TITLE: **Regulation of Abiotic Stress Responses by Ubiqutin Ligases**

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AUTHORS:

M. Kang
H. Abdel-Mageed
Texas Tech University
Dept. of Biological Sciences
Lubbock, TX 79409

M. Fokar
Texas Tech University
Center for Biotechnology and Genomics
Lubbock, TX 79409

P. Payton
USDA/ARS
Cropping Systems Research Laboratory
3810 4th Street
Lubbock, TX 79415

R. D. Allen (Corresponding author)
Texas Tech University
Depts. of Biological Sciences and Plant & Soil Sciences
Lubbock, TX 79409
Phone: 806-742-3722, x 232
Fax: 806-742-2963
Email: randy.allen@ttu.edu

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ABBREVIATIONS: Abscisic Acid, ABA

Regulation of Abiotic Stress Responses by Ubiqutin Ligases

ABSTRACT

Abiotic stress is a primary factor limiting crop productivity world-wide. Understanding the complex regulatory mechanisms that mediate the responses of plants to stressful conditions is necessary if we are to use these systems to optimize stress tolerance and increase crop productivity. We have begun to characterize the functions of a group of genes that encode zinc-finger containing proteins with sequence similarity to mammalian ubiquitin ligases. Expression of these genes in strongly activated in response to abscisic acid and exposure to stressful conditions. Functional analysis of the function of these genes in the model plant Arabidopsis indicates that they up-regulate stress responsive gene expression pathways, which leads to substantial increases in tolerance to abiotic stresses including water deficit. The effectiveness of these genes in promoting stress tolerance is now being tested in transgenic lines of cotton and other crop species.

KEY WORDS:

Cotton, Protein turnover, Stress tolerance, Ubiquitin

Drought induces a variety of plant responses, including changes in gene expression, accumulation of the phytohormone abscisic acid (ABA), production of osmotically active compounds, and the synthesis of protective proteins that scavenge oxygen radicals or act as molecular chaperones (Wang et al., 2003). These responses are controlled by molecular networks that activate stress responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes (Ramachandra-Reddy, 2004). Abiotic stress responses are genetically complex and thus difficult to manipulate. Strategies for engineering abiotic stress tolerance in plants have relied primarily on the expression of genes that encode protective molecules, such as dehydrins, antioxidant enzymes or on enzymes involved in the synthesis of functional and structural metabolites (c.f. Park et al., 2004; Payton et al., 2001; Kornyeyev, 2001; Roxas et al., 2000). More recently, strategies to use genes that are involved in signaling and regulatory pathways for engineering for plant stress responses have been developed and show great promise (Umezawa et al., 2006).

Genetic dissection of plant signal transduction has provided an important framework for the development of a more complete understanding of these complex pathways. Due to amenability of analysis, genetic dissection of plant stress responses has focused primarily on Arabidopsis. Although significant advances have been made in our understanding of abiotic stress tolerance in plants, much of this research has been focused on testing the responses of plants to a single stress treatment applied under controlled conditions. However, in the field, a variety of different stresses can occur simultaneously. Especially in semi-arid regions, plants in the field are likely to be exposed to the combined effects of high irradiance, water deficit and extreme temperatures. Therefore, the responses of plants to abiotic stresses in the field may be very different from those seen in controlled laboratory tests (Moffat, 2002; Zhu, 2002).

Although water deficit and heat stress have been extensively studied (Vierling, 1991; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Queitsch et al., 2000), less is known about how combined stresses impact plant physiology. The combination of drought and heat stress was reported to have greater detrimental effects on the growth and productivity of crops than does individually applied stresses (Savage and Jacobson, 1935; Craufurd and Peacock, 1993; Savin and Nicolas, 1996). Resistance to a combination of drought and heat stress has been a long-standing target for maize breeders (Heyne and Brunson, 1940).

Our experiments indicate that Arabidopsis dual zinc finger- proteins with sequence similarity to the A20 zinc-finger protein of mammalian cells play an important role in integrating stress responses in plants. These results show that expression of *AtA20.5* is increased in Arabidopsis plants in response to a variety of stressful conditions including ABA treatment, osmotic stress, and cold temperatures. Constitutively elevated expression of *AtA20.5* in transgenic plants leads to substantial increases in tolerance to a variety of abiotic stresses including water deficit and osmotic stress.

RESULTS AND DISCUSSION

AtA20 genes were identified through bioinformatic screening for ABA responsive genes with sequence motifs likely to be found in regulatory proteins. The derived amino acid sequences of these genes showed sequence similarity to OsiSAP1 from rice (Mukhopadhyay et al., 2004). This protein contains A20 and AN1 zinc-finger motifs at its N- and C-terminus, respectively. Expression of the *OsiSAP1* gene is induced in response to a variety of environmental stresses including cold, salt, drought, anoxia, wounding and heavy metals. The rice genome contains 18 SAP genes, 12 of which encode putative proteins with both A20-like and AN1-like zinc finger domains (Vij and Tyagi, 2006). Of the 14 SAP-like genes in the

Arabidopsis genome, 10 genes that contain both A20 and AN1 domains were identified. Like OsiSAP1, most of the rice A20-like genes were found to be stress responsive (Vij and Tyagi, 2006). Analysis of microarray data using the Genevestigator database indicated that several of the *AtA20* transcripts are strongly induced by ABA and a range of abiotic stress treatments (Fig. 1). Quantitative real-time PCR analyses confirmed that *AtA20.5* (AT3G12630) expression is strongly induced by osmotic stress, salt stress, and ABA treatment (data not shown).

Over-expression of *OsiSAP1* in transgenic tobacco plants was reported to lead to increased abiotic stress tolerance (Mukhopadhyay et al., 2004). To determine if *AtA20.5* could also confer substantial stress tolerance phenotypes, transgenic Arabidopsis plants that express an *AtA20.5* transgene under control of the CaMV 35S promoter were developed and tested for tolerance to water deficit and osmotic stress. More than 10 independent transgenic *AtA20.5* over-expressing Arabidopsis lines were developed. Expression of *AtA20.5* transcripts in these plants ranged from about 2-fold to more than 10-fold higher than native *AtA20.5* in non-transformed control plants (data not shown). Initial screens for osmotic stress tolerance were carried out with seedlings grown on media containing mannitol. Growth of both shoots and roots of transgenic *AtA20.5*-expressing seedlings was significantly greater than non-expressing control plants under these conditions while growth of *AtA20.5* T-DNA knock-out seedlings was more stress sensitive (Fig. 2). For evaluation of tolerance to water deficit stress, transgenic *AtA20.5*-expressing plants and non-expressing control plants were grown in soil to vegetative maturity in a growth chamber. Water was withheld for three weeks, at which time, the control plants showed severe wilting and desiccation damage. The *AtA20.5*- expressing plants remained turgid and apparently healthy (Fig. 2). When they were re-watered, control plants failed to recover while the *AtA20.5*-expressing plants resumed normal development. Under these conditions,

transgenic *AtA20.5*-expressing plants were able to withstand water deficit for an additional 7 to 10 days before terminal wilting and death occurred. These results clearly show that over-expression of the A20-like protein *AtA20.5* in transgenic Arabidopsis plants leads to easily detectable increases in abiotic stress tolerance.

With declining global water resources and the increased salinization of soil and water, abiotic stress has become a major limiting factor in plant productivity. Drought and salinity are already widespread and are expected to affect more than 50% of all arable lands by the year 2050 (Ashraf, 1994). Therefore, development of drought tolerant crop plants used for food, feed and fiber is critical to meet the ever-expanding demand for these products from ever-diminishing resources. One important strategy for this optimization process will be the development of drought tolerance using molecular genetic and transgenic approaches.

Our results show that members of an evolutionarily conserved gene family that encodes zinc finger proteins are expressed in response to abiotic stress and over-expression of at least one of these proteins provides a substantial increase in stress tolerance in Arabidopsis. Development of transgenic crop plants, including cotton, that express these genes is now underway to determine if they provide improved stress tolerance in these species.

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FIGURE CAPTIONS

Figure 1. Expression of certain Arabidopsis A20-like genes (AtA20) is stress responsive. Data was obtained from the Genevestigator database. Expression of AtA20.5 was confirmed by quantitative real-time PCR analysis (data not shown).

Figure 2. Stress tolerance phenotypes are associated with in *AtA20.5* expression. Left panel: Seedlings of *AtA20.5* knock-out lines (KO) showed increased sensitivity to osmotic stress relative to wild type plants (WT) while growth of AtA20.5 over-expressing transgenic plants (OE) were somewhat less sensitive. Right panel: AtA20.5 over-expressing plants (OE) withstand water deficit stress more effectively than wild type plants (WT). Water was withheld for 21 days.

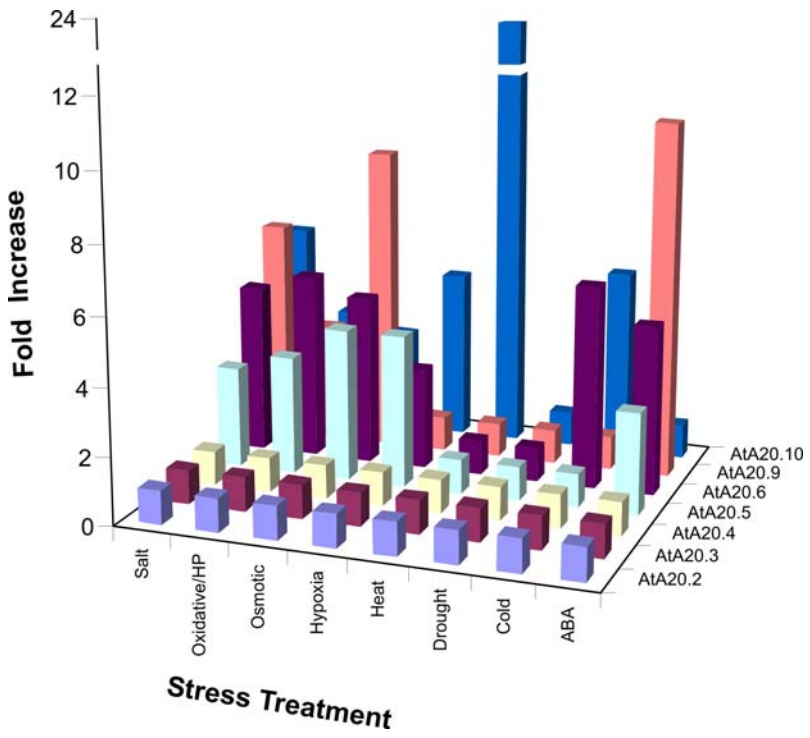
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Figure 1

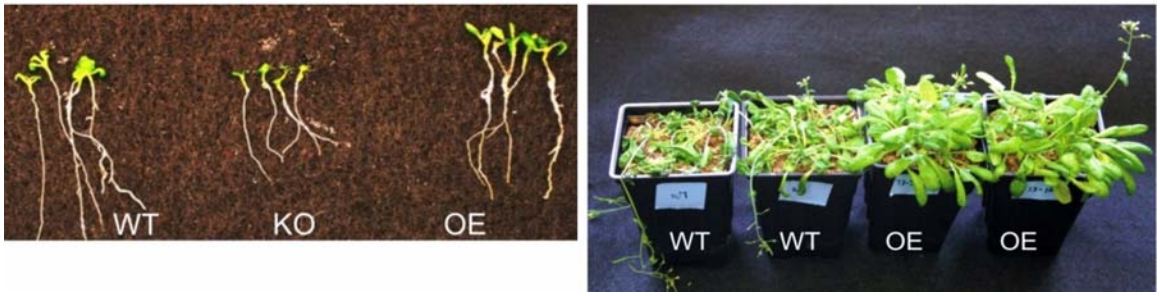

Figure 2