Transcriptional Induction of Two Genes for CCaPs, Novel Cytosolic Proteins, in Arabidopsis thaliana in the Dark

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Ca\textsuperscript{2+}-signaling in downstream effectors is supported by many kinds of Ca\textsuperscript{2+}-binding proteins, which function as a signal mediator and a Ca\textsuperscript{2+}-buffering protein. We found in Arabidopsis thaliana a new type of Ca\textsuperscript{2+}-binding protein, CCaP1, which consists of 152 amino acid residues, and binds 4Ca\textsuperscript{2+} even in the presence of a high concentration of Mg\textsuperscript{2+}. We found two other proteins with similar motifs, CCaP2 and CCaP3. These three proteins had no organelle localization signal and their green fluorescent protein (GFP) fusions were detected in the cytosol. Real-time PCR and histochemical analysis of promoter–β-glucuronidase fusions revealed that CCaP1 was predominantly expressed in petioles while CCaP2 was expressed in roots. CCaP3 was hardly expressed. Expression of CCaP1 and CCaP2 was enhanced in darkness and became maximal after 24h. Immunoblotting revealed petiole-specific accumulation of CCaP1. Expression of CCaP1 and CCaP2 was suppressed by a high concentration of Ca\textsuperscript{2+} and other metal ions. Deletion of sucrose from the medium markedly increased the mRNA levels of CCaP1 and CCaP2 within 2h. Gibberelic acid enhanced the expression of CCaP1 and CCaP2 by 5- and 2.5-fold, respectively, after 6h. CCaP1 and CCaP2 were suppressed in the petiole and the root, respectively, by light and the product of photosynthesis (sucrose) or both. These results suggest that CCaP1 functions as a mediator in response to continuous dark or gibberelic acid.

Keywords: Arabidopsis thaliana — Calcium — Dark — Gibberelic acid.

Abbreviations: CaBP, Ca\textsuperscript{2+}-binding protein; CCaP, cytosolic calcium-binding protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; GUS, β-glucuronidase; SA, salicylic acid.

Introduction

Among the second messengers that encode information and deliver it to downstream effectors, which decode signals and initiate cellular responses, Ca\textsuperscript{2+} serves as the most prominent signal carrier in both plants and animals. Calcium signaling is performed through spatial and temporal propagation of the cytosolic Ca\textsuperscript{2+} concentration, which is mediated by membrane transport systems for Ca\textsuperscript{2+}, such as the Ca\textsuperscript{2+} channel and Ca\textsuperscript{2+}-ATPase, and several kinds of calcium-binding proteins (CaBPs) (Sze et al. 2000, Sanders et al. 2002, Bouché et al. 2005). Also calcium signaling proteins, such as calcium-dependent protein kinases, serve as one of the Ca\textsuperscript{2+} sensor transducer proteins in plants (Dammann et al. 2003, Chehab et al. 2004). CalBPs have been reported as the Ca\textsuperscript{2+} transducer for various eukaryotes. Intracellular communication networks including CalBPs have evolved to convey information about a perceived stimulus to the cellular machinery responsible for mediating the specific response. Some other CalBPs with relatively low affinity and high capacity for Ca\textsuperscript{2+} play a role in maintaining a low resting free Ca\textsuperscript{2+} level in the cytosol.

Plants have various families of CalBPs, such as the EF-hand protein family, the endoplasmic reticulum (ER) luminal CaBP group and the annexin family. The EF-hand family is the largest group of CalBPs and most of its members function as ‘modulator’ proteins that regulate other proteins in a Ca\textsuperscript{2+}-dependent manner. Calmodulin, which contains EF-hand motifs, is a ubiquitous Ca\textsuperscript{2+} sensor protein of 16–18 kDa with no catalytic activity that can, upon binding Ca\textsuperscript{2+}, activate target proteins involved in various cellular processes. Plant cells have multiple calmodulin isoforms including a vacuolar luminal member (Zielinski 1998, Snedden and Fromm 2001, Yamaguchi et al. 2005). Calreticulin is a typical ER luminal CaBP and is functionally involved in Ca\textsuperscript{2+} storage and signaling, chaperone activity, cell adhesion and regulation of gene expression (Krause and Michalak 1997). Annexins have a characteristic ability to bind acidic phospholipid in a Ca\textsuperscript{2+}-dependent manner and play a role in essential cellular processes such as membrane trafficking, ion transport and cytoskeleton rearrangement (Seaton and Dedman 1998). Seven annexin genes have been identified in Arabidopsis thaliana (Clark et al. 2001).

Another acidic CaBP [vacuole Ca\textsuperscript{2+}-binding protein (RVCaB)] was recently identified in radish taproots (Yuasa and Maeshima 2000, 2001). RVCaB was characterized as having high capacity (19 Ca\textsuperscript{2+} mol\textsuperscript{-1}) and low affinity (K\textsubscript{d}, 3.4 mM) for Ca\textsuperscript{2+} and has been estimated to function as a Ca\textsuperscript{2+} buffer and/or Ca\textsuperscript{2+}-sequestering protein in the vacuole (Yuasa and Maeshima 2002).

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We found three predicted acidic proteins with some sequence similarity to RVCaB in a search of the A. thaliana database. These three proteins contained several glutamate-rich motifs, such as V(A)EExEK, which are also found in RVCaB. We determined the proteins AtCCaP1, AtCCaP2 and AtCCaP3 (A. thaliana cytosolic Ca2+-binding protein) (hereafter referred to as CCaP1, CCaP2 and CCaP3). Here we report the tissue- and cell-specific expression patterns, response of gene expression to physiological stimuli and light conditions, and intracellular localization of the proteins, especially of CCaP1 which was induced in darkness.

Results

Identification of CCaP1, CCaP2 and CCaP3 proteins

CCaP1 (DDBJ/GenBankTM/EBI locus ID, At1g62480) was found as a protein with some sequence similarity to RVcaB in a search of the Arabidopsis database. CCaP2 and CCaP3 were found as homologs of CCaP1. CCaP1, CCaP2 (At1g12080) and CCaP3 (At3g59370) are predicted to consist of 152, 138 and 95 amino acid residues, respectively. As shown in Fig. 1, the three proteins showed in common the abundance of glutamate (>30%), valine (>16%), threonine and lysine residues, and lack of cysteine, glycine, tyrosine, arginine and tryptophan residues. However, the overall sequence identity among the three sequences is relatively low (42%, 40/95). Charged residues comprise 41% of CCaP1, 44% of CCaP2 and 45% of CCaP3. CCaP1 and CCaP2 contain a characteristic VEEKK or VEEEEKK motif. Most parts of CCaP1, CCaP2 and CCaP3 were predicted to form helices by the method of Chou and Fasman (1974).

Protein sequence homology search by the UniProt/Swiss-Prot (http://www.ebi.uniprot.org/index.shtml) with a program of PAPIA (http://mbs.ebc.jp/papia-cgi/seqHS_menuJ.pl) gave several proteins with low similarity, such as calsequestrin, neurofilament triplet proteins (NF-H and NF-M) (Park et al. 2004) and a kiwi fruit protein KIWI501. However, CCaPs have no calsequestrin consensus motif [(E/Q)(D/E)GL(D/N)FPxYDGx DRV]. Neurofilaments are intermediate filament proteins with high molecular masses of 160 kDa (NF-M) and 200 kDa (NF-H), and their structures are different from that of CCaPs. KIWI501 was found in developing fruits of kiwifruit as a glutamate/alanine/proline-rich protein (Ledger and Gardner 1994), although its function is unknown. Therefore, the three CCaPs we identified are novel proteins whose function remains unknown.

Ca2+ binding activity of recombinant CCaP1

CCaP1 synthesized in Escherichia coli as a recombinant protein migrated as a protein of 33 kDa (Fig. 2A), which was higher than the molecular mass (18 kDa) predicted from the cDNA. Acidic proteins including RVCaB (Yuasa and Maeshima 2000) have been reported to migrate slowly on SDS–PAGE (Vorum et al. 1998). The antibody raised against the C-terminal sequence of CCaP1 clearly reacted against the purified protein at 31 kDa in the presence of a final ionic strength of 500 mM NaCl and in the absence of SDS or Triton X-100 (Fig. 2B). The high concentration of NaCl was effective for trapping CCaP1 on the transfer membrane. The 31 kDa immunostained band was not detected with the antibody pre-treated with a corresponding antigen peptide (Fig. 2B), indicating the specificity of the antibody to CCaP1.

The purified CCaP1 was clearly stained with the metachromatic cationic carbocyanine dye Stains-all (Fig. 2A), which has been used for identification of CaBPs (Campbell et al. 1983). Most of the CaBPs with acidic motifs to bind Ca2+ stain blue with Stains-all, whereas other proteins stain pink and the color fades away quickly in the light (Yuasa and Maeshima 2000). The blue staining pattern suggested that CCaP1 can bind Ca2+. This property of CCaP1 was revealed by 45Ca2+ overlay analysis (Fig. 2C) in the presence of 5 mM MgCl2 and 60 mM KCl followed by rinsing with 50% ethanol. The purified CCaP1 reproducibly gave a clear positive signal of 45Ca2+, demonstrating the Ca2+-binding capacity of CCaP1 in vitro. In this assay, calmodulin showed a positive signal and immunoglobulin no signal.
Distribution of CCaP1-like proteins among plants

To examine the presence of CCaP1 protein among plants, we tested the immunoreactivity of soluble fractions prepared from several plants. Soluble fractions from radish (*Raphanus sativa*), rose (*Rosa*), broccoli (*Brassica oleracea* var. *italica*), turnip (*Brassica rapa*) and Chinese cabbage (*Brassica rapa*, cv. Pekinensis) showed an immunostained band at 34 kDa with anti-CCaP1 antibody (data not shown). The result suggests that CCaP1 is a protein common to higher plants.

Cytosol localization of CCaP protein

To determine the subcellular localization of CCaP proteins, which are highly charged proteins with no predicted transmembrane domain, we prepared DNA constructs encoding fusion proteins of CCaP1, CCaP2 and CCaP3 with green fluorescent protein (GFP) and expressed them transiently in *Arabidopsis* roots (Fig. 3, left two panels) and suspension-cultured cells (Fig. 3, right two panels). The green fluorescence from GFP was clearly detected in the cytosolic space in both the root and suspension-cultured cells. In the control, the fluorescence of the cells expressing free GFP was dispersed throughout the cytosol and nucleus in both the root and suspension-cultured cells (Fig. 3A–C).

Organ- and cell-specific expression of CCaP genes

The mRNA levels of *CCaP1*, *CCaP2* and *CCaP3* were quantified by real-time PCR and compared in several organs (Fig. 4). *CCaP1* was expressed in all organs examined. In this experiment, the root contained the...
bottom part of hypocotyls. CCaP2 was expressed in shoot, root, stem, flower and silique, but was hardly expressed in the cauline leaf and rosette leaf. In contrast, CCaP3 was expressed predominantly in roots, and the mRNA level in other organs was 55% of that in roots. In the present experiment, we quantified the copy numbers of each transcript, although the data show the relative contents normalized with the 18S rRNA content. The amount of CCaP3 mRNA in whole plants was 51% of that of CCaP1 and CCaP2 when quantitated by semi-quantitative reverse transcription–PCR (RT–PCR).

The putative promoter regions of about 2 kb were fused in-frame with the β-glucuronidase (GUS) gene and transformed into Arabidopsis. Staining for GUS activity in 3-week-old plants of several independent lines revealed cell-specific expression of CCaP genes (Fig. 5). There was strong GUS activity for CCaP1 in the petiole and the main leaf veins (Fig. 5A–C) and weak GUS activity in the root. The GUS activity for CCaP2 and CCaP3 was predominantly detected in the root (Fig. 5D–I). There was strong GUS activity for CCaP2 in endodermis in the stele and for CCaP3 in cambium.

Immunoblotting of cytosolic fractions from petioles and other organs from 3-week-old plants with anti-CCaP1 antibody revealed the petiole-specific accumulation of the protein (Fig. 6). The immunostained band was clearly detected in petiole but not in roots or leaves, from which petioles and the main leaf vein were removed.

Changes in mRNA levels in response to light and darkness

We examined the time-dependent expression in a day/night (12 h/12 h) cycle by real-time PCR analysis of CCaP genes (Fig. 7). The mRNA levels of CCaP1 and CCaP2 were decreased during the day and increased at night, although the amplitude of fluctuations of CCaP1 and CCaP2 mRNAs was smaller than that of Chl a/b-binding protein. The CCaP3 mRNA level hardly changed.

The mRNA levels of CCaP1 and CCaP2, but not that of CCaP3, were markedly increased in the prolonged dark period and reached a maximum after 24 h in darkness. The maximum levels were 20-fold the basal level during the day and the high levels were maintained for 80 h. Unlike Chl a/b-binding protein, they did not show a circadian rhythm (Fig. 8D). The level of CCaP1 protein monitored by immunoblotting was increased during the dark period (Fig. 8E, F). Therefore, the transcript level of CCaP1 might be partly reflected in the protein level.

Changes in mRNA levels in response to salts and metals

The mRNA levels of CCaP1 and CCaP2 in whole plants were decreased by 60% by treatment with 50 mM CaCl2 for 2 h (Fig. 9A). CaCl2 showed a suppressive effect on the mRNA levels even at 5 mM. The suppressive effect of CaCl2 on the mRNA in CCaP2 was nullified at 18 h. The mRNA levels were examined in plants incubated for 48 h with CaCl2 or other metals at concentrations not affecting their growth (Fig. 9B). Unlike CaCl2, NaCl and KCl did not decrease the mRNA levels of three CCaPs. On the contrary, treatment with 100 mM NaCl increased the mRNA levels of CCaP1 and CCaP2 by 150 and 80%, respectively (Fig. 9B). Therefore, the suppression of transcription by CaCl2 might not be due to the ionic strength. Like calcium, magnesium, cadmium, manganese, nickel and zinc also down-regulated the three genes. Interestingly, CoCl2 at 0.1 mM did not show a suppressive effect.

The effect of deficiency of metals on the mRNA levels was also examined by changing the growth medium from the metal-containing medium (at 5 mM) to 0.5× MS medium without the corresponding metal. The mRNA levels of CCaP1 and CCaP2 were increased by 80% after
deletion of Mg\(^{2+}\) for 2 h (data not shown). The mRNA levels of CCaP1 and CCaP2 were relatively constant in the absence of Ca\(^{2+}\).

**Effect of sugars on mRNA levels**

Since carbon source nutrients affect the expression of many genes (Rolland et al. 2002), we examined the effect of four different sugars at a concentration of 150 mM (Fig. 10). Mannitol and sorbitol increased the mRNA level of CCaP1. The CCaP2 mRNA level was increased at 18 h of incubation with glucose, mannitol or sorbitol. The mRNA level of CCaP3 was markedly suppressed by the incubation with these sugars.

The mRNA levels of CCaP1 and CCaP2 were unaffected by sucrose at 150 mM (Fig. 10A). On the other hand, the CCaP1 mRNA was increased 2-fold and remained at a high level when sucrose was depleted from the medium. The CCaP2 mRNA level was increased by 60% at 2 h. The mRNA level of CCaP3 was markedly lowered by sucrose depletion.

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**Fig. 5** Expression profiles of CCaP promoter::GUS fusions in Arabidopsis. Plants expressing each promoter::GUS construct were grown for 21 d and then incubated in GUS staining solution for 12 h. (A and B) Whole plants of CCaP1 promoter::GUS. Two are different lines. (C) Cauline leaf. (D and E) Whole plants of CCaP2 promoter::GUS. (F) Section of a root. (G and H) Whole plants of CCaP3 promoter::GUS. (I) Section of a rosette leaf. Bars = 2 mm (A, B, D, E, G and H) and 100 μm (F and I).

**Fig. 6** Petiole-specific accumulation of CCaP1 in Arabidopsis. Cytosol fractions prepared from roots (lane 1), petioles (lane 2), leaves without a petiole (lane 3) and leaves with a petiole (lane 4) of 3-week-old plants were subjected to immunoblotting with anti-CCaP1 antibody. Recombinant CCaP1 was applied as the positive control (lane 5). The applied amount of the cytosol fraction was 10 μg. The left-hand lane shows molecular sizes (kDa) of the standard proteins.
Response to physiological and physical stimuli

Among the three phytohormones examined, only gibberellic acid increased the mRNA levels of CCaP1, CCaP2 and CCaP3 (Fig. 11). In particular, treatment with gibberellic acid for 6 h increased the transcript level of CCaP1 4-6-fold. Salicylic acid (SA) and ABA showed a suppressive effect on the mRNA level of CCaP3.

The flagellin oligopeptide flg22, which is the most highly conserved region in the N-terminus of eubacterial flagellin (Felix et al. 1999), induces pathological responses in Arabidopsis and other plants (Nuhse et al. 2003, Zipfel et al. 2004). Treatment with the flagellin peptide showed no effect on CCaP1, but decreased the CCaP2 and CCaP3 mRNA levels.

The transcript level of CCaP1 was unaffected by the transfer of 3-week-old plantlets grown at 22°C to either 36 or 4°C, but the mRNA levels of CCaP2 and CCaP3 were increased 2-fold 6 h after transfer to 4°C. Dehydration showed no effect on the three genes.

Discussion

We identified CCaP1 as a Ca2+-binding acidic protein from Arabidopsis. The recombinant CCaP1 protein bound Ca2+ even in the presence of Mg2+ (Fig. 2). CCaP1 and two other proteins, CCaP2 and CCaP3, with a sequence similar to that of CCaP1 lacked putative localization signals for organelles, such as ER, mitochondrion, plastid, vacuole or nuclei. Three CCaPs were strongly suggested to be localized to the cytosol by sequence analysis by PSORT (http://psort.ims.u-tokyo.ac.jp/). The present study expressing GFP-tagged proteins in Arabidopsis roots and cultured cells shows the cytosolic localization of three CCaPs (Fig. 3). CCaP1 was detected only in the cytosolic fraction (100,000 x g, supernatant) but not in any membrane fractions or intracellular organelles by immunoblotting. Thus we estimate that the CCaP functions in the cytosol in Arabidopsis cells. The three CCaPs are rich in glutamate residues, the main acidic residue, and have a relatively high content (≈9%) of valine, threonine and lysine residues (Fig. 1).

The abundance of glutamate residues supports the helical structure of polypeptides as estimated by the Chou–Fasman method (1978). However, CCaP1, CCaP2 and CCaP3 were suggested to be unstructured (unfolded) proteins by sequence analyses by FoldIndex (http://bip.weizmann.ac.il/fldbin/index) (Prilusky et al. 2005) and DisEMBL 1.4 (http://dis.embl.de/) (Linding et al. 2003). The N- and C-terminal tails are highly conserved among CCaPs, and the three proteins have three or four EEEK motifs (variant, EEEK) individually. These EEEK and EEEK motifs and/or the other glutamate-rich regions might be
responsible for binding of Ca\(^{2+}\). The other candidates for Ca\(^{2+}\) binding are several double-glutamate-containing parts in CCaP1, such as DEE and TEE. The three CCaPs commonly lacked cysteine, glycine, tyrosine, arginine and tryptophan residues. The only known protein with such a unique sequences is RVCaB (Yuasa and Maeshima 2000). The properties found in the primary sequences of CCaPs are similar to those of RVCaB, and the amino acid identity
between RVCaB and CCaP1 is 63% (96 in 152 residues). Recently, RVCaB has been suggested to be a naturally unstructured protein (in other words, an intrinsically unstructured protein) by biochemical and physical methods (unpublished data). The tertiary structure of CCaP1 remains to be determined by crystallography and/or biochemical methods. Structural information might provide insight into the biochemical mechanism of the calcium binding.

Expression profiles of CCaP genes were examined by real-time PCR (Fig. 4) and the promoter–GUS assay in Arabidopsis plants (Fig. 5). The CCaP1 mRNA was found in the shoot and root of 3-week-old plants in RT–PCR analysis. The promoter–GUS analysis revealed that CCaP1 is predominantly expressed in the petiole of 3-week-old plants and slightly expressed in the root. High expression was observed around the petiole, such as the shoot apex, hypocotyl and leaf main vein. Immunoblot analysis revealed specific accumulation of CCaP1 in petioles (Fig. 6). CCaP1 was scarcely detected in the root or in the leaf with the main vein removed. Thus, the present study revealed the petiole-specific expression and accumulation of CCaP1. In contrast to CCaP1, CCaP2 is expressed in the root and the basal part of the stem (Figs. 4, 5) whereas CCaP3 is expressed specifically in the root. The results of the semi-quantitative analysis of mRNA levels were roughly consistent with those of the promoter–GUS assay. Thus, we concluded that each CCaP is expressed and accumulated in the cytosol in a tissue-specific manner.

The most interesting finding is that CCaP1 and CCaP2 genes, but not CCaP3, were markedly induced by a dark period of >9.5 h (Fig. 8) although the mRNA levels of CCaP1 and CCaP2 were only moderately enhanced during the dark period in a day/night cycle (Fig. 7). The mRNA levels increased continuously during the long dark period, reaching a maximum after 24 h in darkness, thus without a diurnal fluctuation.

Expression of CCaP1 was suppressed when plants were exposed to high concentrations of CaCl₂ (Fig. 9) although CCaP1 has a Ca²⁺-binding capacity. This is not due to a high concentration of Cl⁻, since NaCl treatment increased the mRNA level of CCaP1. The negative response of CCaP1 to the exogenous Ca²⁺ is in contrast to that of RVCaB (Yuasa and Maeshima 2000) and suggests that CCaP1 may not be a ‘buffer’ protein for Ca²⁺. The low protein content of CCaP1 in the petiole estimated by the immunoblot analysis may also remove the possibility of a Ca²⁺-buffering function. Biochemical analysis of the kinetics of Ca²⁺ binding is required for understanding the physiological meanings of the association of CCaP1 with calcium ions in plants.

The depletion of sucrose from the growth medium enhanced the CCaP1 mRNA level (Fig. 10). The CCaP2 mRNA also increased moderately under the same conditions. Treatment with gibberellic acid, but not with SA or ABA, increased the mRNA levels of CCaP1 and CCaP2 by 500 and 150%, respectively (Fig. 11).
It is not clear whether or not the endogenous content of gibberellic acid increases in Arabidopsis in a long dark period. In pea seedlings, the higher growth rate in a long dark period could be due to increased responsiveness to gibberellic acid in the dark, because there is no evidence for up-regulation of genes for gibberellic acid biosynthesis (Sponsel and Hedden 2004). Thus, darkness for 4–12 h may increase the endogenous level of gibberellic acid or the sensitivity of the target genes in the petiole and the root of Arabidopsis. In general, the sucrose level in plant tissue is low at night. Therefore, the stimulation of gene expression under sucrose-depleted conditions may be physiologically related to the marked increase in the mRNA level of CCaP1 and CCaP2 in darkness.

In conclusion, of the three CCaPs we found, CCaP1 was a novel CaBP whose primary sequence was different from that of known CaBPs. In Arabidopsis, petioles rapidly extend under conditions of a long dark period as a shade avoidance response. Genetic analyses of Arabidopsis have indicated that phytochromes (PhyA, PhyB, PhyD and PhyE) play a predominant role in mediating the shade avoidance responses (Quail et al. 1995, Brutnell 2006). CCaP1 may be
related to this shade avoidance response in Arabidopsis. Further studies are needed on the kinetic properties of CCAp1 to Ca2+, and the physiological phenotypes of gene-deleted mutants of CCAp1 and CCAp1-overexpressing mutants in relation to phytochromes.

Materials and Methods

Plant materials

Seeds of A. thaliana (Col-0) were germinated on sterile gel plates containing Murashige–Skoog (MS) salt, 2.5 mM MES-KOH (pH 5.7), 2% (w/v) sucrose and 0.25% Gellan gum (Wako Pure Chemical Ind., Osaka, Japan) (standard medium, 1x MS-sucrose) at 22°C under continuous light (90 μmol m⁻² s⁻¹). The effects of metal ions, sugars and phytohormones were examined on sterile gel plates containing 0.5x MS-sucrose supplemented with the corresponding metal. For examination of phytopathological responses, plantlets were treated with fgl22 SA ABA GA3 in the air at saturated humidity. Oragns or whole plants frozen in liquid nitrogen were homogenized with a mortar and pestle. RNA extracted using an extraction kit (Clontech, Mountain View, CA, USA) was treated with DNase I and an RNase-free DNase Set (Qiagen, Valencia, CA, USA). Protein concentration was determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples were subjected to 40 cycles of PCR (94°C, 10 s incubation). Standard plasmids that contained a sequence for the most highly conserved region in the N-terminal (QRLSTGSRNSAKDDAAQLGQA) of eubacterial flagellin (Felix et al. 1999, Kobae et al. 2006). In some cases, seeds germinated on agar plates were grown in vermiculite in pots under continuous light. Arabidopsis (Col-0) suspension-cultured cells (also called ‘Deep’ cells; obtained from Dr. Masaaki Umeda of the University of Tokyo, Japan) were cultured in MS medium at 22°C in the dark.

Preparation of antibodies and immunoblotting

For antibody production, we synthesized a peptide corresponding to the C-terminal region of Arabidopsis CCAp1 (positions 139–152, VEASVTAPVEKAD) (Fig. 1, overlined sequence). The peptide was linked with carrier protein (keyhole limpet hemocyanin) and injected into rabbits. The antibody and peptide were prepared by Operon Biotechnologies (Tokyo, Japan). Protein samples were subjected to SDS-PAGE and immunoblotting. The blots were visualized with horseradish peroxidase-coupled protein A and Western blotting detection reagents (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Protein concentration was determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, CA, USA). Cytosolic fractions were prepared from 3-week-old plants. Tissue homogenates were centrifuged at 10,000×g for 20 min and the supernatants were centrifuged at 104,000×g for 30 min. The obtained supernatant was concentrated by adding trichloroacetic acid at a final concentration of 15%. The precipitate was rinsed with cold ethanol, suspended in SDS solution, and then immunoblotted together with recombinant CCAp1 protein.

RNA preparation and mRNA quantification

Organs or whole plants frozen in liquid nitrogen were subjected to 40 cycles of PCR (94°C, 10 s incubation). Real-time PCR analysis was performed with an iCycler iQ Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using the iQ SYBR Green Supermix (Bio-Rad) as described previously (Kamiya et al. 2005). Primer sets used for real-time PCR were: 5’-CGGTAAACAGATCTCAACCGG-3’ (forward) and 5’-CTTCCGGAGTTTATCACTTGA-3’ (reverse) for CCAp1; 5’-CCAAAACAGACGTGAGAAGA-3’ (forward) and 5’-TTTCTACATCGTGGTCTCCGGA-3’ (reverse) for CCAp2; 5’-TCCCTTTCTTCTGTTGGAGAG-3’ (forward) and 5’-TACGACTACAACTATGTTCAT-3’ (reverse) for CCAp3; and 5’-CGGCTACCACATCCAAAGGAA-3’ (forward) and 5’-TGCTGAAATTTCCGCGGCT-3’ (reverse). The specificity of these primers was confirmed by PCR. Sample mixtures incubated for 30 s at 94°C were subjected to 40 cycles of PCR (94°C, 30 s; 54.3–63°C, 30 s; 72°C, 30 s), and heated at 55–94.5°C with a 0.5°C interval (10s incubation). Standard plasmids that contained a sequence of each CCAp were prepared. The standard curves for each CCAp was generated by using these standard plasmids and gene-specific primers. The copy numbers of the products were calculated from the threshold cycles of triplicate real-time PCR assay using the standard curves. Relative mRNA contents were normalized with the 18S rRNA content. Values are expressed as means±SD calculated for three assays.

Fig. 11 Effects of phytohormones, flagellin oligopeptide, dehydration, and high and low temperatures on mRNA levels of CCAps. Plants grown for 3 weeks under continuous light in 0.5x MS-sucrose were treated for 6 h with 1 μM flagellin oligopeptide (flg22), 100 μM salicylic acid (SA), 100 μM ABA or 100 μM gibberellic acid (GA3) in the air at saturated humidity. Plants were incubated for 6 h at 36°C (high temperature) or 4°C (low temperature). mRNA levels were determined and expressed as a ratio to the control value at time 0.
Transient transformation of Arabidopsis roots and cultured cells with GFP-tagged CCaPs

GFP fusion proteins were constructed by amplifying cDNAs for CCaP1, CCaP2 and CCaP3 by PCR using the primer sets as follows: 5'-CACCATGGCCACCATCGAGGTGTT-3' (forward) and 5'-CTCATAGCTTCTCTCTACTGTG-3' (reverse) for CCaP1; 5'-CACCATGGCAGGGCTGTTAGGTTG-3' (forward) and 5'-CTCTCGGTTTCTCTACAC-3' (reverse) for CCaP2; and 5'-CACC ATGGCCACCATCGAGGTGTT-3' (forward) and 5'-CTCTGGT GCTTCCTGCAC-3' (reverse) for CCaP3. The resulting fragments were inserted into pENTR/D-TOPO (Invitrogen) followed by the destination vector pGWB5 or New-pUGW5 (developed by Dr. Tsuyoshi Nakagawa, Shimane University, Japan) to generate a fusion construct with GFP at the C-terminus of CCaP1, CCaP2 or CCaP3.

For transient expression in roots, 1 μm gold particles coated with the GFP-CCaP fusion construct were bombarded onto Arabidopsis roots at a distance of 6 cm using an IDERA Particle Gun (Tanaka Co., Sapporo, Japan) at 600 mHg. Transformed roots were stored in the dark overnight at 23°C. Procedures used for expression in Arabidopsis suspension-cultured cells (Deep cells) were as described previously (Kobae et al. 2004, Ishikawa et al. 2005). The GFP fluorescence was visualized with a Fluoview FV500 confocal laser scanning microscope (Olympus).

Promoter–GUS constructs and histochemical analysis

Putative promoters of CCaP1 (−1,641 to +12 from the predicted start codon), CCaP2 (−1,711 to +12) and CCaP3 (−1,911 to +15) were amplified from genomic DNA. The fusion product was produced by ligating DNA fragments with the predicted start codon), Promoter–GUS constructs and histochemical analysis

FV500 confocal laser scanning microscope (Olympus).

Calcium-45 overlay assay

Purified preparations of IgG, calmodulin and CCaP1 were blotted onto a poly(vinylidene difluoride) membrane using a slot blot apparatus (Bio-Rad). The membrane sheet was washed twice with 10 mM MES-KOH, pH 6.5, 5 mM MgCl2 and 60 mM KCl; incubated in the same buffer (1 ml) supplemented with 1 mM CaCl2 and 3.7 MBq of 45Ca2+ (as CaCl2) at 25°C for 30 min; washed twice in 10 ml of 50% (v/v) ethanol; and dried at room temperature. An autoradiogram of the 45Ca2+-labeled proteins on the membrane was obtained by exposure to an X-ray film for 3 d at −80°C.

Acknowledgments

We are grateful to Dr. Tsuyoshi Nakagawa (Shimane University, Japan) for providing the Gateway vectors New-pUGW5 and pGWB203 that he developed, to Dr. Masaaki Umeda (University of Tokyo, Japan) for his gift of A. thaliana ‘Deep’ cells, and to Dr. Yoichi Nakanishi for his valuable advice. This work was supported by Grants-in-Aid for Scientific Research 18380064, 16085204 and 14COEA02 (to M.M.) from the Ministry of Education, Sports, Culture, Science and Technology of Japan.

References


(Invitrogen) and then ligated into the pET23b expression vector (Novagen, Madison, WI, USA). After confirmation of the nucleotide sequences, the expression vector was introduced into E. coli BL21(DE3) (Novagen). Transformants were grown in LB broth supplemented with 50 mg ml−1 ampicillin for 3 h at 30°C after induction with 0.5 mM isopropylthio-β-D-galactopyranoside.

Cells were harvested by centrifugation and suspended in 20 mM Tris-acetate (pH 7.5) containing 20% (v/v) glycerol, 0.2 mg ml−1 DNase, 0.4 mg ml−1 lysozyme, 10 mM 2-mercaptoethanol and protein inhibitor cocktail (0.5× Complete, EDTA-free) (Roche Applied Science, Mannheim, Germany). The cells were disrupted by sonication for 12.5 min on ice. After removal of cell debris by centrifugation at 104,000×g for 30 min, the supernatant was applied to a Hi Trap Q Sepharose HP column (GE Healthcare, 5 ml) equilibrated with 20 mM Bis-Tris-acetate (pH 7.5), 1 mM dithiothreitol (DTT) and 5% (v/v) glycerol, and then eluted with an NaCl concentration gradient (0–150 mM). The peak fractions of CCaP1 were collected and applied to a Hi Load Superdex 75 column (GE Healthcare, 1.6 × 60 cm) equilibrated with 20 mM Bis-Tris-acetate (pH 7.5), 1 mM DTT, 150 mM NaCl and 5% (v/v) glycerol.

T1 plants for the GUS analysis were incubated with 90% (v/v) acetone for 30 min at −20°C and then washed with 100 mM phosphate buffer (pH 7.0). The tissues were infiltrated with a solution containing 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 20% (v/v) methanol and 0.3% (w/v) Triton X-100 under vacuum for 30 min, and then incubated at 37°C until blue histochemical staining was confirmed (6–24 h). The stained tissues were incubated sequentially in 30, 50 and 70% ethanol for 1 h, dehydrated, embedded in Histo Resin Plus (Leica, Heidelberg, Germany) and sectioned with a microtome. Root sections were counter-stained with 0.01% safranine O (Waldeck GmbH & Co. KG, Muenster, Germany) before observation.

Preparation of recombinant CCaP1

cDNA for CCaP1 was amplified by PCR with the primers 5'-GGAATTCACAATGGCCACCATCGAGGTGTT-3' (forward) and 5'-ATAGTTAGCCGCGCTGCTCATACGCTTCCTCCAC TG-3' (reverse; EcoRI and NotI sites are underlined) using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA fragment was inserted into a plasmid vector pZErO™-2

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(Received September 18, 2006; Accepted November 14, 2006)