Plant Molecular Biology **25**: 791–798, 1994.

Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in *Arabidopsis thaliana* L.: identification of three ERDs as HSP cognate genes

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Accepted in revised form 15 April 1994

Key words: Arabidopsis thaliana L., dehydration stress, differential screening, HSP cognates

Abstract

In *Arabidopsis thaliana* L., accumulation of abscisic acid (ABA) began to increase 2 h after plants had been subjected to dehydration stress and reached maximum levels after 10 h. Differential hybridization was used to isolate 26 *Arabidopsis* cDNAs with gene expression induced by a 1 h dehydration treatment. The cDNA clones were classified into 16 groups based on Southern blot hybridization, and named ERD (*e*arly-*r*esponsive to *d*ehydration) clones. Partial sequencing of the cDNA clones revealed that three ERDs were identical to those of HSP cognates (Athsp70-1, Athsp81-2, and ubiquitin extension protein). Dehydration stress strongly induced the expression of genes for the three ERDs, while application of ABA, which is known to act as a signal transmitter in dehydration-stressed plants, did not significantly affect the ERD gene expression. This result suggests that these HSP cognates are preferentially responsive to dehydration stress in *A. thaliana*, and that signaling pathways for the expression of these genes under conditions of dehydration stress are not mainly mediated by ABA. We also discuss the possible functions of these three ERD gene products against dehydration stress.

Introduction

Unlike animals, higher plants are immobile and therefore must cope with numerous environmental stresses solely through biochemical and physiological changes. Dehydration is one of the most common environmental stresses that soil plants are exposed to and it affects the growth and development of plants through alternations in metabolism and gene expression [15, 18]. Abscisic acid (ABA), a plant hormone, plays an important role in these processes [9]. In a previous study, the accumulation of ABA began to increase rapidly in plants subjected to dehydration stress, and the resultant high levels of ABA induced the expression of several new genes [3]. Considerable research has been done on the mechanisms of ABA-mediated response in plants, and several ABA-responsive cDNAs and genes have been isolated and characterized [3, 27]. However, there are few reports on the dehydration stress-induced expression of genes in plants prior to the onset of ABA accumulation.

Arabidopsis thaliana L. has been used as a model

plant to study both the molecular processes and genetic interactions of signal transduction, growth, and development in plants. In order to analyze the early response of plants to dehydration stress at the molecular level, we used differential screening to isolate 26 cDNA clones containing *Arabidopsis* genes expressed within 1 h after the plants were subjected to dehydration. We describe how we obtained these ERD (*e*arly-*r*esponsive to *d*ehydration) cDNA clones and discuss the possible functions of the gene products from three ERD clones against dehydration stress in plant cells.

Materials and methods

Plant materials

Seeds of *Arabidopsis thaliana* L. (Columbia ecotype) were sown on vermiculite beds and grown at 22 °C for 4 weeks under continuous illumination of ca. 2500 lux.

Dehydration

Whole *Arabidopsis* plants were harvested, washed gently to remove vermiculite from the roots, weighed, and subjected to dehydration on chromatography paper (3MM, Whatman, Maidstone, UK) at 22 °C and 60% humidity, under dim light. The plants were then weighed again and frozen in liquid nitrogen.

Extraction and quantification of ABA

Extraction of ABA was performed as previously described [12], but without the gel permeation chromatography step. Quantification of ABA was also performed as previously described, using ELISA [12].

Isolation of RNA from A. thaliana

RNA was isolated from whole *Arabidopsis* plants that were harvested prior to bolting as previously described [11].

Preparation of a cDNA library from A. thaliana *dried for 1 h*

Poly(A)$^+$ RNA, prepared from 4-week-old *Arabidopsis* plants that had been subjected to dehydration-stress for one hour, was used to construct a cDNA library in λZAPII (Stratagene, La Jolla, CA) utilizing the cDNA Synthesis System Plus and Cloning System from Amersham (Aylesbury, UK). The oligo dT primer was used as the primer for synthesis of first-strand cDNA.

Differential screening of the cDNA library

Double-stranded cDNA was synthesized from poly(A)$^+$ RNA prepared from *Arabidopsis* plants that had been harvested before or after exposure to one hour of dehydration stress, using the cDNA Synthesis System Plus and Cloning System from Amersham. Ten thousand plaques from the primary cDNA library constructed from *Arabidopsis* plants dried for one hour were plated (10^3 plaques per 154 cm^2 dish), transferred onto nylon filters (Dupont, Boston, MA), and differentially screened with [^{32}P]-labelled (Random Primed DNA labelling kit, Boehringer, Mannheim, Germany) synthesized cDNAs and cDNAs for RD clones [32]. Hybridization was performed according to the instructions from the manufacturer of the nylon filters. *In vivo* excision of the recombinant phage was performed according to the instructions from Stratagene.

Hybridization of RNA and DNA

Hybridization of RNA and DNA were performed as previously described [11]. The 3′-non-coding regions of clone 7 and clone 30 were amplified by PCR, [^{32}P]-labelled, and used as specific probes to detect mRNAs for Athsp81-2 and Athsp70-1, respectively.

Analysis of DNA sequences

Plasmid DNA templates were prepared using the Automatic Plasmid Isolation System Model

PI-100 (KURABO, Osaka, Japan) and sequenced using the DNA Sequencer Model 373A (ABI, San Jose, CA). Nucleotide sequences were analyzed using the GENETYX software system (Software development Co., Tokyo, Japan).

Heat shock and cold shock

For heat shock or cold shock treatment, whole *Arabidopsis* plants grown on vermiculite beds were transferred to incubators that had been set at either 42 °C or 4 °C.

Treatment with ABA

Four-week-old *Arabidopsis* plants, grown on vermiculite beds, were sprayed with a 0.1 mM solution of ABA and incubated for 0, 1, 2, 5, 10, or 24 h at 22 °C.

Results

Estimation of relative water content (RWC) and accumulation of ABA during dehydration stress in A. thaliana

To identify the genes with products involved in early acclimation of plants to dehydration stress, we started by measuring both relative water content (RWC) and ABA accumulation in *Arabidopsis* during dehydration stress (Fig. 1). When *A. thaliana* plants were subjected to dehydration stress, they lost water gradually, reaching a plateau of 90% water loss after 10 h of dehydration stress. Accumulation of ABA began to increase after 2 h of dehydration stress, and stressed plants accumulated levels of ABA four times higher than those found in unstressed plants after being exposed to dehydration stress for 10 h. Thus, dehydration exposure for 1 h was determined as the point for differential screening.

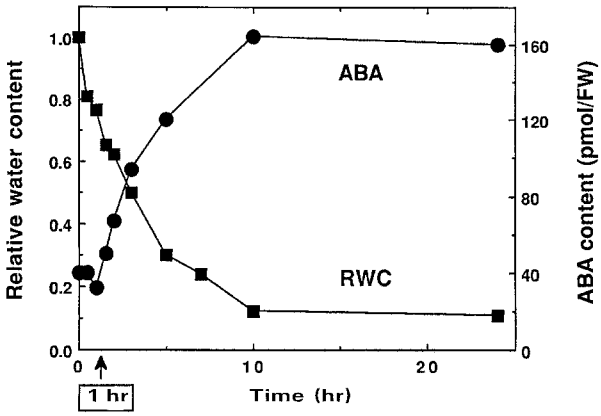

Fig. 1. Relative water content and accumulation of ABA in *Arabidopsis* plants under conditions of dehydration stress. Dehydration-stressed *Arabidopsis* plants were collected at the times indicated, and both relative water content (RWC) and ABA content were determined. ABA content was estimated by ELISA. FW: fresh weight.

Isolation of cDNA clones corresponding to genes induced by dehydration for 1 h

A cDNA library was constructed with poly(A)$^+$ RNA isolated from plants dehydrated for 1 h, and was differentially screened using cDNA prepared with poly(A)$^+$ RNA from unstressed plants and cDNA prepared with poly(A)$^+$ RNA from plants subjected to dehydration stress for 1 h. As a result of the first screening, 78 out of 10^4 plaques in the library displayed a stronger hybridization signal with [^{32}P]-labelled cDNA from dehydration-stressed plants. These phage clones were rescreened differentially using cDNA from RD clones [32] in addition to both previously used cDNAs. These RD clones consisted of nine different cDNAs which had been isolated from a cDNA library prepared from plants dehydrated for 10 h. Finally, 26 clones were found that hybridized strongly with cDNA from plants dehydrated for 1 h but not with cDNAs from unstressed plants or RD clones. The plasmid regions of these 27 phage clones were excised *in vivo* and used to transform *Escherichia coli* cells. The cDNA fragments from the resultant plasmids were analyzed by Southern blot hybridization, which led to the classification of these 26 cDNA clones into 16 distinct ERD groups (Table 1).

Table 1. General characteristics of the ERD clones. Members of the 16 groups are indicated together with the insert sizes of the clones, which represent the longest from each group.

ERD	Insert size (kb)	Isolated clones
ERD 1	3.0	10, 73
ERD 2	2.2	19, 20, 30, 67
ERD 3	2.1	65
ERD 4	2.0	3
ERD 5	1.8	2
ERD 6	1.7	58
ERD 7	1.4	69
ERD 8	1.3	6, 7
ERD 9	1.2	49
ERD 10	1.1	4
ERD 11	1.0	43
ERD 12	1.0	27
ERD 13	1.0	64
ERD 14	0.8	16
ERD 15	0.8	24, 25
ERD 16	0.6	34, 40, 74, 76, 77

Northern analysis demonstrated that gene expression in all of these ERDs was induced by a 1 h dehydration stress, while the level of mRNA for *rbcS* remained constant (Fig. 2).

Identification of three ERDs as HSP cognates

The ERD clones were partially sequenced using either the M13-20 primer or reverse primer, and each obtained sequence (ca. 200 bp) was compared to DNA sequences listed in GenBank, which revealed that sequences from three of the ERD groups (ERD2: clones 19, 20, 30 and 67; ERD8: clones 6 and 7; and ERD16: clones 34, 76 and 77) were identical to cDNAs for hsp70-1, hsp81-2, and the ubiquitin extension protein (UBQ1) of *A. thaliana*, respectively [4, 29, 31].

Northern blot analysis of the three ERD clones

Dehydration-induced gene expression in a representative clone from each of the three ERD groups was analyzed by northern blot hybridization (Fig. 3). Differing increments of mRNA accumulation were detected during dehydration stress, when whole cDNA regions of clones 7, 30, and 34 were used as probes. While high levels of ERD16 mRNAs were detected after 10 h of dehydration, high mRNA levels from ERD2 and

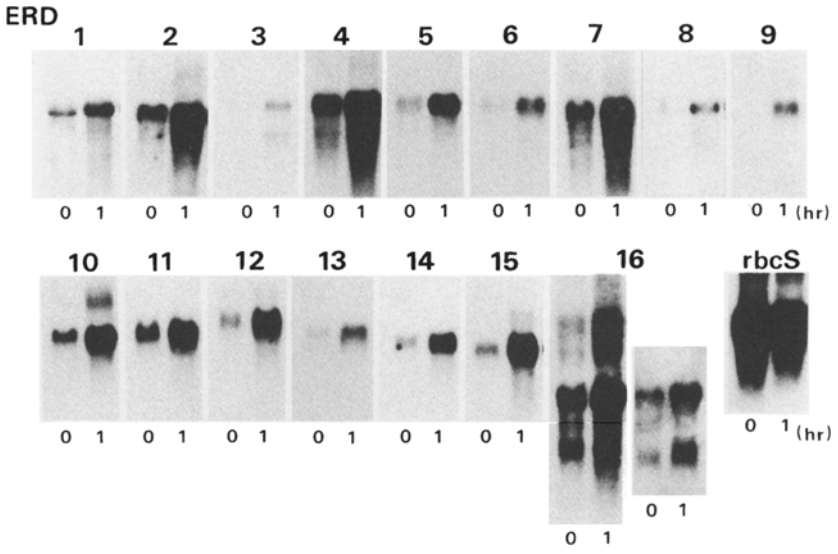

Fig. 2. Induction of expression of the ERD genes by dehydration stress. Total RNA was extracted from one-month-old *Arabidopsis* plants before or after 1 h of dehydration stress. Ten μg of each RNA were separated on agarose gels, blotted onto nitrocellulose filters, and probed with [^{32}P]-labelled cDNAs of the indicated ERDs. For ERD16, both the ubiquitin coding region (left) and the entire coding region for ubiquitin extension protein (right) were used as probes. cDNA for *rbcS* was used as a control.

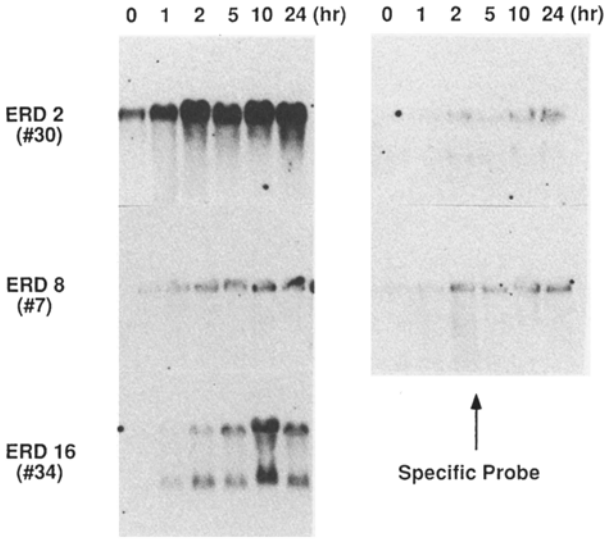

Fig. 3. Northern blot analysis of the three ERD mRNAs during dehydration stress. One-month-old *Arabidopsis* plants were subjected to dehydration stress and were collected at 0, 1, 2, 5, 10, and 24 h after treatment. Ten μg of total RNA was loaded in each lane. Entire cDNA regions from the indicated clones were used as probes (left). For ERD2 and ERD8, their 3′-non-coding regions were also used as specific probes (right). All filters in Fig. 3 were less exposed than in Fig. 2, which avoided the overexposure of ERD2 blot.

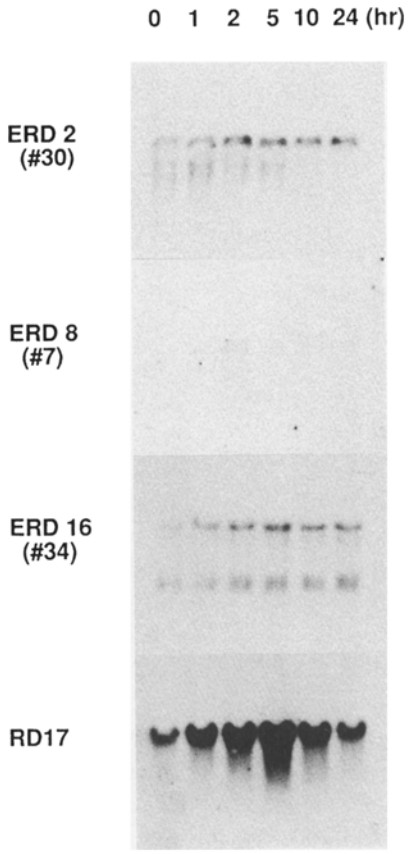

Fig. 4. Effects of ABA on expression of genes for the three ERD mRNAs in *Arabidopsis* plants. One-month-old *Arabidopsis* plants were sprayed with 0.1 mM ABA and collected at the times indicated. RNA was also hybridized to the RD17 clone, a homologue of *rab16* and dehydrins, as a positive control for induction by ABA. Ten μg of total RNA were laded per lane.

ERD8 were observed after dehydration for 2 h, and these remained high during a 24 h dehydration. Similar induction kinetics were also observed when the 3′-non-coding regions of ERD2 and ERD8 clones were used as probes, which are specific for mRNAs for hsp70-1 and hsp81-2, respectively.

Application of ABA, which is known to accumulate in plants under conditions of dehydration stress and to induce the expression of several genes in *Arabidopsis* plants, did not strongly induced the expression in the three ERDs as compared to dehydration, while it strongly induced expression of the gene for RD17, a homologue of the rice rab16 clone and of maize dehydrin (Fig. 4).

Discussion

In order to analyze the early response of plants to dehydration stress at the molecular level, we isolated 26 *Arabidopsis* cDNA clones containing genes induced by a 1 h exposure to dehydration stress. These clones were then classified into 16 groups based on Southern blot hybridization (Table 1). Partial sequencing of the clones revealed that some of them were identical to cognates of HSP clones, namely Athsp70-1, Athsp81-2, and the ubiquitin extension protein (UBQ1). Gene expression in these three clones was induced by exposure to dehydration stress for 1 to 24 h (Fig. 3). In the ERD16 group, two hybridizing bands were detected. Based on size, the lower band appeared to correspond to the UBQ1 mRNA [4]. The upper band may

correspond to other ubiquitin-related transcripts or to unspliced forms of UBQ1 mRNA. At least three additional hybridizing bands were detected by northern blot analysis when cDNA for the ubiquitin-coding region was used as a probe (Fig. 2), suggesting that the expression of other ubiquitin-related genes could also be induced by a short period of dehydration stress.

Because the specific probes corresponding to 3′-non-coding region of Athsp70-1 and Athsp81-2 are much shorter than the cDNAs which we used as probes, the signals obtained by these specific probes were weaker than those obtained by the cDNA probes (Fig. 3). However, the induction kinetics obtained by these specific probes are quite similar as those obtained by the whole cDNAs, which implied that signals detected by the whole cDNAs represented those for Athsp70-1 and Athsp81-2 mRNAs, though we cannot exclude another possibility of the existence of other hsp70 or 81 genes whose expression is inducible by dehydration stress.

In plants, it is well known that levels of ABA increase after exposure to dehydration stress, and the accumulated high levels of ABA alter the expression of several genes involved in protection against dehydration stress [5, 8, 16]. However, the induced expression of genes for Athsp70-1, Athsp81-2 and UBQ1 during dehydration stress did not appear to be mainly due to high levels of endogenous ABA, since ABA levels did not begin to increase until 2 h after the plants had been subjected to dehydration stress, while mRNAs for these three genes appeared only 1 h after subjection (Fig. 1, 3). Furthermore, the fact confirms that the levels of induction of expression of genes for these three clones by exogenously applied ABA was too weak as compared to those induced by dehydration stress (Fig. 4). Therefore, under conditions of dehydration stress the expression of these three genes seemed to be not induced mainly by endogenous ABA, but by other factors. A potential inducer could be denatured proteins. Denatured proteins were reported to induce the expression of hsp genes [1], and the loss of water from plant cells will lead to the denaturation of proteins.

While clones from the three ERD groups exhibit high homologies to cDNA clones of heat shock proteins from a variety of animals, plants, and fungi, such as chicken hsp70 [21], yeast hsp 90 [7], and human UbA52 [2], their gene expression did not appear to be significantly affected by heat shock. Wu *et al.* reported that heat shock at 37 °C for 2 h increased Athsp70-1 mRNA only 4- or 5-fold [31]. Takahashi *et al.* reported that the level of Athsp81-2 mRNA increased only modestly after heat-shock at 35 °C for 2 h [29]. The abundance of ubiquitin extension protein mRNAs was not affected by a 2 h incubation at 37 °C [4]. We obtained similar results (data not shown). Furthermore, the expression of these three genes was not induced by cold stress (4 °C) as strongly as by dehydration stress (data not shown). Thus, the gene expression induced by dehydration stress in genes for these three clones appear to be significant. It seems likely that the gene products from these three clones work preferentially when plants are subjected to dehydration stress.

The physiological role of HSP70 as a molecular chaperone is well known [6]. HSP70 plays a role in the unfolding and renaturation of denatured proteins formed during stress conditions [23, 25, 26]. HSP90 is another molecular chaperone, which stimulates protein folding by preventing non-native proteins from participating in unproductive, intermolecular interactions [30]. HSP90 also facilitates the phosphorylation of eIF-2α by casein kinase II (CK II), leading to the arrest of mRNA translation housekeeping genes [19, 20, 28]. Ubiquitin extension protein genes encode a ubiquitin monomer followed by one of two unrelated proteins [2, 17, 22]. In *Arabidopsis* plants, such extension proteins are known to be constituents of the ribosome and free extension proteins, no longer associated with ubiquitin, are accumulated [4]. The best characterized function of ubiquitin is as a covalently bound recognition signal for proteolysis [10, 24].

The present paper describes the cDNA cloning of genes whose expression was induced after a short period of dehydration stress. In addition, we demonstrated that several of the cDNAs were

identical to cDNAs for heat shock protein cognate genes. As for other ERD cDNA clones, we recently reported three of them, namely ERD1, 11, and 13, were homologous to cDNAs for ATP-dependent protease regulatory subunit (ClpA) in *E. coli*, *parB*, an auxin-inducible gene in tobacco, and glutathione *S*-transferase III in maize, respectively [13, 14]. Further characterization of these ERD clones will enable us to better understand the early molecular mechanisms that plants use to cope with dehydration stress.

Acknowledgements

This work was supported in part by the Special Coordination Fund of the Science and Technology Agency of the Japanese Government and by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan. T. Kiyosue was supported by a fellowship from the Science and Technology Agency of Japan.

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