the presence of calreticulin in plant cells and its developmental regulation in fertilization.

INTRODUCTION

Calreticulin and calsequestrin are two major Ca^{2+} storage proteins that have been identified in many animal species (Michalak et al., 1992; Milner et al., 1992). Calsequestrin is the major Ca^{2+} binding protein in the sarcoplasmic reticulum in skeletal and cardiac muscle (MacLennan et al., 1985), where release of Ca^{2+} from calsequestrin through a specific Ca^{2+} channel is primarily responsible for muscle contraction. In the smooth muscle sarcoplasmic reticulum and nonmuscle endoplasmic reticulum (ER), however, calreticulin is the major Ca^{2+} binding protein (Milner et al., 1991; Opas et al., 1991). In nonmuscle cells (Walz and Baumann, 1989) as well as in many types of plant cells (Hepler and Wayne, 1985), the ER is responsible for the regulation of cytosolic free Ca^{2+} concentration. Many cell types respond to external stimuli by elevating the cytosolic free Ca^{2+} concentration through the release of Ca^{2+} from an internal store. In sea urchin, for example, the intracellular free Ca^{2+} concentration is elevated at fertilization (Whitaker and Patel, 1990).

Both calsequestrin and calreticulin have a high-capacity, low-affinity Ca^{2+} binding domain. Calsequestrin binds 30 to 50 mol of Ca^{2+} per mol of protein with a low affinity of ~1 mM (MacLennan et al., 1983), and calreticulin binds ~25 mol of Ca^{2+} per mol of protein with a low affinity of ~2 mM (Ostwald and MacLennan, 1974; Baksh and Michalak, 1991). In addition, calreticulin also has a high-affinity, low-capacity binding site that binds 1 mol of Ca^{2+} per mol of protein with a high affinity of 1 to 10 μM (Baksh and Michalak, 1991; Milner et al., 1992). As a result of their calcium binding property, the two proteins stained blue with the cationic carbocyanine dye Stains-all and had an anomalous mobility in the Laemmli SDS gel buffer system (Campbell et al., 1983; Milner et al., 1991). These two "diagnostic" biochemical properties have been widely used in identifying Ca^{2+} binding proteins from different sources.

Amino acid sequences for both calsequestrin and calreticulin have been deduced from cDNA clones of several mammalian species (Fliegel et al., 1987, 1989; Scott et al., 1988; Smith and Koch, 1989). More recently, deduced amino acid sequences for calreticulin have been reported in nematode (Smith, 1992a), marine snail (Kennedy et al., 1992), clawed frog (Treves et al., 1992), and fruit fly (Smith, 1992b). The deduced amino acid sequence of calreticulin has a zonal characteristic: a nearly neutral N domain is followed by a proline-rich central domain (P domain), which is followed by a short hydrophobic sequence before the polyacidic C domain (Fliegel et al., 1989; Smith and Koch, 1989; Michalak et al., 1992). Within the P domain, there are three KPEDWD sequence repeats that are highly conserved among various animal species. Using recombinant calreticulin, Baksh and Michalak (1991) demonstrated that the P domain is responsible for the high-affinity, low-capacity Ca^{2+} binding and that the highly acidic C domain is responsible for the low-affinity, high-capacity Ca^{2+} binding.

There is evidence that calreticulin may have other intracellular functions besides the apparent Ca^{2+} binding role (Michalak et al., 1992). Burns et al. (1992) found that calreticulin gene expression is elevated in stimulated mouse splenocytes and human lymphocytes and suggested that calreticulin may be involved in the signaling pathway for the induction of Ca^{2+}-dependent processes in the activation of T lymphocytes. Nakamura et al. (1992) reported that rabbit antisera raised

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against rat calreticulin immobilized mouse sperm and inhibited in vitro fertilization of mouse eggs.

Attempts have been made to identify calsequestrin and calreticulin in plants. Chou et al. (1989) and Krause et al. (1989) reported a calsequestrin-like 56-kD protein in the extract of *Streptanthus tortuosus* cell cultures and spinach leaves. In germinating pear pollen, Allen and Tiwari (1991) identified a protein cross-reacting with calreticulin antibody. More recently, Menegazzi et al. (1993) identified a calreticulin-like protein from spinach leaves. The N-terminal amino acid sequence of this protein is very similar to animal calreticulins. In this study, we report the identification and characterization of two different cDNA clones encoding plant calreticulin in barley.

RESULTS

Nucleotide and Deduced Amino Acid Sequences

Among cDNA clones expressed exclusively or at elevated levels after fertilization, we identified two clones (CRH1, GenBank accession number L27348, and CRH2, GenBank accession number L27349) that are homologous to animal calreticulins. Both clones are incomplete in the 5′ region that presumably encodes the N-terminal signal sequence. CRH1 (1472 bp) and CRH2 (1450 bp) differ in the 3′ untranslated region and the first 45 bp of the 5′ coding region. In the 5′ region, the first 37-bp sequence of CRH1 is not present in CRH2, and the first 45-bp sequence of CRH2 is not present in CRH1. In the 3′ region, CRH1 has an extra 30-bp sequence before the poly(A) tail. The nucleotide sequence of both clones share 60 to 70% identity in the entire coding region with various animal calreticulin clones.

CRH1 encodes 412 amino acid residues and CRH2 encodes 415 amino acid residues. The first 18 amino acid residues in CRH1 and the first 21 amino acid residues in CRH2 are likely to be a part of the N-terminal signal sequence. The prediction is consistent with the N-terminal sequence of the spinach calreticulin-like protein (Menegazzi et al., 1993), which is 70% identical to the first 20 residues of the predicted mature protein. CRH1 and CRH2 differ in the putative N-terminal signal sequences. The first 10 residues of CRH1 are not present in CRH2, and the first 13 residues of CRH2 are not present in CRH1. However, the two clones are identical in the predicted mature protein, which has 394 residues with a calculated molecular mass of 45 kD and an estimated pI of 4.3.

The deduced amino acid sequences share 50 to 55% identity with calreticulins of various animal species, including nematode (Smith, 1992a), fruit fly (Smith, 1992b), marine snail (Kennedy et al., 1992), clawed frog (Treves et al., 1992), rabbit (Fliegel et al., 1989), rat (Nakamura et al., 1993), mouse (Smith and Koch, 1989), and human (Rokeach et al., 1991), and exhibit the same zonal characteristic. The N-terminal region (residues 19 to 202 in CRH1 and 22 to 205 in CRH2) is nearly neutral, containing almost the same amount of acidic and basic residues. This region is followed by a proline-rich region (residues 203 to 303 in CRH1 and 206 to 306 in CRH2), which contains 18 of the total 23 proline residues. Within this region, there are two nine-residue repeats: AKKPEDWDD. These two repeats also contain the first and the third KPEDWD repeats that are found in animal calreticulins. The second KPEDWD repeat found in animal calreticulins is replaced by KPEGYD in the CRH clones. The proline-rich region is followed by a short stretch of predominantly hydrophobic residues (residues 304 to 333 in CRH1 and 307 to 336 in CRH2). The C-terminal region (residues 334 to 412 in CRH1 and 337 to 415 in CRH2) is highly acidic: in the last 54 residues, 32 are acidic (21 D + 11 E), whereas eight are basic (7 K + 1 H). The C-terminal region ends with the ER retention signal HDEL.

Sequence alignment between the deduced amino acid sequences of CRH1 and rabbit calreticulin (Fliegel et al., 1989) is presented in Figure 1. The first 17 residues of rabbit calreticulin are the N-terminal signal sequence, which is not present in mature protein (Fliegel et al., 1989). The alignment reveals several consensus sequences between the two proteins, each ranging from eight to 11 residues. There are three such consensus sequences in the N-terminal region, one in

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C-  LLRRLALLALASVAAVAADVFFQEKF--EDGWESRWVKSEWKKD 42
    **.   **.* ..**... *.*.*.*  .***..**..*. *.*
R- MLLPVPLLLGLLGLAAAEPVVYFKEQFLDGDGWTERWIESKHKSD 45

C- ENMAGEWNHTSGKWHGDAE-DKGIQTSEDYRFYAISAEYPEFSNK 86
     *..  .***..**.* ***.***.* ****.** ...****
R- ---FGKFVLSSGKFYGDQEKDKGLQTSQDARFYALSARFEPFSNK 87

C- DKTLVLQFTVKHEQKLDCGGGYVKLLGGDVDQKKFGGDTPYGIMF 131
   ...**.********..*********.....***.. **..*.***  .
R- GQPLVVQFTVKHEQNIDCGGGYVKLFPAGLDQKDMHGDSEYNIMF 132

C- GPDICGYSTKKVHTILTKNGKNHLIKKDVPCETDQLSHVYTLIIR 176
   ****** .*****.*.. .*** **.**..*..*...*.****.*
R- GPDICGPGTKKVHVIFNYKGKNVLINKDIRCKDDEFTHLYTLIVR 177

C- PDATYSILIDNEEKQTGSIYEHWDILPPKEIKDPEAKKPEDWDDK 221
   **.**.. ***.. ..**. ..**.****.****.*.******..
R- PDNTYEVKIDNSQVESGSLEDDWDFLPPKKIKDPDASKPEDWDER 222

C- EYIPDPEDVKPEGYDDIPKEVTDPDAKKPEDWDDEEDGEWTAPTI 266
   . *.**.* ***..*. *....***********.* ****..*.*
R- AKIDDPTDSKPEDWDK-PEHIPDPDAKKPEDWDEEMDGEWEPPVI 266

C- PNPEYKGPWKQKKIKNPNYQGKWKAPMIANPDFQDDPYIYAFDSL 311
   .******.**...*.**.*.*.*  * *.**..  .*. ***.**.
R- QNPEYKGEWKPRQIDNPDYKGTWIHPEIDNPEYSPDANIYAYDSF 311

C- KYIGIELWQVKSGTLFDNILITDDAALAKTFAEETWAKHKDAEKA 356
    .*..********.***.***.*.*.*..*..***.  *.***.
R- AVLGLDLWQVKSGTIFDNFLITNDEAYAEEFGNETWGVTKTAEKQ 356

C- AFD-----------EAEKKKEEEDASKAGEDDDDLDDEDADDED 389
    *            *..*.****.*....**.** .***.*.**
R- MKDKQDEEQRLKEEEEEKKRKEEEEAEEDEEDKDDKEDEDEDEED 401

C- KDDKAGSDAEDDKDSDDEKHDEL 412
   **.      *........ .***
R- KDE------EEEEAAAGQAKDEL 418
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Figure 1. Amino Acid Sequence Comparison between CRH1 and Rabbit Calreticulin.

C indicates CRH1 and R denotes rabbit calreticulin (Fliegel et al., 1989). Identical residues are marked by asterisks. Similar residue substitution is denoted by dots. Gaps are denoted by dashed lines. An overall 55% identity and 72% similarity were found between the two sequences.

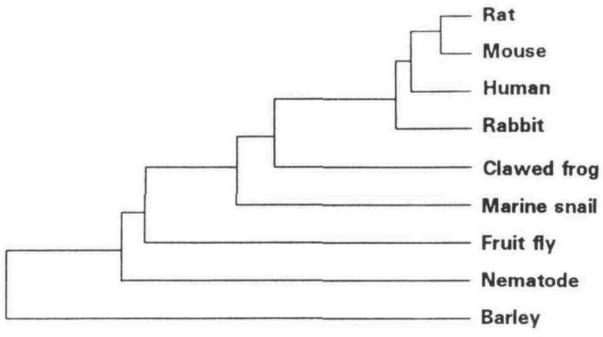

Figure 2. Calreticulin Dendrogram for Nine Different Species.

The deduced amino acid sequences from nine species were used in the dendrogram construction. Sequence identity between barley calreticulin and animal calreticulins ranges from 50 to 55%.

the proline-rich region, and one in the hydrophobic region (Figure 1). Extensive sequence variation was found in the highly acidic C-terminal region. Sequence comparisons among plant, nematode, fruit fly, marine snail, clawed frog, rabbit, rat, mouse, and human calreticulins showed that the two consensus sequences of DCGGGYVK and MFGPDICG in the N-terminal region are invariant, whereas the other consensus sequences are highly conserved. The three KPEDWD repeats in the proline-rich region are also highly conserved but not invariant. The acidic C-terminal region was also less conserved in the multiple comparison.

Using the clustering method of Feng and Doolittle (1987), a dendrogram was constructed with the deduced amino acid sequences from the nine species, as shown in Figure 2. Based on the clustering, plant calreticulin is more closely related to nematode calreticulin than to the other animal calreticulins. However, the percent identity between the two predicted proteins for plants and nematodes (54%) is similar to the sequence identities between plant and other animal calreticulins (50 to 55%). When compared with mammalian calreticulins, the percent identity for nematode, fruit fly, marine snail, and clawed frog is 64, 67, 74, and 80, respectively. More than 90% sequence identity was found among mammalian calreticulins.

Expression and Ca^{2+} Binding of Recombinant Protein

A fragment encoding the P and C domains and most of the N domain was cloned into the pQE30 expression vector. The recombinant protein was overexpressed in *Escherichia coli* when induced by isopropyl-β-D-thiogalactopyranoside. As shown in Figure 3A, purified recombinant protein migrated on a SDS–polyacrylamide gel with a mobility corresponding to ~52 kD. The predicted molecular mass of the recombinant protein is 44 kD, which is slightly smaller than the predicted size of the native mature protein (45 kD). The recombinant protein stained blue with Stains-all, while all the molecular mass marker proteins stained red or pink. The anomalous mobility on SDS–polyacrylamide gels and the blue staining with Stains-all are characteristic of calcium binding proteins. We further tested the calcium binding ability of the recombinant protein by the $^{45}Ca^{2+}$ overlay technique using nitrocellulose membranes. As shown in Figure 3B, calmodulin (a high-affinity calcium binding protein) and the recombinant protein bound Ca^{2+} after a high-stringency wash with 50% ethanol, whereas ovalbumin did not bind any Ca^{2+} under the same conditions. The signal of calmodulin was reduced because it is a small protein that binds poorly to nitrocellulose membranes in alcohol solution. These results provide direct evidence that the gene product of CRH1 and CRH2, as predicted, is an authentic calcium binding protein.

Restriction Fragment Length Polymorphism Mapping of CRH1 and CRH2

With a panel of five barley cultivars (Dicktoo, Morex, Steptoe, Harrington, and TR306) and five restriction enzymes (DraI, EcoRI, EcoRV, HindIII, and XbaI), moderate restriction fragment

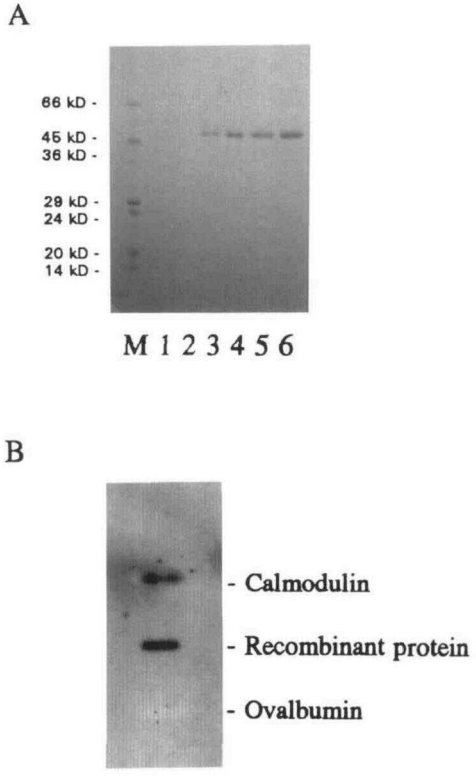

Figure 3. SDS-PAGE and Calcium Binding Analyses of the Recombinant Protein.

(A) SDS-PAGE followed by staining with Stains-all. Lanes 1 to 6 contain recombinant protein from different column fractions; lane M, molecular mass markers, which are given at left in kilodaltons.
(B) $^{45}Ca^{2+}$ overlay on a slot blot. Each slot was loaded with 2 μg of protein.

length polymorphisms (RFLPs) were detected in genomic DNA blots using CRH1 and CRH2 as probes. All five restriction enzymes detected polymorphisms among the five cultivars. The two clones gave the same RFLP patterns for most of the major bands, but CRH1 detected an extra major band and more minor bands. CRH1 also gave a higher background signal.

We used RFLP mapping to determine the chromosome location of the two genes. CRH1 alone was used as a probe for the mapping because it detected both the common bands with CRH2 and the unique band. In the doubled-haploid progeny of Steptoe × Morex, a 1:1 ratio was observed for the unique CRH1 band (χ^2 = 1.5, P > 0.2). Using 297 previously mapped markers in this cross (Kleinhofs et al., 1993), the RFLP locus was mapped to the terminus of the minus arm of chromosome 1, which is tightly linked to the catalase gene (*cat3*). A portion of the linkage map is presented in Figure 4A. In the doubled-haploid progeny of Dicktoo × Morex, two parental and two recombinant RFLP patterns with a 1:1:1:1 ratio were observed (χ^2 = 0.5, P > 0.9). Using 80 previously mapped markers in this cross (P. Hayes and F. Chen, unpublished data), the unique CRH1 band was again mapped to chromosome 1, which is 21.4 centimorgans from the RFLP marker *ABG461* (Kleinhofs et al., 1993) and common to both populations. The common band that was detected by both CRH1 and CRH2 was mapped to the minus arm of chromosome 2, which is 6.4 centimorgans from the RFLP marker *BCD410* (Heun et al., 1991). The linkage map is presented in Figure 4B. CRH1 was assigned to the chromosome 1 locus and CRH2 to the chromosome 2 locus based on their differential hybridization to the genomic restriction fragments.

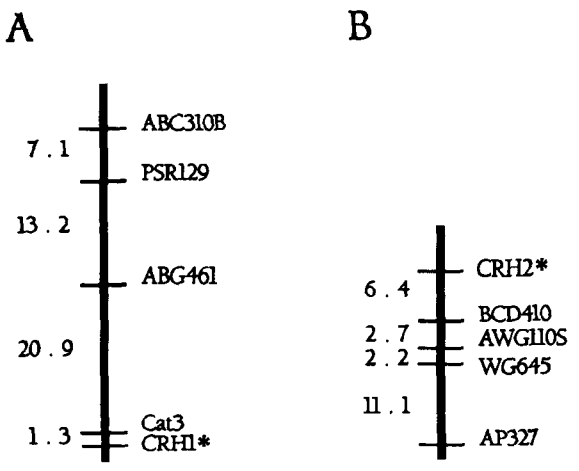

Figure 4. Linkage Maps for CRH1 and CRH2.

(A) A portion of the minus arm of chromosome 1.
(B) A portion of the minus arm of chromosome 2.
The map units are given in centimorgans. CRH1 and CRH2 are indicated by asterisks.

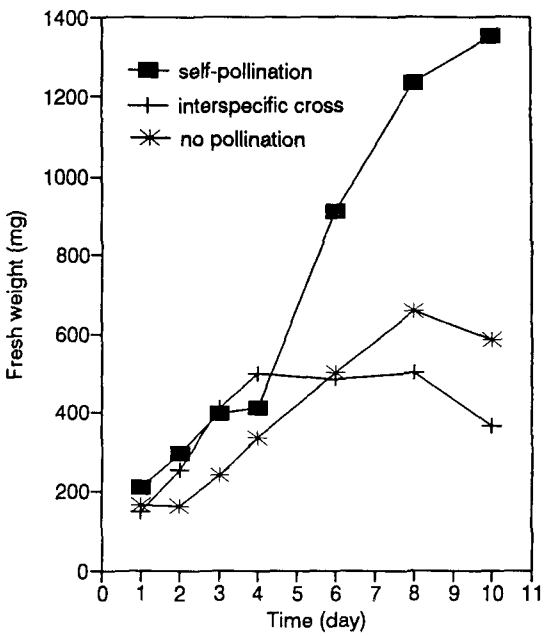

Figure 5. Growth Rates of Caryopses and Unpollinated Ovaries.

The time course was defined by days after pollination for caryopses and days after initial receptiveness for unpollinated ovaries. The initial receptiveness usually occurs 2 days after emasculation. Fresh weight was defined as the number of milligrams per 100 ovaries or caryopses.

Elevated Gene Expression after Pollination

Barley is a self-pollinated crop. Fertilization and the first division in the zygote occur within 24 hr after pollination (Engell, 1988). The interspecific cross between barley and *Hordeum bulbosum* results in the selective chromosome elimination of *H. bulbosum* during early embryogenesis (Kasha and Kao, 1970). We studied the gene expression of CRH1 and CRH2 using both compatible (self-pollination) and partially incompatible (interspecific cross) pollen sources.

The growth rates of caryopses and unpollinated ovaries are presented in Figure 5. In the first 4 days after pollination, there was little difference between self-pollination and the interspecific cross. However, the fresh weight of self-pollinated caryopses increased dramatically after day 4, but no increase in fresh weight was observed for the interspecific cross after day 4. The growth arrest was due to the selective elimination of *H. bulbosum* chromosomes (Jensen, 1977). In unpollinated ovaries, growth was slow as compared to self-pollination. The maximum fresh weight (660 mg per 100 ovaries) of these unpollinated ovaries was observed at day 8.

Elevated levels of calreticulin transcripts in pollinated ovaries were detected in RNA gel blots using CRH1 and CRH2 as probes (data not shown). However, a detailed quantitation was precluded by an excessive trailing background in the RNA

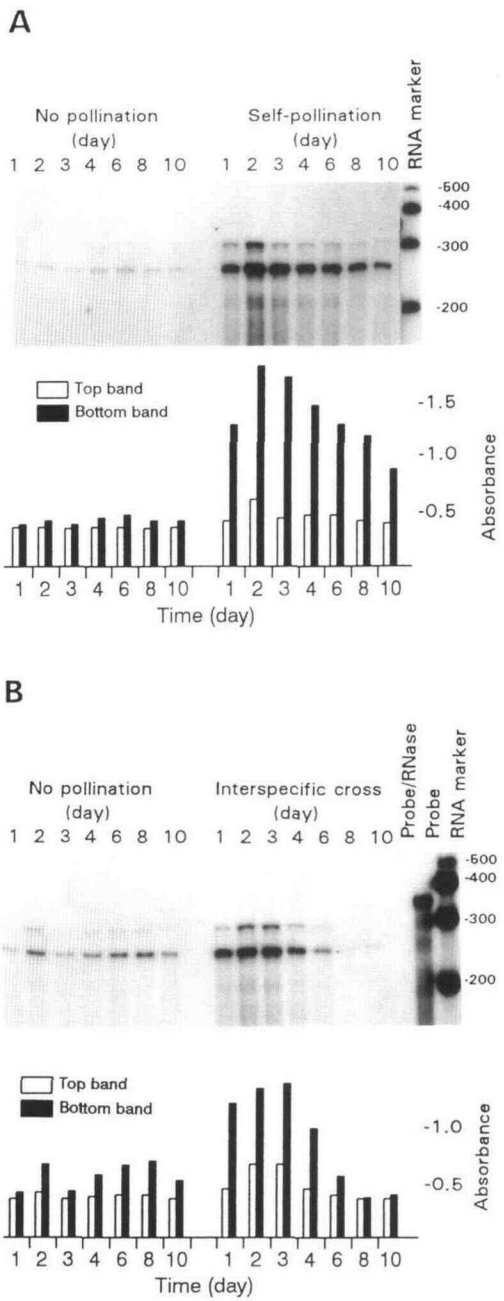

Figure 6. RNase Protection Analysis of Calreticulin Transcripts in Barley Ovaries.

(A) A comparison between unpollinated and self-pollinated ovaries. **(B)** A comparison between unpollinated and the interspecific cross ovaries.

Total RNA was used at 4 μg per assay. The upper band is the fragment protected by CRH2 transcripts, and the lower band is the fragment protected by CRH1 transcripts in **(A)** and **(B)**. The expected size is 316 bases for the upper band and 271 bases for the lower band. The expected size for the full-length probe is 363 bases including the sequence transcribed from the vector. Numbers at right indicate the size of the RNA markers in bases. The RNA markers were transcribed from an RNA marker template set (Ambion, Austin, TX) using T7 RNA polymerase. The graphical representation of the data was obtained by scanning the autoradiograph with a densitometer at 633 nm. Peak absorbance was used for the comparison. The time course is as described in Figure 5.

gel blots. We attributed the excessive background of the CRH clones to specific in vivo transcript degradation rather than the quality of total RNA preparation because when the same blots were probed with other, unrelated clones we observed no such background. Cross-hybridization of CRH1 and CRH2 in the RNA gel blots also precluded a comparison between CRH1 and CRH2.

The need for quantitation of individual transcripts led us to use ribonuclease protection assays. The antisense RNA probe was transcribed from the first 316 bp of the 5′ end of the CRH2 cDNA insert. It was expected to be fully protected by CRH2 transcripts and partially protected by CRH1 transcripts because the two clones, as described above, only differed in the first 45 bp in the 5′ region. As shown in Figure 6, the two predicted fragments were detected in the ribonuclease protection assays. The level of CRH2 transcripts (upper band) was lower than that of CRH1 transcripts (lower band) in almost all samples, particularly in self-pollinated ovaries and the interspecific cross ovaries at the early stages (days 1 to 3). Using reverse transcriptase–polymerase chain reaction assays, we also found that CRH1 transcripts were present at a higher level than CRH2 transcripts in self-pollinated ovaries (data not shown).

A slight elevation in CRH2 transcription was observed after pollination. In contrast, there was a substantial increase in the CRH1 transcript level after pollination. As shown in Figure 6A, a more than threefold increase in CRH1 transcripts was observed in self-pollinated ovaries at day 1 and a more than fourfold increase at day 2 and day 3 as compared to the unpollinated ovaries. The level then dropped gradually, but was still about twofold over the level of the unpollinated ovaries at the end of the time course (day 10). The change in CRH1 transcript levels, however, was not paralleled by the ovary growth rate over the time course. Higher levels of CRH1 transcripts were found in the first 4 days, whereas a drastic increase in the ovary growth rate occurred after day 4 (Figure 5). The elevation of CRH1 transcripts in the interspecific cross differed from that of self-pollination in the magnitude and duration. As shown in Figure 6B, a threefold increase in CRH1 transcripts was observed in the interspecific cross at day 1 as compared to the unpollinated ovaries. However, only a small further increase was observed at day 2 and day 3. The level then decreased drastically at day 4 and dropped to that of the unpollinated ovaries at day 6. In unpollinated ovaries, a low level of expression was observed for both CRH1 and CRH2 throughout the time course, although there was a small deviation between days.

Detection of Native Calreticulin in Ovary Extracts

Total ovary protein extracts were analyzed by SDS-PAGE followed by staining with Stains-all. As shown in Figure 7A, while most of the bands stained pink or yellow with Stains-all, a blue-staining protein with a mobility corresponding to ~55 kD was detected in the ovary extracts. The intensity of the blue band increased in pollinated ovaries. We proceeded to test the calcium binding ability of this protein band by $^{45}Ca^{2+}$ overlay on a protein gel blot. As shown in Figure 7B, Ca^{2+} binding was detected on a protein band with the same mobility as the blue-staining band after a high-stringency wash with 50% ethanol; however, the signal was weak. Using immunoblotting, a protein with the same mobility was detected by the polyclonal antibody raised against the recombinant protein, as shown in Figure 7C. The apparent molecular mass of this band was slightly larger than that of the recombinant protein. This is consistent with the difference between the predicted molecular mass of the native mature protein (45 kD) and that of the recombinant protein (44 kD). These results showed that the 55-kD blue-staining protein is likely to be the native calreticulin in barley.

DISCUSSION

The remarkable similarity between the CRH clones and animal calreticulins in both the deduced amino acid sequences and the protein zonal structure showed that calreticulin is highly conserved between animals and plants. Based on the 20 residue N-terminal sequence in the spinach calreticulin-like protein reported by Menegazzi et al. (1993), the sequence identity between barley, a monocotyledon, and spinach, a dicotyledon, is 70%. This percent identity is similar to those between mammals and nonmammals. The results of Stains-all staining and $^{45}Ca^{2+}$ overlay provided direct evidence that plant calreticulin has a similar, if not identical, Ca^{2+} sequestering function.

The presence of the ER retention signal HDEL suggests that the gene product of CRH1 and CRH2 is likely to be an ER luminal protein. The sequence of the first 18 amino acid residues (LLRRLALLALASVAAVAA) in CRH1 is characteristic of an N-terminal signal sequence. However, the sequence of the first 21 amino acid residues (RIGKKALESRLVCASVAAVAA) in CRH2 is somewhat variant from a typical N-terminal signal sequence because it has more charged residues. Different N-terminal signal sequences in two otherwise identical mature proteins have not been reported in animal calreticulins. The biological significance of this feature is yet to be characterized.

Although calreticulin is highly conserved between animals and plants, we were able to detect moderate RFLPs on the genomic DNA blots of the five barley cultivars using CRH1 and CRH2 as probes. Among the five restriction enzymes, DraI detected more polymorphisms than any of the others. The polymorphisms, however, are likely a result of genomic DNA

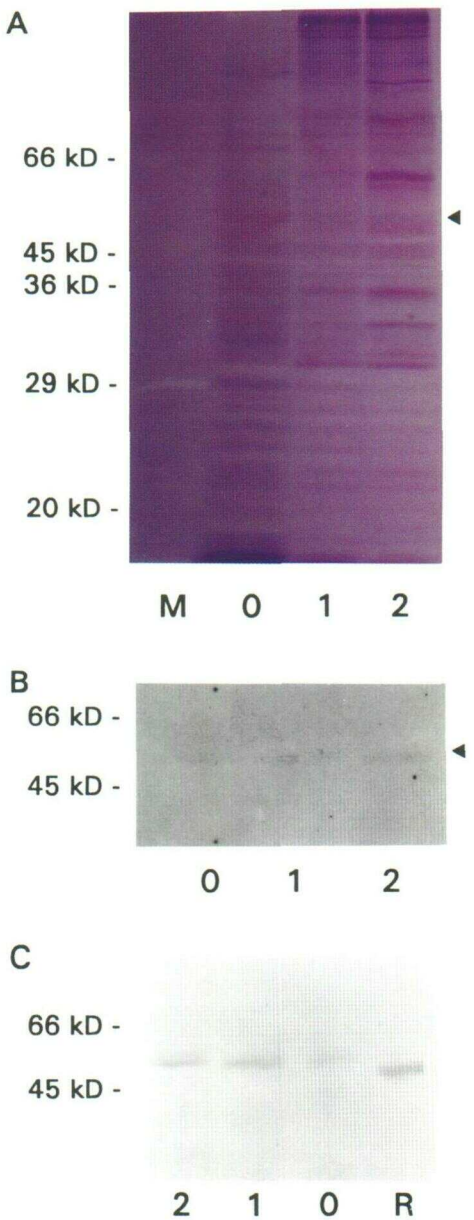

Figure 7. Analysis of Ovary Protein Extract.

(A) SDS-PAGE followed by staining with Stains-all.
(B) $^{45}Ca^{2+}$ overlay on a protein gel blot.
(C) Immunoblotting.
For Stains-all staining and the $^{45}Ca^{2+}$ overlay, each lane was loaded with 400 μg of total ovary protein extract. For immunoblotting, each lane was loaded with 50 μg of total ovary protein extract. The recombinant protein (0.5 μg) was included in immunoblotting. A polyclonal antibody raised against the recombinant protein was used at 1:5000 dilution. Immunodetection was conducted under high-stringency conditions. Lane M, molecular mass markers; lane R, recombinant protein; lanes O, unpollinated ovaries; lanes 1, ovaries 1 day after self-pollination; lanes 2, ovaries 2 days after self-pollination. The blue-staining band in **(A)** and the $^{45}Ca^{2+}$ binding band in **(B)** are indicated by arrowheads. Numbers at left indicate molecular mass in kilodaltons.

sequence variation in the introns and/or flanking regions. There are no restriction sites for DraI in the cDNA sequences, but multiple bands that are polymorphic among the five cultivars were detected by DraI. The different nucleotide sequences between CRH1 and CRH2 in the 5′ and 3′ regions were manifested by the different RFLP patterns detected by the two probes. The results of RFLP mapping showed that the extra major band detected by CRH1 is independent of the major band detected by both CRH1 and CRH2. The use of previously mapped markers in the two mapping populations of Steptoe × Morex and Dicktoo × Morex allowed us to place the two RFLP loci on the barley linkage maps.

The ribonuclease protection assays showed that the level of CRH1 transcripts was substantially elevated 1 day after pollination. Opas et al. (1991) and Michalack et al. (1991) reported that calreticulin was up-regulated in proliferating skeletal muscle myoblasts and down-regulated after cellular fusion and the formation of myotubes. In barley, the first division of the zygote occurs at 22 to 24 hr after pollination (Engell, 1988). An early study by Pope (1937) showed that the division of the endosperm started earlier than the zygote and that there were about eight endosperm cells 15 hr after pollination. If the elevation only occurred locally within the embryo sac, there must be extremely high transcription of the CRH1 gene because when compared to the whole ovary, the number of cells within an embryo sac is minimal at 24 hr after pollination. The elevation we observed was based on whole ovary extracts. Cell activity outside the embryo sac at this stage is largely unknown. In situ hybridization and more detailed time course studies are needed to localize the elevated calreticulin gene expression within the ovary.

Gene expression was apparently affected from the outset in the interspecific cross between *H. vulgare* and *H. bulbosum*, although there was virtually no difference in the growth rate between self-pollination and the interspecific cross during the first 4 days. The two pollination treatments differed in the genome of male gametes and thus the genome of fertilization products. The smaller initial elevation in the interspecific cross may have been a result of gene dosage and/or the partial incompatibility between the two species. The deficiency in gene expression after day 4 is likely a result of growth arrest.

METHODS

Plant Materials

Barley (*Hordeum vulgare*) cultivar Morex was grown under greenhouse conditions. Florets emasculated 2 to 3 days before flowering were pollinated either with pollen from Morex plants ("self-pollination") or with *H. bulbosum* Cb2924 (interspecific cross). For the control, florets were left unpollinated. All spikes were isolated with glassine bags. Growth rates were measured by the fresh weight (mg) per 100 ovaries or caryopses.

RNA Isolation and cDNA Library Construction

Total RNA was isolated using the procedures of guanidine thiocyanate and phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated from total RNA using the PolyATract system (Promega). Poly(A)$^+$ RNA (5 μg) from 3-day-old fertilized ovaries of Morex was used in cDNA synthesis using the λ ZAP cDNA synthesis kit (Stratagene). Size-selected cDNA was ligated to the Uni-ZAP XR vector (Stratagene) and then packed with the Gigapack (Stratagene) in vitro λ packaging system.

Differential Screening and DNA Sequence Analysis

Plaque lifts were prepared using Magna lift membranes (Micron Separations Inc., Westborough, MA). Membrane treatment and hybridization were performed as recommended by the manufacturer. A total of 20,000 plague-forming units from the primary cDNA library were screened with first strand cDNA probes synthesized with 0.5 μg of poly(A)$^+$ RNA from unpollinated ovaries and pollinated ovaries, respectively, using oligo(dT)$_{12-18}$ as primers. Selected plaques were isolated and subsequently converted to pBluescript SK− (Stratagene) by in vivo excision. Plasmid DNA prepared by the Magic minipreps (Promega) was sequenced by the dideoxy method of Sanger et al. (1977) on an autosequencer (ABI model 373A, version 1.2.0; Foster, CA). Each base was determined at least once on both DNA strands. Gaps were filled by primer walking. DNA sequence data were analyzed using the computer programs of the Genetics Computer Group, Inc. (Madison, WI).

Restriction Fragment Length Polymorphism Mapping

Restriction fragment length polymorphism (RFLP) mapping was conducted using the two doubled-haploid barley genome mapping populations derived from the F_1 population of Steptoe × Morex and Dicktoo × Morex, respectively. The population of Steptoe × Morex had 150 lines and 297 markers (Kleinhofs et al., 1993). The population of Dicktoo × Morex had 100 lines and 80 markers (P. Hayes and F. Chen, unpublished results). DNA extraction and hybridization were conducted as described by Chen et al. (1994). Genomic DNA (10 μg per lane) was restricted with DraI and probed with the CRH1 cDNA insert. Linkage analysis was performed using Mapmaker/Exp 3.0 (Lander et al., 1987). The recombination fraction was converted to centimorgans by the Kosambi function (Kosambi, 1944).

Ribonuclease Protection Assay

A subclone containing the first 316 bp from the 5′ end of the CRH2 cDNA insert in pBluescript SK− (Stratagene) was digested with EcoRI and then transcribed from the T7 promoter in the presence of α-^{32}P-UTP using a MAXIscript transcription kit (Ambion, Austin, TX). Full-length transcripts were isolated by gel purification. The radiolabeled antisense RNA was used to detect calreticulin transcripts in barley ovaries using a ribonuclease protection assay kit (Ambion). Protected fragments were separated in an 8 M urea polyacrylamide gel (0.75 mm thick, 5% acrylamide).

Expression of Recombinant Protein

A fragment encoding the last 370 residues of the predicted protein was cloned to the SacI site of the pQE30 (Qiagen Inc., Chatsworth, CA) expression vector. The construct was expressed in *Escherichia coli* host strain M15 (pREP4). Recombinant protein was purified through a 6 × histidine tag using Ni-NTA (nickle nitrilotriacetic acid; Qiagen Inc.) columns under the denaturing condition. Purification was conducted according to the protocol provided by the manufacturer. Purified recombinant protein was then analyzed by SDS-PAGE and stained with 1-ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-*d*]-thiazolium bromide (Stains-all) according to Campbell et al. (1993). The $^{45}Ca^{2+}$ binding assay was conducted as described by Maruyama et al. (1984). Membranes were washed with 50% ethanol for 5 min. Bovine calmodulin and chicken egg ovalbumin (Sigma) were used as positive and negative controls, respectively. Purified recombinant protein was used to raise polyclonal antibody in a rabbit.

Ovary Protein Analyses

Ovaries were ground to a fine powder in liquid nitrogen and then extracted with a buffer (150 mM Tris-HCl, pH 8.0, 10 mM KCl, and 1 mM EDTA). The homogenate was centrifuged at 14,000 rpm for 10 min, and the supernatant was collected for SDS-PAGE analysis. Protein concentration was determined by the method of Bradford (1976), using bovine γ-globulin as the standard (Bio-Rad). SDS-PAGE (1.5 mm thick, 12% acrylamide) was run at 12°C and 2.2 mA/cm. Gels were stained with Stains-all as described by Campbell et al. (1983). For $^{45}Ca^{2+}$ overlay and immunodetection, proteins were electroblotted to nitrocellulose membranes. $^{45}Ca^{2+}$ overlay was conducted as described by Maruyama et al. (1984). Membranes were stained with amido black after autoradiography to determine the band position. Immunodetection was performed under high-stringency conditions as described by Birkett et al. (1985). Polyclonal antibody raised against the recombinant protein was used at a 1:5000 dilution. Anti-IgG alkaline phosphatase conjugate (Promega) was used as the secondary antibody.

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