Novel Plant Ca$_{2+}$-binding Protein Expressed in Response to Abscisic Acid and Osmotic Stress*

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A cDNA corresponding to an mRNA which accumulates in germinating rice seeds in response to the phytohormone abscisic acid was isolated by differential hybridization. Northern blotting indicated that the mRNA also accumulates in vegetative tissues in response to treatment with abscisic acid and to osmotic stress. Sequencing identified a major open reading frame encoding a novel protein of 27.4 kDa. The identity of the open reading frame was confirmed by comparing the translated RNAs and by immunoprecipitation. Western blotting of cellular extracts indicated that the protein is associated with microsomal or membrane fractions. Data base searches indicated that it contains a conserved Ca$_{2+}$-binding, EF-hand motif and that related proteins are similarly expressed in Arabidopsis thaliana. A fusion protein purified from Escherichia coli contains the putative EF-hand region was shown to bind Ca$_{2+}$ in blot binding assays. These data identify a novel gene family encoding proteins involved in the response of plants to abscisic acid and osmotic stress.

Plants growing in many environments must respond and adapt to osmotic stress caused by drought, salinity, and temperature extremes. Stress tolerance involves changes in gene expression and solute metabolism (1–4). The complexity of these responses indicates that they may be mediated by multiple signaling pathways, one of which is modulated by the phytohormone abscisic acid (ABA) (5, 6). Although varied, plant responses to ABA indicate that the hormone acts as an osmoregulatory signal which inhibits normal growth, and potentiates stress tolerance or adaptation (reviewed in Refs. 7 and 8). For example, ABA levels rise developmentally during late embryogenesis and enhance dormancy and desiccation tolerance (9, 10). Similarly, osmotic stress in vegetative tissues leads to increased ABA levels which regulate stomatal closure, thereby reducing transpiration, and which promote the expression of genes involved in stress tolerance (11, 12).

Molecular studies of ABA action focus on the mechanism(s) by which the hormone regulates specific gene expression and on the functions of these ABA-responsive genes. Two specific components in the ABA signaling pathway(s) are known. One is a transactivator which regulates ABA-responsive genes in seed tissues (13, 14), perhaps in concert with basic leucine zipper proteins binding to a G-box element (15, 16). The other encodes a Ca$_{2+}$-modulated, protein phosphatase 2C which may integrate ABA and Ca$_{2+}$ signals with phosphorylation-dependent responses in seed and vegetative tissues (17, 18). The involvement of this phosphatase supports evidence indicating that Ca$_{2+}$ acts as a messenger in ABA mediated processes (19, 20).

The contribution of downstream, ABA-responsive genes to an integrated set of physiological responses to ABA or osmotic stress remains obscure, in part because many of these genes encode proteins of unknown function. Some of them may function as molecular chaperones during dehydration (21), in osmolyte metabolism (22), or in regulatory processes such as nuclear protein trafficking (23). We describe here another family of ABA-responsive genes encoding novel proteins with an EF-hand, Ca$_{2+}$-binding domain (24). The properties and patterns of regulation of these proteins appear to be similar among distantly related species, indicating that they are involved in the response of plants to ABA and osmotic stress.

**EXPERIMENTAL PROCEDURES**

Isolation and Sequence Analysis of the cDNA and Encoded Protein—Methods used in cDNA library construction and differential screening have been described (25). Both strands of the cDNA were sequenced with a commercial sequencing protocol and reagents (U.S. Biochemical Corp., Sequenase version 2.0) using primers synthesized on an Applied Biosystems model 380A. Sequences in DNA and protein data bases were searched for similarity to the cDNA and encoded protein using programs of the University of Wisconsin GCG-package (26–28). Two secondary structure prediction algorithms were also used (29, 30).

**RNA Blot Analysis—**Conditions for the growth and treatment of rice (Oryza sativa, var. Indica, cv. IR64) and for the isolation of total RNA have been described (25, 31). RNAs were separated in formaldehyde gels, blotted to nitrocellulose, and hybridized to the random-primed cDNA probe labeled according to the Mega-Prime kit (Amersham Corp.). Replicate gels were stained with ethidium bromide to control for equal loading of RNA.

In Vitro Protein Synthesis and SDS-PAGE—Isolation of poly(A) RNA, in vitro translation using reticulocyte lysate and immunoprecipitation with protein A-Sepharose have been described (32). In vitro transcription from the cDNA was performed according to the T7 Message Machine kit (Ambion). Hybridization selection of poly(A) RNA was performed as described previously (32, 33). SDS-PAGE utilizing 12.5% gels was performed (34).

Production of Recombinant Proteins, Western and Ca$_{2+}$-binding Blots—The open reading frame was synthesized using a 5’ BamH I linker-primer (5’-TATAGGATCCTATGGCGGAGGCGGCGTAGC) and a 3’ EcoRI linker-primer (5’-TATAGATTCTACTTGCCTTCTCATTGTC) by PCR containing 20% glycerol (35). This fragment was directionally cloned in frame at the C terminus of glutathione S-trans-
from ABA-treated, rice seedling mRNA. Sequencing revealed isolated by differential screening of a cDNA library constructed Tricine (pH 7.9), 50 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 5 mg/ml HEPES, 10% dithiothreitol, and 5 μM Ca2⁺. 45Ca2⁺ was produced as negative and positive between MBP and the hippocalcin EF-hand 1 (37) were similarly processed (New England Biolabs). pMal-2c-encoded MBP and a fusion 0.3 mM IPTG and incubated for 2.5 h. The fusion protein was purified by chromatography, 2 and used as a control protein on Western blots. Hormone and Osmoregulated Plant Protein with EF-hand— Figure 1. Sequence of the cDNA and encoded protein. The start (nucleotide 84) and stop (nucleotide 816) codons and a putative polyadenylation signal (1017) are underlined. that the cDNA contains a major, GC-rich (57%) open reading frame (ORF) of 732 nucleotides (84–815) encoding a protein of 244 amino acids (Fig. 1). Flanking the ORF is an 83-nucleotide 5'-untranslated region (43%) lacking in-frame stop codons, and a 236-nucleotide 3'-untranslated region (37%) containing a putative polyadenylation sequence AATAAA 30 nucleotides upstream of the site of poly(A) addition. Primer extension (data not shown) indicated that the G nucleotide denoted +1 corresponds to the 5' end of the mRNA, and that the cDNA is full length. The longest ORF encodes a protein with a molecular mass of 27.4 kDa. We call this protein EFA27 (EF-hand, abscisic acid responsive, see below). Two lines of evidence indicate that it is 27.4 kDa. We call this protein EFA27 (EF-hand, abscisic acid responsive, see below). Two lines of evidence indicate that it is transcribed in embryos, but not to significant levels in endosperm, during late embryogenesis (Fig. 2A, lanes 1–6). However, it accumulated if 200 mM salt was added to the hydroponic medium or if the plants were removed from the medium and air-dried (Fig. 2A, lanes 8–11).

Two lines of evidence indicate that EFA27 mRNA also accumulates in response to the phytohormone ABA in embryo and/or seedling tissues. First, the cDNA was isolated by differential screening for ABA-induced mRNAs from a library con-

RESULTS

Isolation of the Rice cDNA— A 1051-base pair cDNA was isolated by differential screening of a cDNA library constructed from ABA-treated, rice seedling mRNA. Sequencing revealed

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FIG. 1. Sequence of the cDNA and encoded protein. The start (nucleotide 84) and stop (nucleotide 816) codons and a putative polyadenylation signal (1017) are underlined. that the cDNA contains a major, GC-rich (57%) open reading frame (ORF) of 732 nucleotides (84–815) encoding a protein of 244 amino acids (Fig. 1). Flanking the ORF is an 83-nucleotide 5'-untranslated region (43%) lacking in-frame stop codons, and a 236-nucleotide 3'-untranslated region (37%) containing a putative polyadenylation sequence AATAAA 30 nucleotides upstream of the site of poly(A) addition. Primer extension (data not shown) indicated that the G nucleotide denoted +1 corresponds to the 5' end of the mRNA, and that the cDNA is full length. The longest ORF encodes a protein with a molecular mass of 27.4 kDa. We call this protein EFA27 (EF-hand, abscisic acid responsive, see below). Two lines of evidence indicate that it is 27.4 kDa. We call this protein EFA27 (EF-hand, abscisic acid responsive, see below). Two lines of evidence indicate that it is transcribed in embryos, but not to significant levels in endosperm, during late embryogenesis (Fig. 2A, lanes 1–6). However, it accumulated if 200 mM salt was added to the hydroponic medium or if the plants were removed from the medium and air-dried (Fig. 2A, lanes 8–11).

Two lines of evidence indicate that EFA27 mRNA also accumulates in response to the phytohormone ABA in embryo and/or seedling tissues. First, the cDNA was isolated by differential screening for ABA-induced mRNAs from a library con-
structured with poly(A) RNA from ABA-treated, germinating embryos (25). Second, in vitro translation experiments indicated that EFA27 mRNA accumulated in germinating embryos following ABA treatment (Fig. 3, see below). To determine whether the expression of the mRNA could also be induced by ABA in vegetative tissues, the hormone was added to the hydroponic medium at a final concentration of 50 μM. Fig. 2B (lanes 1–8) indicates that the mRNA accumulated some 20–30-fold in both roots and shoots after 6 h of treatment. EFA27 mRNA accumulated in shoots for up to 48 h, although its accumulation in roots was transient and was not detectable after 24 h (Fig. 2B, lanes 4–7).

In conclusion, the Northern blot data indicated that EFA27 accumulates in tissues in response to osmotic stress and ABA. Its pattern of expression is therefore similar to that of RAB16, a member of another family of ABA-responsive genes (25, 41).

Confirmation of the EFA27 Open Reading Frame—Hybridization selection of cellular poly(A) RNA with the cDNA followed by in vitro translation indicated that several related mRNAs accumulate in rice (Fig. 3, lanes 1–3). These mRNAs encode proteins of approximately 29, 27, 25, and 24 kDa based upon their relative electrophoretic mobilities. The 27-kDa polypeptide was apparently encoded by EFA27 mRNA, as shown by its comigration with a polypeptide produced by in vitro transcription and translation of the EFA27 cDNA (Fig. 3, lanes 3 versus 4). This indicated that the 27-kDa polypeptide was not derived from the larger one of 29 kDa and confirmed the identification of the EFA27 ORF. Further confirmatory evidence was provided by raising antibodies against a fusion product in vitro. The resulting antisera specifically recognized the expected 27-kDa polypeptide synthesized in vitro from total poly(A) RNA isolated from ABA-treated seedlings (Fig. 4, lanes 1–4). This polypeptide was not detected among total products of RNAs from untreated seedling. This confirmed that EFA27 mRNA accumulated in response to ABA, as did mRNA encoding RAB16 used as a positive control (Fig. 4, lanes 5 and 6 (25)).

Characteristics of EFA27 and Identification of a Ca²⁺-Binding Domain in It and in Similar Proteins—DNA and protein data bases were searched for sequences with similarity to EFA27. Comparison with entries of the Prosite data base indicated that the EFA27 C-terminal half contains five casein kinase 2 phosphorylation site signatures (Ser¹⁴⁵, Ser¹⁹⁷, and Ser²²⁷ and Thr¹⁶⁸ and Thr¹⁷⁴) and a tyrosine kinase signature (Tyr¹⁴⁷) (42).

The BLAST algorithm (27) identified several partial cDNAs from Arabidopsis thaliana encoding proteins with similarity to EFA27. While we have yet to confirm these cDNA sequences, it is unlikely that the correction of potential inaccuracies in them would significantly decrease similarities between the proteins they encode and EFA27. An alignment between them and EFA27 is shown in Fig. 5A. The N-terminal region of EFA27 is most similar to Z27053, while the two threonine sites (Thr¹⁶⁸ and Thr¹⁷⁴) are not overlapped by the Arabidopsis cDNAs.

The data bases were then searched for sequences similar to regions of EFA27 with the greatest similarity to these Arabidopsis cDNAs. The region Ser⁹⁶–Tyr⁹⁸ was thereby found to contain the EF-hand consensus sequence, a conserved Ca²⁺-binding motif (24, 28). Fig. 5B shows an alignment of EFA27 with the Arabidopsis sequences, EF-hand 1 of Arabidopsis calmodulin 3 (43), and the EF-hand consensus (24). A secondary structure algorithm (29) predicted the conserved EF-hand, helix-turn-helix/loop-helix structure for all of these sequences.

The ability of the EFA27 EF-hand domain to bind ⁴⁵Ca²⁺ was tested in a protein blot assay. To this end, EFA27 residues
51-103 were fused in frame with the E. coli MBP, and the ability of the fusion protein to bind $^{45}$Ca$^{2+}$ was examined following affinity purification from E. coli extracts, SDS-PAGE, and protein blotting (38). Fig. 6A shows the Coomassie-stained pattern following SDS-PAGE of 0.5 and 5 μg of MBP (lanes 1 and 2, negative control), MBP/EFA27 EF-hand (lanes 3 and 4), and of MBP/hippocalcin EF-hand 1 (lanes 5 and 6, positive control) (37). Fig. 6B shows that, as expected, $^{45}$Ca$^{2+}$ was not bound by MBP, but was bound by both of the EF-hand fusion proteins. This suggests that the EF-hand sequence of EFA27 and presumably those encoded by the Arabidopsis cDNAs bind Ca$^{2+}$ under physiological conditions.

The apparent insolubility of the heterologous GST/EFA27 fusion protein in E. coli could be due to the accumulation of improperly folded or aggregated recombinant protein. Alternatively, hydrophobic regions of EFA27 could make the fusion protein insoluble. The sequence of EFA27 was therefore analyzed with algorithms which predict hydropathy and secondary structure. Two secondary structure algorithms predicted that residues Leu96–Tyr116 may form a $\alpha$-helical, trans-membrane domain (29, 30). To examine the possibility that EFA27 is associated with membranes, antibodies against GST/EFA27 were used to probe cell extracts and fractions prepared from ABA-treated quarter-seeds and seedling leaves (Fig. 7A). These experiments detected EFA27 among total, SDS-solubilized proteins from ABA-treated quarter-seeds (Fig. 7A, lane 2). It was not detected in a supernatant protein fraction solubilized with an aqueous buffer, but was detected among the insoluble proteins pelleted by centrifugation (Fig. 7A, lanes 3 and 4). In extracts of ABA-treated seedling leaves, EFA27 was detected in the homogenate (lane 5) and in a fraction derived from it containing microsomal membranes (lane 7), but not in the soluble fraction (lane 6). As a control, the same fractions were probed with an antibody against RAB16, a soluble, ABA-responsive protein (25). As expected, RAB16 was detected in total and soluble fractions of quarter-seeds and leaves (Fig. 7B, lane 3 and 6), but not in insoluble or microsomal membrane fractions (Fig. 7B, lanes 4 and 7). These experiments suggest that EFA27 is not a soluble, cytosolic protein and that it may associate in vivo with membranes or with membrane-associated proteins.

**DISCUSSION**

We are studying the molecular mechanism(s) of action of the plant hormone ABA. Part of this work entails the characterization of ABA-responsive genes with the aim of understanding the functions of the proteins they encode. Here we present the characterization of a rice cDNA isolated by differential expression of the encoded sequence tags. In EMBL data bases expressed sequence tags: EMBL account no. Z27503 (ATTS1925), Z17677 (ATTS0251), Z47401 (ATTS4423), and Z29900 (ATTS2344). Residues identical or conserved between EFA27 and one other sequence are identified, and the consensus is given for residues showing identity with EFA27. EFA27 and Z29900 stop codons are noted with an asterisk. (B) comparison of the EF-hand motif in EFA27 with related proteins. EMBL Z27503 and Z17677 are as noted in A; PIR Z22503 is Arabidopsis calmodulin 3 (EF-hand 1); EF-hand is the conserved EF-hand motif (24) where $n$ is hydrophobic, $*$ is any residue, and $x, y, z$ contain oxygen in their side chains.

**Fig. 4.** SDS-PAGE of translation products of poly(A) RNA from germinating seeds immunoprecipitated with antisera against EFA27 and RAB16 fusion proteins.

**Fig. 5.** Sequencing alignment of EFA27 and open reading frames translated from data base sequences. A, the amino acid sequence of EFA27 compared with those encoded by the longest open reading frames of four Arabidopsis partial cDNAs in the EMBL data base expressed sequence tags: EMBL account no. Z27503 (ATTS1925), Z17677 (ATTS0251), Z47401 (ATTS4423), and Z29900 (ATTS2344). Residues identical or conserved between EFA27 and one other sequence are identified, and the consensus is given for residues showing identity with EFA27. EFA27 and Z29900 stop codons are noted with an asterisk. (B) comparison of the EF-hand motif in EFA27 with related proteins.
Positive control, recombinant RAB16 (0.5 ng in kilodaltons. This protein exhibits homology to previously described ABA or osmotic stress-responsive plant proteins. However, its sequence is similar to several polypeptides encoded by A. thaliana-expressed sequence tags (partial-length cDNAs) isolated from a library derived from dry seed mRNAs. These similarities include a region containing an EF-hand, a Ca\(^{2+}\)-binding motif. We have therefore initially called the protein EFA27 (EF-hand, ABA-responsive, 27 kDa). To our knowledge, it is the first example of an EF-hand protein whose expression is responsive to ABA and osmotic stress. Hybridization selection and in vitro translation experiments suggested that several other proteins related to EFA27 show similar patterns of expression. These findings indicate that EFA27 is a member of a small gene family whose members are expressed in response to ABA and osmotic stress in phylogenetically distant species.

The EFA27 EF-hand region was shown to bind \(^{45}\)Ca\(^{2+}\) using binding blots. Like other EF-hand proteins, the EFA27 motif is predicted to form a helix-loop-helix structure, and the most highly conserved residues of the EF-hand motif are also present in the EFA27 EF-hand. Invariant Asp and Glu residues forming part of a Ca\(^{2+}\)-coordinated site are present in positions 10 and 21. UV absorption at 260 nm, and in positions 12, 14, and 18 are also often involved in Ca\(^{2+}\) coordination either directly or via a water molecule. Other invariant residues are also conserved in the EFA27 motif including Gly at position 15, which permits a sharp bend in the Ca\(^{2+}\)-binding loop and a hydrophobic residue (Val) in position 17, which attaches the Ca\(^{2+}\)-binding loop to the hydrophobic core of the structure. Most EF-hands also have a conserved pattern of hydrophobic residues in the two helices at positions 2, 5, 9, 12, 22, 25, 26, and 29. These are involved in structural stabilization and contribute to packing EF-hands against each other in proteins in which they are repeated. The EFA27 EF-hand has such hydrophobic residues in some, but not all, of these positions. This incomplete pattern may be allowed because EFA27 contains only a single EF-hand. The pattern of hydrophobicity is also less conserved in the EF-hand of another protein, G14 from Arabidopsis. This protein also has a single EF-1 hand and has been shown to bind \(^{45}\)Ca\(^{2+}\) in vitro (44).

EF-hand proteins have been characterized from many organisms and function in regulating a variety of cellular activities. Ca\(^{2+}\) may function as a cytosolic second messenger by modulating the activity of EF-hand proteins in the cytosol or in membranes facing the cytosol. In plant cells, cytosolic Ca\(^{2+}\) levels change in response to ABA treatment (45), and an EF-hand protein phosphatase was recently shown to be part of an ABA-signaling pathway (17, 18). These findings clearly implicate Ca\(^{2+}\) as a second messenger in ABA-dependent signaling. The presence of an EF-hand in EFA27 suggests that its activity or function may be modulated by changes in Ca\(^{2+}\) levels affected by upstream signaling events. EFA27 activity or function may also be modulated by phosphorylation at tyrosine kinase and/or casein kinase 2 sites in the C-terminal region. For example, phosphorylation has been shown to affect the Ca\(^{2+}\)-binding properties of proteins, including lipocortins (46) and possibly the Arabidopsis 14-3-3 protein homologue GF14 (44). Furthermore, phosphorylation at casein kinase 2 signature sites has been shown to affect an activity of another ABA responsive protein, RAB16 (23). This suggests that phosphorylation by this class of kinase may be involved in the post-translational modification of ABA-responsive proteins.

EFA27 was detected by Western blotting in SDS-soluble and in membrane-enriched cellular fractions. The EFA27 sequence was examined for the presence of membrane spanning regions. Two algorithms (20, 29) predicted that residues Leu-96–Tyr-116 may form a hydrophobic, \(\alpha\)-helical membrane spanning region. This region contains no charged side chains and has a high content of Leu, Phe, and Val, residues which make up about 50% of the residues of transmembrane protein chains (47). Membrane spanning regions were also predicted by the algorithm in the corresponding regions of the Arabidopsis ho-
mologues. Application of the positive inside rule (48) indicates that the regions of EFA27 and one of the Arabidopsis polypeptides (Z17677) C-terminal to these putative membrane anchors would be cytosolic. This suggests that the EFA27 N-terminal and EF-hand are extracellular or intraorganellar while all of the polypeptides potential phosphorylation sites are cytosolic. While speculative, such a structure would be consistent with a role for EFA27 in some form of Ca$^{2+}$ modulated membrane or transmembrane signaling. We are conducting further immunological and biochemical studies to determine the cell type and subcellular localization of EFA27 in plants.

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