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The Expression of a Novel Gene Encoding an Ankyrin-Repeat Protein, DRA1, is Regulated by Drought-Responsive Alternative Splicing

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***Abstract*—**Drought stress is a critical environmental factor that adversely affects crop productivity and quality. Because of their immobile nature, plants have evolved mechanisms to sense and respond to drought stress. We identified a novel locus of *Arabidopsis*, designated *DRA1* (*drought responsive ankyrin1*), whose disruption leads to increased drought-stress tolerance. *DRA1* encodes a transmembrane protein with an ankyrin-repeat motif that has been implicated in diverse cellular processes such as signal transduction. RT-PCR analysis revealed that there were at least two splicing variants of *DRA1* transcripts in wild-type plants. In response to drought stress, the levels of *DRA1* transcripts retaining second and third introns were increased, whereas these introns were removed under unstressed conditions. These results suggest that DRA1 protein may negatively regulate plant drought tolerance and that the expression of *DRA1*is regulated in response to drought stress by alternative splicing.

***Keywords*—**Alternative splicing, ankyrin repeat, *Arabidopsis*, drought tolerance.

I. Introduction

ANKYRIN-REPEAT domains are present in a great variety of proteins in prokaryotes, eukaryotes and some viruses and often mediate protein–protein interactions [1]. Proteins containing ankyrin repeats are involved in diverse cellular functions.

In plants, ankyrin-repeat proteins are involved in plant responses to biotic and abiotic stresses. Because of the importance of ankyrin-repeat proteins in plants, genome-wide localization, phylogenetic relationships and expression profiles have been analyzed in *Arabidopsis* [2] and rice [3]. In the *Arabidopsis* genome, 105 genes encoding ankyrin-repeat proteins have been identified. Becerra et al. [2] classified these genes in 16 groups based on their structural similarity. The most abundant group contains 37 genes encoding proteins with ankyrin repeats and transmembrane domains (named the AtANKTM family), and three of these genes, *ACD6*, *BDA1* and *ITN1* have been functionally characterized as mediators of stress responses so far [4]-[7].

ACD6 and BDA1 proteins are proposed to act as a plasma membrane (PM)-localized signaling components that control defense responses against pathogens [4]-[6]. We previously demonstrated that ITN1 protein was also localized to the PM and that this protein negatively regulated plant tolerance to salt stress. ITN1 functions as a PM anchor of a nuclear protein RTV1 and partially inhibits the nuclear transport of RTV1, although possible effects of ITN1-RTV1 interaction on salt tolerance remain unclear [7], [8]. These findings raise a possibility that each member of the AtANKTM family may function as signaling components in responses to various environmental factors through interaction with (or release of) their respective partners.

Drought is a critical environmental factor that adversely affects crop productivity and quality. Because of their immobile nature, plants have evolved mechanisms to sense and respond to drought [9]. For example, on the onset of drought, stomatal aperture rapidly close to avoid water deficit. A number of signaling components that regulate plant responses to drought have been identified. Recently, it has been demonstrated that alternative splicing is an important mechanism for the regulation of expression of some of these components, such as *OsDREB2B* [10]. In the absence of stress, the cells are provided with the non-functional *OsDREB2B* transcripts, which can be rapidly converted to the full-length, fully functional transcripts by changing the splicing pattern of the gene, and therefore avoiding the time necessary for transcriptional activation and pre-mRNA accumulation. In other cases, the generation of transcript isoforms carrying premature termination codons by alternative splicing probably leads to nonsense-mediated decay or translation of truncated proteins with functional roles.

In this study, a member of AtANKTM was identified as a negative regulator of drought tolerance. In response to drought stress, the expression of the gene was regulated through alternative splicing.

II. Materials and Methods

A. Plant Materials and Growth Conditions

All lines of *Arabidopsis thaliana* described here were derived from the Columbia wild type. The *SAIL_237_F09* line is available from the Arabidopsis Biological Resource Center at Ohio State University. Unless otherwise stated, plants were routinely grown at 22°C under continuous white light on solid MS medium [11] containing 1% w/v sucrose and 0.5% w/v gellan gum.

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B. Drought Tolerance Assay

Three-week-old plants were transferred to dry filter papers and exposed to dehydration for 24 h. Subsequently, plants were rehydrated for 3 days. Electrolyte leakage from leaves was measured as described previously [12].

C. RT-PCR Analysis

Total RNAs were isolated from 3-week-old plants using RNeasy Plant Mini Kit (QIAGEN) and reverse transcribed using PrimeScript II 1st strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. We used 0.4 µl of the reverse transcription reactions as templates in 10 µl PCR reactions. RT-PCR was performed using specific primers for *DRA1* (5'-CACCATCTGGCTTCTTGAATG-3' and 5'-TCAAAGGTTGGAGGAAGATCC-3') and *ACT2* (5'-GTGCCAATCTACGAGGGTTTC-3' and 5'-AAAACAATGGGACTAAAA-3') and PCR reactions as follows: *DRA1*, 94°C for 2min, then 30 cycles of 94°C for 30 sec, 59°C for 30sec and 72°C for 1min; *ACT2*, 94°C for 2min, then 22 cycles of 94°C for 30sec, 48°C for 30sec and 72°C for 30sec.

D. Sequence Analysis

DNA fragments amplified by RT-PCR were inserted into pMD20 (Takara) and sequenced.

III. Results and Discussion

A. Drought Tolerant Phenotype of an Arabidopsis Mutant Deficient in At4g03500

In the *Arabidopsis* genome, 37 genes encoding proteins with ankyrin repeats and transmembrane domains (named the AtANKTM family) have been identified [2]. Each member of the family may function as signaling components in responses to various environmental factors, such as *ACD6*, *BDA1* and *ITN1* [4]-[7]. To understand the possible involvement of AtANKTMs in plant drought tolerance, we screened for drought-tolerant or –sensitive mutants among *Arabidopsis* T-DNA insertional mutants of the *AtANKTMs* gene. A mutant line, *SAIL_237_F09*, survived after 24 h of dehydration and subsequent 3 d of rehydration, although almost all the wild-type plants died under the same conditions (Fig. 1). A similar result was also obtained when tissue damage was monitored in leaves by electrolyte leakage, an indicator of plasma membrane damage (Fig. 2). Thus, the *SAIL_237_F09* line is more tolerant to drought stress than wild-type plants. In the mutant, T-DNA was inserted in *At4g03500* gene and the expression of the gene was suppressed (Fig. 3). We have designated this gene as *DRA1* (*drought responsive ankyrin1*). These results suggested that *DRA1* might negatively affect plant drought tolerance in the wild-type plants. Recently, it was reported that a mutant allele of *At4g03500* (SALK_057550C) showed enhanced tolerance to osmotic stress [13]. Because both drought and osmotic stresses are known to cause water deficit in plant cells, *DRA1* may function as a negative regulator of cellular responses to water deficit stress.

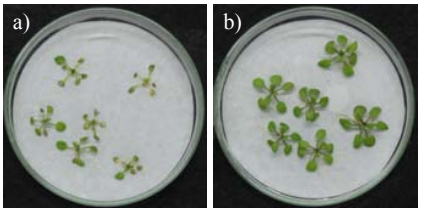

Fig. 1 Phenotypes of wild-type (a) and *SAIL_237_F09* (b) plants exposed to dehydration for 24h and rehydrated for 3 days

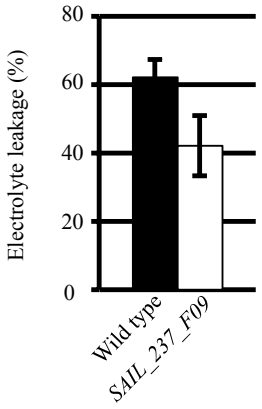

Fig. 2 Electrolyte leakage from leaves of *SAIL_237_F09* (white bar) and wild-type (black bar) plants used in Fig. 1. Data are means ± SE (n = 4)

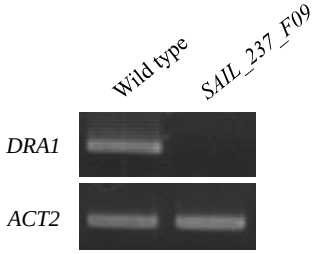

Fig. 3 *DRA1* expression in *SAIL_237_F09* mutant and wild-type plants RT-PCR analysis was performed on total RNAs. The PCR-amplified region is illustrated in Fig. 4 *ACT2* was used as an internal control

B. Structure of DRA1

According to TAIR (http://www.arabidopsis.org/), the *DRA1* gene consists of 3119 bp between the start and stop codons. It is organized into four exons and three introns (Fig. 4). DRA1 encodes an AtANKTM protein. The deduced DRA1 protein consists of 652 amino acids with a predicted molecular mass of 71971 Da. The N-terminal region (100 amino acids) does not match any peptides with known functions. The middle part contains nine ankyrin repeats, based on the SMART protein domain prediction program (http://smart.embl-heidelberg.de). The C-terminal region contains four predicted transmembrane helices. In *Arabidopsis*, three of *AtANKTM* genes, *ACD6*, *BDA1* and *ITN1* have been functionally characterized. Their deduced amino acid sequences show 35.6%, 21.6% and 18.1%

identity with the DRA1 protein, respectively.

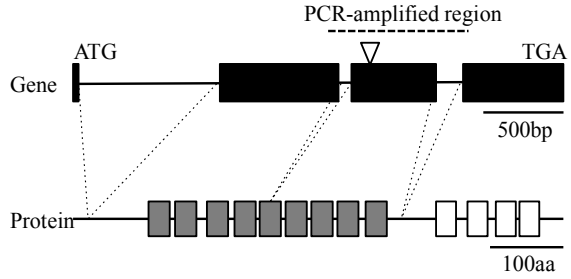

Fig. 4 The structures of the *DRA1* gene (upper) and the predicted DRA1 protein (lower). In the gene structure, black boxes indicate exons and lines indicate untranslated regions and introns. The triangle indicates the T-DNA insertion site in *SAIL_237_F09*. The broken horizontal line indicates the region amplified by PCR for Figs. 3 and 5. In the protein structure, gray boxes indicate ankyrin repeats and white boxes indicate transmembrane helices. Broken lines between the two structures connect the exons to their encoded protein regions. The "aa" is an abbreviation for "amino acids"

C. Drought-Responsive Alternative Splicing of DRA1

To investigate the expression pattern of *DRA1*, we performed RT-PCR using RNA extracted from shoots of the wild-type plants exposed to drought for various periods of time (Fig. 5). The region amplified by RT-PCR experiments was shown as a broken horizontal line in Fig. 4. The predicted length of the region with no intron is 688bp. Under unstressed conditions, only the expected length of DNA fragment was detected (a lower band in Fig. 5). However, under drought conditions, a longer length of DNA fragment was also detected (about 1000bp, an upper band in Fig. 5) in addition to the lower band. After 24 h of dehydration, only the upper band was detected. This result suggests that the expression of *DRA1*is regulated in response to drought stress by alternative splicing that generates at least two types of transcripts.

To investigate the drought-responsive change in the splicing of *DRA1*transcripts, the two PCR fragments in Fig. 5 were sequenced. The lower band contained no predicted introns, suggesting that *DRA1* transcripts might be fully spliced under unstressed conditions. On the contrary, the upper band retained second and third introns. The second intron contained a new stop codon. It is possible that the upper band is derived from a nonfunctional form of *DRA1*transcripts retaining introns.

Considering the drought-tolerant phenotype of the *dra1*-deficient mutant, the functional form of *DRA1* reduces plant drought tolerance. Accumulation of the nonfunctional form of *DRA1* transcripts may result in the suppression of *DRA1* function under drought stress conditions and the immediate induction of *DRA1* expression, once the adverse stress conditions have been withdrawn. Alternatively, the *DRA1* transcripts with second and third introns may encode a functional protein distinct from the full-length DRA1 protein. To investigate this possibility, we attempt to clone and sequence the full-length *DRA1* transcripts isolated from *Arabidopsis* plants with or without drought treatment. The elucidation of these sequences can enhance our understanding of physiological roles of alternative splicing of *DRA1* transcripts.

A detailed study is required on the modes of action of *DRA1* in plant drought tolerance. A dissection of the biochemistry of the DRA1 protein, possibly including a study of its protein–protein interactions, will provide valuable information regarding the precise roles of AtANKTM family proteins in the fundamentally important adaptations of plants to abiotic stresses.

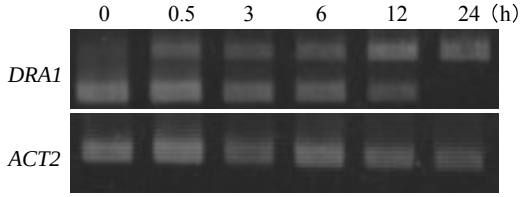

Fig. 5 *DRA1* expression in response to drought treatment. RT-PCR analysis was performed on total RNAs isolated from 3-week-old *Arabidopsis* plants that were exposed to dehydration for 0.5, 3, 6, 12, and 24h. The PCR-amplified region is illustrated in Fig. 4. *ACT2* was used as an internal control

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