The Involvement of Zinc-Finger Proteins in the Abiotic Stress-Response Network of Arabidopsis thaliana

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ABSTRACT

Even though plants have many defense and acclimation mechanisms, environmental stresses are still the major cause for crop loss worldwide. All environmental stresses together are estimated to reduce average yields of major crop plants by more than 50% worldwide, whereas yield loss caused by pathogens is estimated at 10-20%. According to USDA, the US loses about $17-19 billion in yield because of environmental stress every year. Increasing abiotic threats such as ozone and drought that accompany global warming force us to understand and manipulate the stress response mechanisms of plants.

The regulation of stress responses in plants involves many different factors such as kinases, transcription factors, signaling molecules, and repressors. Recent studies emphasized the importance of repressors in the stress response and defense activation of plants. Some of these repressors are members of the C2H2 zinc finger gene family and contain ERF-associated amphiphilic repression (EAR) domain. Recent studies performed with certain zinc finger family members revealed that some of them are key elements of the stress response pathway of Arabidopsis. In our study, we investigated involvement of three zinc finger proteins in the stress response of Arabidopsis: Zat7, Zat10 and Zat12.

Although previous studies have suggested that EAR motif-containing C2H2-type zinc finger proteins are involved in the stress response network of Arabidopsis, it was not clear whether the EAR motif is involved in this function. Our data showes that transgenic plants constitutively expressing Zat7 had enhanced tolerance to salinity. Deletion or mutation of the EAR motif of Zat7 abolished this tolerance. Moreover, a yeast-two hybrid analysis revealed that the EAR motif plays role in protein-protein interaction. Our
research with Zat10, another EAR motif-containing C2H2-type zinc finger protein, suggested that Zat10 acts as both a positive and a negative regulator of plant defenses,

Recent studies suggested that Zat10 and Zat12 act in a coordinated manner in response to cold stress in Arabidopsis. To analyze the relationship between Zat10 and Zat12 during cold stress, we obtained a Zat10/Zat12 double knockout line. This line showed enhanced sensitivity to cold when compared to wild type, Zat10-knockout or Zat12-knockout plants. Our data suggests that there are at least two different cold stress response pathways in Arabidopsis. One pathway involves Zat10 and the other one involves Zat12. These pathways are linked to each other but act at different time points during the cold stress response.

More than 25% of plant genes encode proteins with unknown functions. Although research has suggested that they might play important roles in plants, their function remains unknown. To begin the characterization of these proteins, we choose 41 genes that are up-regulated in response to endogenous oxidative stress. These were stress screened by both over-expressing them in Arabidopsis and in yeast that lacks reactive oxygen species (ROS) scavenging ability. More than 70% of the proteins enhanced the tolerance of transgenic plants to oxidative stress whereas 90% of the proteins did not enhance the tolerance of transgenic plants to other stresses tested. All proteins but one didn’t change the response of the yeast to oxidative stress. This study suggested that these proteins are highly specific to plant oxidative stress response pathway.

Our study showed that the stress response network of Arabidopsis is highly complex and possibly contains unknown members and pathways that require further investigation. We have also demonstrated that EAR motif containing zinc-finger proteins are key
members of stress response network of Arabidopsis and at least some of them act through their EAR motif.
Dedication

To my beloved mother, Nimet. I owe everything to you. Thanks for your unconditional love and support.

Your daughter,

Sultan
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Chapter 1

Introduction

I. The zinc finger network of plants

II. Proteins of unknown function
I. The Zinc Finger Network of Plants

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Abstract

The zinc finger domain enables different proteins to interact with, or bind DNA, RNA, or other proteins, and is present in the proteomes of many different organisms. Proteins containing zinc finger domain(s) were found to play important roles in eukaryotic cells regulating different signal transduction pathways and controlling processes such as development and programmed cell death. There are many types of zinc finger proteins, classified according to the number and order of the Cys and His residues that bind the Zinc ion. Among these, C2H2 type zinc finger proteins, with 176 members in Arabidopsis thaliana, constitute one of the largest families of transcriptional regulators in plants. They are mostly plant-specific and contain a conserved QALGGH sequence within their zinc finger domain. Recent studies revealed that C2H2 zinc finger proteins could function as key transcriptional repressors involved in the defense and acclimation response of plants to different environmental stress conditions. Here we highlight recent functional characterization studies of different C2H2 proteins in Arabidopsis, and suggest that many of these proteins function as part of a large regulatory network that senses and responds to different environmental stimuli.
Introduction

Zinc finger proteins play a critical role in many cellular functions, including transcriptional regulation, RNA binding, regulation of apoptosis, and protein-protein interactions. They are classified into several different types including C2H2, C2C2, C2HC, C2C2C2C2, and C2HCC2C2 based on the number and order of the Cys and His residues that bind the Zinc ion in the secondary structure of the finger (Sanchez-Garcia and Rabbitts, 1994; Klug and Schwabe, 1995; Mackay and Crossley, 1998). Among the different zinc finger types, C2H2-type zinc finger proteins are one of the best studied and most abundant in eukaryotes (Laity et al., 2001). According to in silico analysis, ~3% of all genes in mammals, ~0.8% of all genes in Saccharomyces cerevisiae and ~0.7% of all genes in Arabidopsis are C2H2-type zinc finger proteins (Englbrecht et al., 2004). Even though many of these proteins are thought to mainly bind DNA, some are also thought to bind RNA and protein, and a sub class of zinc finger proteins is thought to specifically bind RNA (Iuchi, 2001). In this review we will focus on the C2H2 class of plant zinc finger protein and present new findings regarding their role in regulating signal transduction events in plants.

The C2H2-zinc finger motif was first discovered in the Xenopus oocytes transcription factor TFIIIA about 17 years ago (Miller et al., 1985). Early studies suggested that it associates with 5S rRNA within a 7S particle in Xenopus (Picard and Wegnez, 1979), but later studies suggested that it might bind to DNA and regulate the expression of the 5S rRNA gene (Pelham and Brown, 1980). Nowadays, some C2H2-zinc finger proteins are still referred to as TFIIIA-type zinc finger proteins. The first report of C2H2-zinc finger proteins in plants occurred in 1992 for the DNA-binding protein of petunia, ZPT2-1.
(previously named as EPF1) (Takatsuji et al., 1992). Soon after, WZF1 was reported in wheat as a DNA-binding zinc finger protein that interacts with a cis element of histone genes (Sakamoto et al., 1993). Soon afterwards, many other TFIIIA-type zinc-finger proteins have been reported from different plant species including wheat, petunia, Arabidopsis and rice.

C2H2-type zinc finger proteins contain one of the best characterized DNA-binding motifs found in eukaryotes. This motif consists mostly of about 30 amino acids and includes two conserved Cys and two conserved His residues bound to one zinc ion tetrahedrally, and is represented as CX_{2-4}CX3FX5LX2HX_{3-5}H (Please see example in Figure 1) (Pabo et al., 2001). Each finger forms two $\beta$ strands and one $\alpha$ helix. A recent in silico analysis revealed that there are 176 C2H2-type zinc finger proteins in Arabidopsis thaliana with only 33 of them conserved with other eukaryotes and 81% of them plant-specific (Engbrecht et al., 2004). Two main structural features, found in most of the C2H2-type plant zinc finger proteins, distinguish them from other eukaryotes (Takatsuji, 1999): i) In multiple fingered plant C2H2-type proteins, the zinc finger domains are separated by long spacers that vary in length and sequence from protein to protein (Sakamoto et al., 2004), whereas in yeast and animals, the C2H2-type fingers are mostly clustered and separated by a short spacer (6-8 amino acids) known as an H-C link (Klug and Schwabe, 1995). ii) Most of the plant zinc finger proteins have an invariant QALGGH motif in the zinc finger helices, while animal and yeast lack this motif (Takatsuji, 1999). In vitro binding analysis revealed that the conserved QALGGH motif in plants plays a critical role in DNA binding activity. It has been shown that each amino acid of this conserved sequence is essential for the DNA-binding activity of C2H2-type
zinc finger proteins (Kubo et al., 1998). Thus, substitution of any of the A, L, G, G or H residues of the first finger of the ZPT2-2 protein resulted in a complete loss of DNA-binding ability of ZPT2-2, whereas substitution of the Q residue significantly reduced DNA-binding ability of ZPT2-2 (Kubo et al., 1998). Another study performed with substituting the second G residue of the SUPERMAN protein, which contains only one C2H2-type zinc finger, to D resulted in loss of function of SUPERMAN (Sakai et al., 1995).

Englbrecht et al., 2004 used different criteria, including zinc finger position and sequence, to divide all Arabidopsis thaliana C2H2-type zinc finger proteins into three different sets (A, B and C), each divided in to different subsets (e.g., C1, C2 and C3), that in turn is divided into different families and subclasses (Englbrecht et al., 2004). Pair-wise distance analysis revealed that A1 and C1 family members have smaller pair-wise distances than C2 and C3 subsets (Englbrecht et al., 2004). Many members of subset C2 and subset C3 are involved in ancient cellular pathways such as RNA metabolism whereas almost all members of A1 and C1 families are plant- and Arabidopsis- specific and are involved in processes such as development and stress responses, suggesting that C2 and C3 subsets are evolutionary older than A1 and C1 families (Englbrecht et al., 2004). Among the plant-specific C2H2-type zinc finger proteins, A1 with 24 members and C1 with 64 members were the largest and evolutionary youngest families (Englbrecht et al., 2004). A1 family members consists of tandemly organized zinc finger domains whereas, C1 family members have either one isolated or two to five dispersed zinc finger domains (Englbrecht et al., 2004).
C2H2-type zinc finger proteins play a crucial role in many metabolic pathways as well as in stress response and defense activation in plants. Recent studies emphasized the importance of C2H2-type zinc finger proteins with a putative repression activity to the defense and stress response of plants. Most of these proteins are thought to acquire their repression activity via their ERF-associated amphiphilic repression (EAR) domain (described below). Recent studies performed with the C1 C2H2-type zinc finger family suggested that these proteins play key roles in different developmental pathways, as well as in the defense and stress response pathways of Arabidopsis. These will be described in detail below.

C1 Family

The C1 family of plant zinc finger proteins contains 64 members and is one of the largest and evolutionary youngest zinc finger families (Englbrecht et al., 2004). C1 family members contain either one isolated or two to five dispersed C2H2-type zinc fingers (indicated by the acronym “i”; e.g. C1-2i), and are classified according to their number of zinc fingers; 1i donates one finger, 2i two finger, etc. (Englbrecht et al., 2004). The C1 subclasses include C1-1i (33 members), C1-2i (20 members), C1-3i (8 members), C1-4i (2 members) and C1-5i (1 member) (Englbrecht et al., 2004). Among these subclasses, members of C1-1i and C1-2i are some of the most investigated plant C2H2-type zinc finger proteins.
**C1-1i Subclass**

C1-1i with 33 members is the largest subclass of the C1 family. Members of C1-1i consist of only one C2H2-type zinc finger domain, most of which contain the conserved QALGGH motif (Englbrecht et al., 2004). In recent years, many members of the C1-1i subclass were investigated. One of these proteins is Telomerase Activator 1 (TAC1, At3g09290). Studies showed that in the presence of endogenous auxin TAC1 can induce telomerase expression in non-cycling cells (Ren at al., 2004). A later study showed that BT2, a calmodulin binding protein, is also required for TAC1-related telomerase expression (Ren et al., 2007). Recent studies suggested that TAC1 might play a role in the auxin-signaling pathway, involves in telomerase induction. Another C1-1i subclass member investigated was Glabrous Inflorescence Stems (GIS, At3g58070). GIS plays a role in shoot maturation in Arabidopsis (Gan et al., 2006). It plays a role in trichome initiation downstream of giberellin (GA)-signaling pathway during inflorescence development (Gan et al., 2006). Cytokinrin induced trichome initiation requires two other members of C1-1i subclass: ZFP8 (At2g41940) and GIS2 (At5g06650) (Gan et al., 2007). Both proteins are also required for giberellin-induced trichome initiation, which is interesting because several papers have suggested that giberellin and cytokinin work antagonistically (Gan et al., 2007). Even though GIS, GIS2 and ZFP8 seem redundant in function, they are all regulated differentially during giberellin and cytokinin signaling. Other redundantly working members of C1-1i subclass include JAGGED (At1g68480) and NUBBIN (At1g13400). Both proteins play a role in microsporangia growth of anthers and the valves that are close to the apical region of gynoecium that encloses the ovules (Dinneny et al., 2006). They play a role in specifically defining cell layer numbers.
and differentiation of adaxial cell types of the carpel walls of gynoecium (Dinneny et al., 2006). One of the most investigated C1-1i subclass member is SUPERMAN (SUP, At3g23130). It has been proposed that SUP maintains the boundary between the third and fourth whorls of the flower (Sakai et al., 1995). SUP can bind to DNA through its zinc finger domain and two basic regions that surround the domain suggesting that SUP acts as a transcription factor (Dathan et al., 2002). Later studies showed that other SUPERMAN-like proteins play a role in the development of *Arabidopsis thaliana*. One of these proteins is RABBIT EARS (RBE, At5g06070). RBE has been proposed to play a role in early development of the organ primordia of the second whorl and maintain the boundaries of homeotic gene expression between whorls (Takeda et al., 2004; Krizek et al., 2006). Several studies have suggested that RBE might play a role as a repressor and obtain this ability through its EAR domain (Krizek et al., 2006). KNUCKLES (KNU, At5g14010), also encodes a SUPERMAN-like protein suggested to play role as a transcriptional repressor of cellular proliferation (Payne et al., 2004).

**C1-3i, C1-4i and C1-5i Subclasses**

Subclass C1-3i consists of eight C2H2-type zinc finger proteins all with three dispersed zinc finger domains (Englbrecht et al., 2004). Among them only Zat1 (At1g02030) was previously characterized (Meissner and Michael, 1997). C1-4i subclass has two members with four dispersed zinc finger domains and C1-5i has only one member which has five dispersed zinc fingers (Englbrecht et al., 2004). To the best of our knowledge, the function(s) of both C1-4i and C1-5i subclass members are unknown at present.
**C1-2i Subclass**

The C1-2i subclass contains 20 members including Zat5, Zat6, Zat7, Zat8, Zat10, Zat11, Zat12, Zat13, Zat14, Zat15, Zat16, Zat17, Zat18, AZF1, AZF2, AZF3, At5g04390, At1g02040, At2g26940, and At4g04404 that show extensive homology at their first and second zinc finger domains (Figure 1) (Englbrecht et al., 2004). Most of these proteins were isolated by homology-based cloning (Meissner and Michael, 1997), and all members consist of two dispersed C2H2-type fingers (Meissner and Michael, 1997; Englbrecht et al., 2004).

Eighteen of the C1-2i subclass members contain the invariant QALGGH motif in both their zinc finger helices (Figure 1) (Englbrecht et al., 2004). However, it is unclear at this point whether members that lack this motif are different in their function from members that contain it. Other than the zinc finger domains, most members also share several putative nuclear localization sequences and an ERF-associated amphiphilic repression (EAR) motif (L/FDLNL/F(x)P) that is thought to have an active repression activity and is found at the C-terminus of the proteins (Figure 1C) (Meissner and Michael, 1997; Englbrecht et al., 2004). A neighbor joining tree analysis performed for the different C1-2i members revealed that several of these members could be the result of recent gene duplication (e.g. Zat10 and Zat6, Zat11 and Zat18, and Zat7 and Zat8; Figure 1D).

The ERF-associated amphiphilic repression (EAR) motif was first identified in the AP2/ERF domain proteins (Ohta et al., 2001). AP2/ERF (or ERF proteins) domain proteins are plant-specific transcription factors that consist of a DNA binding domain named the ERF domain (Allen et al., 1998; Hao et al., 1998; Fujimoto et al., 2000). ERF
proteins bind to the core sequence of a conserved ethylene-responsive element (GCC box) that is found in the promoters of many defense and stress response genes (Ohme-Takagi and Shinshi, 1995; Kazan, 2006). Many genes that encode ERF proteins are thought to play a role in plant growth, development and response to biotic or abiotic stresses (Ohta et al., 2001; Kazan, 2006). *In silico* analysis identified over 124 genes that contain the ERF domain in plants (Riechmann et al., 2000).

Homology studies showed that there are two different classes of ERF proteins: class I ERFs and class II ERFs (Fujimoto et al., 2000). Class I ERF proteins act as activators of transcription (Ohta et al., 2001). Members of class I ERFs include tobacco ERF2, ERF4 and JERF1, Arabidopsis AtERF1, AtERF2, AtERF5, ERF1, CBF1, DREB1 and DREB2, periwinkle ORCA2 and ORCA3, and tomato Pti4 (Stockinger et al., 1997; Zhou et al., 1997; Liu et al., 1998; Solano et al., 1998; Menke et al., 1999; Fujimoto et al., 2000; van der Fits and Memelink, 2000; Ohta et al., 2001; Wu et al., 2007). Class II ERFs include NtERF3, AtERF3, AtERF4, AtERF7 and LeERF3b (Fujimoto et al., 2000; Ohta et al., 2001; Song et al., 2005; Chen et al., 2007). This class of ERFs is thought to play a role as active repressors (Ohta et al., 2001). Active repressors include an independent repressor domain that represses transcription directly by chromatin modifications such as histone deacetylation or methylation, whereas passive repressors do not include an independent repressor domain and repress transcription indirectly by either DNA-protein or protein-protein interactions (Thiel et al., 2004; Kazan, 2006). Studies revealed that class II ERF repressors contain a conserved motif ‘L/FDNL/F[x]P’ named as ERF-associated amphiphilic repression (EAR) motif (Ohta et al., 2001). Mutation in the EAR motif of ERF3 abolished its repression activity, as tested with reporter gene expression (Ohta et
al., 2001). It was previously reported that chimeric transcription factors fused to the EAR motif act as dominant repressors (Hiratsu et al., 2003). Latest studies showed that beside repression activity, the EAR motif is also required for protein-protein interaction (Ciftci-Yilmaz et al., 2007, Szemenyei et al., 2009).

Recent studies suggested that EAR-motif containing repressors play a key role in plant defense and stress response mechanisms by transcriptional repression of different defense or stress response related genes in the absence of stress (Kazan, 2006). For instance, the EAR repressor AtERF4 negatively regulates the expression of PDF1.2 that encodes an antifungal peptide belonging to the family of plant defensins by modulating ethylene and jasmonic acid responses (McGrath et al., 2005; Yang et al., 2005). Another EAR repressor, NIMIN1 represses the expression of the pathogenesis-related PR-1 gene that encodes a defense protein induced in response to pathogens or salicylic acid in plants (Kazan, 2006). In accordance, over-expression of the NIMIN1 protein in Arabidopsis resulted in suppression of PR-1 expression and elevated pathogen susceptibility, whereas suppression of NIMIN1 resulted in constitutive expression of PR-1 after salicylic acid treatment (Weigel et al., 2005). It has also been shown, by fusion of the EAR-motif to different DNA binding domains, that it could actively repress transcription of several genes in vivo (Hiratsu et al., 2003; Matsui et al., 2004). These result suggested that EAR motif-containing C2H2-type zinc finger proteins could act as repressors in plants (Ohta et al., 2001, Kazan, 2006). Key members of this group include Zat6, Zat7, Zat10/STZ, Zat12, AZF1, AZF2, and AZF3 which are C1-2i subclass members. Several of these proteins are thought to play a role in the response of plants to different biotic and abiotic insults. In accordance with this hypothesis, transcriptome profiling analyses has shown
that the steady-state transcript level of many of these zinc finger proteins is elevated during different stress conditions (Figure 2). Genetic and biochemical studies of these proteins are described below.

**Zat10/STZ**

Zat10 was first identified as a cDNA that rescues yeast calcineurin null mutants (Lippuner et al., 1996). Calcineurin is Ca\(^{2+}\)/Calmodulin dependent protein that plays a role in modulating ion channels required for tolerance to Na\(^+\) and Li\(^+\) ions (Nakamura et al., 1993). In accordance, expression of Zat10/STZ in salt sensitive yeast cells rescued the phenotype of these cells (Lippuner et al., 1996). Moreover, it also enhanced the tolerance of wild type yeast cells to high concentrations of Na\(^+\) and Li\(^+\) (Lippuner et al., 1996).

Zat10 is a TFIII-type stress-response protein consisting of two C2H2-type zinc fingers. Because of Zat10’s a high structural similarity with different ZPT2-related proteins, it was thought to bind to DNA in a similar manner. ZPT2-related proteins bind to two tandemly repeated AGT core sequences separated by 10 bps (Takatsuji et al., 1994). Investigations showed that Zat10 recognizes either AGT and ACT core sequences separated by 3 bp, or ACT and AGT core sequences separated by 4 bp (Sakamoto et al., 2004). The DNA binding preference of Zat10 is slightly different from that of ZPT2-type and can be summarized as A[G/C]T-\(X_{3,4}\)-A[G/C]T (Sakamoto et al., 2004). Expression studies showed that Zat10 is expressed in all parts of Arabidopsis in response to different stresses including salinity, high light and cold (Lippuner et al., 1996; Meissner and Michael, 1997; Sakamoto et al., 2000; Sakamoto et al., 2004; Mittler et al., 2006).
Zat10 contains an EAR motif at its C-terminus suggesting that Zat10 might play role as repressor. First evidence for Zat10’s repression activity was revealed in 2001 (Ohta et al., 2001). Both full length Zat10 and the repressor domain of Zat10 that included the EAR motif repressed transcription of a luciferase reporter gene through interacting with EP2-type promoter which was shown to bind ZPT2-type proteins (Ohta et al., 2001). Full length Zat10 and its repression domain were also shown to repress the transcription of luciferase when fused to AtERF5 that is a Class I ERF protein (Ohta et al., 2001). Soon after, another study showed that Zat10 can bind to the RD29A promoter and repress its transcription (Lee et al., 2002). RD29A is a classical stress response gene and this finding suggested that Zat10 could regulate RD29A transcription during stress (Lee et al., 2002).

Studies also indicated that Zat10 might be involved in Jasmonic acid signaling as a negative regulator of this pathway (Pauwels et al., 2008). Jasmonic acid signaling pathway play a key role in many biological processes including development, biotic and abiotic signaling (Pauwels et al., 2008).

Recent studies suggested that Zat10 play a dual role in the response of plants to abiotic stress. Transgenic plants that constitutively express Zat10 were found to be more tolerant to drought stress, osmotic stress, salt and heat stresses (Sakamoto et al., 2004, Mittler et al., 2006). Interestingly, Zat10 knockout and RNAi lines were also more tolerant to osmotic and salinity stresses (Mittler et al., 2006). Over-expressing Zat10 enhanced transcription of ascorbate peroxidase 1 and 2 (APX 1, 2) and Iron superoxide dismutase 1 (FSD1), which are known to play role in scavenging reactive oxygen species in plant (Mittler et al., 2006). Zat10 might enhance the transcription of these genes by directly activating their transcription, or repressing a repressor of these genes. Taken
together these data suggest that Zat10 is required for stress tolerance and possibly play a dual role as both an activator and a repressor of stress response genes. Even though different studies revealed that the EAR motif of Zat10 can repress the expression of different reporter genes \textit{in vivo} (Ohta et al., 2001; Lee et al., 2002; Sakamoto et al., 2004), to the best of our knowledge, direct genetic evidence for the role of this domain in the Zat10-controlled stress response pathway(s) of Arabidopsis was not presented.

Recent studies showed that over-expression of CBF3 (C-repeat binding factor 3, also known as dehydration-responsive element-binding protein 1A or DREB1A) in Arabidopsis resulted in enhanced expression of Zat10 (Maruyama et al., 2004). CBF3 is a member of the CBF (C-repeat binding factor) regulon that plays a role in cold acclimation by activating expression of COR (cold responsive) genes (Chinnusamy et al., 2007). Moreover, a decrease in CBF3 expression as a result of ice1 (inducer of CBF expression 1) mutation resulted in a decrease in Zat10 expression in response to cold (Chinnusamy et al., 2007). Transient expression assays showed that Zat10 can suppress the expression of RD29A which is regulated by the CBF regulon suggesting that Zat10 might act downstream of the CBF regulon and play a role in the regulation of a subset of COR genes (Lee et al., 2002; Chinnusamy et al., 2007). Interestingly, studies suggested that Zat12 acts as a negative regulator of the CBF regulon, therefore functioning upstream to Zat10 that is regulated by the CBF regulon (Chinnusamy et al., 2007). Transcriptome analysis revealed that the expression patterns of Zat10 and Zat12 is similar during several different stresses (Figure 2) (Mittler et al., 2006). Both transcripts are elevated in response to cold stress, salinity, UV-B, oxidative stress, osmotic stress and
genotoxic stress (Figure 2). These findings could suggest that Zat10 and Zat12 function in a coordinated manner during different stresses.

Zat12

Zat12 was first identified by homology cloning (Meissner and Michael, 1997). It consists of two C2H2-type zinc finger domains with a 22 amino acid inter-finger region, and belongs to the C1-2i subclass (Englbrecht et al., 2004). Following its initial identification, Zat12 was found to be a light stress-response protein (Vogel et al., 2005). Later studies suggested that Zat12 is involved in the cold and oxidative stress response of Arabidopsis (Iida et al., 2000; Rizhsky et al., 2004; Davletova et al., 2005). Zat12 is required for the expression of the defense enzyme cytosolic ascorbate peroxidase 1 (APX1) during oxidative stress (Iida et al., 2000). It is also required for the expression of two important oxidative stress response proteins: Zat7 and WRKY25 in Arabidopsis during oxidative stress (Iida et al., 2000). This data suggested that Zat12 expression is essential for reactive oxygen metabolism in Arabidopsis.

Transgenic plants that constitutively express Zat12 are more tolerant to high light, osmotic and oxidative stresses, and Zat12 antisense and knockout plants are more sensitive to light, osmotic stress and salinity (Vogel et al., 2005; Iida et al., 2000; Davletova et al., 2005). Moreover, expression of Zat12 in transgenic plants was found to elevate the expression of 42 different transcripts involved in the response of plants to high light and osmotic stress (Davletova et al., 2005). Transcriptome analysis showed that Zat12 expression is elevated in response to many different abiotic stresses (Figure 2). Nevertheless, studies Zat12 gain and loss-function studies suggested that Zat12 is
required for stress tolerance to only a few of these stresses (Davletova et al., 2005). Because of the extensive overlap between the transcriptome of plants subjected to hydrogen peroxide stress and the transcriptome of plants expressing Zat12 (Davletova et al., 2005), it was suggested that Zat12 expression might be associated with the response of plants to ROS accumulation during abiotic stresses (Davletova et al., 2005).

Transcriptome analysis of the cold response in Arabidopsis suggested that 302 genes are up-regulated and 212 genes are down-regulated in response to low temperature (Rizhsky et al., 2004). Most of the genes highly regulated by low temperature were assigned to two main regulons: the CBF regulon and a regulon controlled by Zat12 (Rizhsky et al., 2004). Moreover, it was suggested that Zat12 negatively regulates the CBF regulon (Rizhsky et al., 2004).

Zat12 contains an EAR motif at the C-terminal and this motif might function as a repressor domain (Ohta et al., 2001; Englbrecht et al., 2004; Rizhsky et al., 2004; Davletova et al., 2005). It has been suggested that Zat12 suppresses the expression of the key cold stress response transcription factors CBF1, CBF2 and CBF3 in response to stress (Rizhsky et al., 2004). Microarray analysis revealed that over-expression of Zat12 resulted in the repression of several transcripts (Rizhsky et al., 2004; Davletova et al., 2005). Moreover, Zat12 loss-of-function lines showed enhanced tolerance to heat stress suggesting that Zat12 might function as repressor (Davletova et al., 2005). Even though several lines of evidence suggest that Zat12 has a repression activity, most likely through its EAR motif, at present direct function-structure studies that support this hypothesis were not reported.
Zat7

Zat7 is C2H2–type zinc finger protein consisting of two zinc fingers with a conserved QALGGH sequence and an EAR motif (Englbrecht et al., 2004). Zat7 was initially identified as an oxidative stress response protein in knockout APX1 plants subjected to internal oxidative stress (Iida et al., 2000). Transgenic plants that constitutively express Zat7 have enhanced tolerance to salinity stress and the EAR motif of Zat7 was shown to be required for this tolerance (Ciftci-Yilmaz et al., 2007). Moreover, in contrast to transgenic plants that constitutively express Zat7 and show enhanced tolerance to salinity, transgenic plants that constitutively express a Zat7 protein that lacks a functional EAR motif showed enhanced sensitivity to salinity (Ciftci-Yilmaz et al., 2007). Yeast-two-hybrid experiments suggested that the EAR motif is also required for protein-protein interactions (Ciftci-Yilmaz et al., 2007). These studies showed that Zat7 interacts with stress response and defense related proteins such as the transcription factor WRKY70 and the miRNA transport protein HASTY through its EAR domain (Ciftci-Yilmaz et al., 2007). Interestingly, the expression of Zat7, WRKY70 and HASTY is up-regulated in knockout APX1 plants which are more tolerant to salinity stress suggesting that these three proteins are part of a salinity stress signaling pathway (Ciftci-Yilmaz et al., 2007). Transgenic plants that constitutively express Zat7 showed enhanced tolerance to cold stress but increased sensitivity to osmotic stress (Ciftci-Yilmaz et al., 2007). These findings could suggest a complex mode of regulation for zinc finger proteins during different stresses.

Previous studies suggested that constitutive expression of an EAR motif containing zinc finger proteins, such as Zat10, Zat7 or Zat12 resulted in growth suppression of
plants. Because plants with suppressed growth are typically more tolerant to abiotic stresses, it was suggested that growth suppression in these transgenic plants might be the reason for their enhanced tolerance to different abiotic stresses (Rizhsky et al., 2004; Mittler et al., 2006). Nevertheless, the studies of Ciftci-Yilmaz et al., 2007 clearly demonstrated that growth suppression in Zat7 over-expressing plants could be distinguished from enhanced tolerance to stress. These studies also suggested that the EAR motif is not responsible for growth suppression in transgenic plants expressing Zat7 (Ciftci-Yilmaz et al., 2007).

AZF1-AZF2-AZF3

AZF1 (Arabidopsis zinc-finger protein 1), AZF2, and AZF3 were first identified by homology cloning (Sakamoto et al., 2000). They all contain two canonical C2H2-type zinc fingers separated by a long spacer, and a conserved EAR motif (Sakamoto et al., 2000; Englbrecht et al., 2004). AZFs show similarity to Zat10 both structurally and functionally (Sakamoto et al., 2000; Englbrecht et al., 2004; Sakamoto et al., 2004). Similar to Zat10, AZF2 also binds to two canonical A[G/C]T sequences (Sakamoto et al., 2004). Transient expression analysis revealed that all AZFs have repression activity possibly through their EAR domain (Sakamoto et al., 2004). Expression analysis indicated that AZF1 and AZF3 expression is mainly restricted to roots, whereas AZF2 is expressed at various levels at all organs of Arabidopsis with high expression in roots (Sakamoto et al., 2000; Sakamoto et al., 2004)

All AZFs are involved in the water-stress response of Arabidopsis (Sakamoto et al., 2000). AZF1 responds rapidly to salt and cold stresses but appear to be ABA-independent
AZF2 contains an ABRE (ABA-responsive element) in its promoter region (Sakamoto et al., 2000; Sakamoto et al., 2004), is strongly induced by ABA and salt, and likely to be ABA-dependent (Sakamoto et al., 2000; Sakamoto et al., 2004). Interestingly, all AZFs are induced by ethephon which produces ethylene and hydrogen peroxide (Alonso and Ecker, 2001; Sakamoto et al., 2004).

**Zat6**

Zat6 expression is enhanced in response to cold and osmotic stresses (Figure 2), nevertheless, it is not clear whether Zat6 function is required for tolerance to these stresses. Recent studies suggested that Zat6 is involved in root development and phosphate homeostasis in Arabidopsis (Devaiah et al., 2007). Enhanced expression of Zat6 in Arabidopsis resulted in repression of primary root growth and a subsequent change in phosphate acquisition, whereas suppressing Zat6 expression resulted in lethality (Devaiah et al., 2007). Moreover, enhanced expression of Zat6 repressed the expression of several phosphate response genes such as At4 and Pht1;1 during phosphate starvation (Devaiah et al., 2007). It is possible that the EAR domain of Zat6 is involved in transcriptional repression during phosphate starvation and development of primary root growth.

**Conclusion**

With 176 members, the H2C2-zinc finger protein family constitutes one of the largest families of transcriptional regulators in Arabidopsis (Englbrecht et al., 2004). To date, many different studies have shown that C2H2-zinc finger proteins are required for
key cellular processes including transcriptional regulation, development, pathogen defense, and stress responses. A recent study of the Oryza sativa (rice) C2H2 type zinc family identified 189 members of this family and demonstrated that at least 26 of them respond to different environmental stresses (Agarwal et al., 2007). Interestingly, the expression of 12 rice C2H2 proteins is upregulated in response to different environmental stresses, as well as during different phases of reproduction. Recent genetic studies pointed to possible interactions between different zinc finger proteins during stress. Expression of Zat7 in transgenic plants, for example, enhanced the tolerance of plants to cold stress, but decreased the tolerance of plants to osmotic stress (Ciftci-Yilmaz et al., 2007). In contrast, expression of Zat7 proteins with a mutated or truncated EAR domain in transgenic plants had no effect on abiotic stress tolerance (Ciftci-Yilmaz et al., 2007). Could Zat7 interact with other zinc finger proteins during osmotic stress and disrupt their function? In addition to possible protein-protein interactions between different zinc-finger proteins, a cascade of zinc finger proteins could be activated during stress. Thus, for example, during oxidative stress Zat12 is required for the expression of Zat7 (Iida et al., 2000), and during cold stress Zat12 functions upstream to Zat10 (Chinnusamy et al., 2007). Could different zinc finger proteins interact with each other in a hierarchical or a combinatorial manner to regulate transcription? What then is the order of regulation? Future studies attempting to address these questions could shed much needed light on the mode of action of different zinc finger proteins and how they regulate different process in plants. The in silico analysis shown in Figures 1D and 2 reveals some very interesting relationships between different C1-2i members. Not all members appear to respond to different biotic and abiotic insults, demonstrating a high degree of specificity for different
Zat proteins. Zat6 and Zat10 that appear to be highly related and may be the result of recent gene duplication (Figure 1D), appear to respond in a similar manner to the different stresses studied in leaves and roots (Figure 2). In contrast, Zat10 and Zat12 that appear to respond similarly to different stresses (Figure 2) are much less similar and appear to be more distinct (Figure 1D).

In addition to studying the basic functions of different zinc finger proteins, applied applications of different zinc finger proteins should be considered. Thus, different zinc finger proteins or domains of different proteins, such as the EAR domain, could be used to alter development, or enhance the tolerance of transgenic plants to different stress conditions. Nevertheless, before such applications could be considered, the exact mode of action of different zinc finger proteins should be elucidated because simply over-expressing them in transgenic plants could result in deleterious side effects including decreased tolerance to other unrelated stresses, or suppressed growth. Although this review was mainly focused on the C1 family, other families of zinc finger proteins were recently reported to be involved in basic process in plants, for example the A3 subclass AtCZS protein that was reported to chromatic structure in Arabidopsis (Krichevsky et al., 2007)
Acknowledgments

This work was supported by funding from The National Science Foundation (IBN-0420033 and NSF-0431327), The Nevada Agricultural Experimental Station (publication No. 03055517), and The NIH IDeA Network of Biomedical Research Excellence (INBRE, RR-03-008).
Figure Legends

Figure 1. Conserved sequences in the first and second zinc fingers, and the core EAR motif of C1-2i subclass representatives. (A and B) Conserved sequences within the zinc finger domains of C1-2i subclass representatives. (C) EAR core sequence of C1-2i subclass representatives. (D) A neighbor joining tree of C1-2i Arabidopsis proteins. Alignments were performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html).

Figure 2. In silico DNA chip analysis of selected C1-2i subclass representatives in response to different biotic or abiotic stresses. (Up) Relative expression of C1-2i subclass members in leaves of Arabidopsis exposed to different stresses. (Down) Relative expression of C1-2i subclass members in roots of Arabidopsis exposed to different stresses. In silico analysis of DNA chip data obtained from (https://www.genevestigator.ethz.ch) was conducted according to (Mittler et al., 2006). Due to tandem duplication of Zat7 (Englbrecht et al., 2004), ATH1 252567_at was used to measure its expression.
**Figure 1**

**A. First zinc finger domain**

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**EAR Motif**

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D.
Figure 2
II. Proteins of Unknown Function

18% to 38% of all eukaryotic proteomes sequenced to date contain proteins with obscure features (POFs) (Gollery et al., 2006). A recent study comparing 10 different sequenced proteomes, including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Arabidopsis thaliana, Oryza sativa, Drosophila melanogaster, Anopheles gambiae, Caenorhabditis elegans, Mus musculus, Rattus norvegicus, and Homo sapiens*, revealed that 60% of the POFs were species specific whereas only 7.5% of proteins with defined features (PDFs) were species specific (Gollery et al., 2006). POFs were found to be in their expression level, participation in protein-protein interaction and association with mutant phenotypes (Gollery et al., 2006). On the other hand, they had more predicted disordered structure, were shorter and more hydrophilic than PDFs (Gollery et al., 2006). Over 5,000 POFs have been identified in Arabidopsis (Luhua et al., 2008). Species specificity of most of the POFs suggests that they could be involved in processes unique to species. Some of these proteins could be involved in known pathways and networks and could possess novel functions. Some of them could be involved in new and undiscovered pathways.

In Luhua et al., 2008, 41 Arabidopsis proteins with unknown function that are up-regulated in response to internal oxidative stress were chosen for functional characterization (Davletova et al., 2005; Luhua et al., 2008). We used the Yap1Δ yeast strain to clone and analyze plant genes. In previous reports, yeast complementation was proven to be an effective method to characterize plant genes (Mowla et al., 2006;
Babiychuk et al., 1995; Belles-Boix et al., 2000; Forment et al., 2002; Kushnir et al., 1995). YAP1 plays a key role in the expression of many oxidative stress-responsive genes including ROS scavenging enzymes in yeast (Lee et al., 1999). YAP1 deletion strains showed increased sensitivity to oxidative stress (Kuge and Jones, 1994). Plant genes were also characterized by constitutively expressing them in Arabidopsis and subjecting them to abiotic stresses (Luhua et al., 2008).

70% of transgenic plants expressing proteins with unknown function conferred tolerance to oxidative stress whereas 90% of transgenic plants did not confer tolerance to other abiotic stresses tested (Luhua et al., 2008). Two Arabidopsis specific POFs and one Arabidopsis and *Brassica* specific protein with unknown function showed enhanced tolerance to oxidative stress (Luhua et al., 2008). Only one of the proteins, an Arabidopsis specific POF, tested in yeast enhanced the sensitivity of the yeast to oxidative stress and none of the proteins tested enhanced the response of yeast to the oxidative stress. These findings suggested that the oxidative stress response of Arabidopsis contains many different proteins and pathways which are still unknown today.
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drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis.

Plant Cell 10, 1391-1406


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Nakamura T., Liu Y., Hirata D., Namba H., Harada S., Hirokawa T. and Miyakawa T. (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. 12, 4063-4071


Chapter 2

The EAR-Motif of the C2H2 Zinc-Finger Protein Zat7 Plays a Key Role in the Defense Response of Arabidopsis to Salinity Stress

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Abstract

Cys2/His2-type zinc finger proteins, which contain the EAR transcriptional repressor domain, are thought to play a key role in regulating the defense response of plants to biotic and abiotic stress conditions. Although constitutive expression of several of these proteins was shown to enhance the tolerance of transgenic plants to abiotic stress, it is not clear whether the EAR-motif of these proteins is involved in this function. In addition, it is not clear whether suppression of plant growth, induced in transgenic plants by different Cys2/His2 EAR-containing proteins, is mediated by the EAR-domain. Here we report that transgenic Arabidopsis plants constitutively expressing the Cys2/His2 zinc-finger protein Zat7 have suppressed growth and are more tolerant to salinity stress. A deletion or a mutation of the EAR-motif of Zat7 abolishes salinity tolerance without affecting growth suppression. These results demonstrate that the EAR-motif of Zat7 is directly involved in enhancing the tolerance of transgenic plants to salinity stress. In contrast, the EAR-motif appears not to be involved in suppressing the growth of transgenic plants. Further analysis of Zat7 using RNAi lines suggests that Zat7 functions in Arabidopsis to suppress a repressor of defense responses. A yeast two-hybrid analysis identified putative interactors of Zat7 and the EAR-domain, including WRKY70 and HASTY, a protein involved in miRNA transport. Our findings demonstrate that the EAR-domain of Cys2/His2-type zinc finger proteins plays a key role in the defense response of Arabidopsis to abiotic stresses.
Introduction

Transcriptional repressors are emerging as central regulators of development and stress responses in different organisms. By suppressing defense responses and keeping developmental programs under control they are thought to prevent excessive waste of resources and the activation of programmed cell death due to metabolic imbalances or runaway response pathways (Cowell, 1994; Thiel et al., 2004; Eulgem, 2005; Kazan, 2006). Transcriptional repressors were shown in some cases to be activated by the same signals they control, generating an efficient regulatory circuit. They can also be constantly present in cells, but removed in response to a specific signal by proteolytic degradation (Cowell, 1994; Thiel et al., 2004; Eulgem, 2005; Kazan, 2006).

At least two main classes of transcriptional repressors have been described in eukaryotes: passive and active repressors. Passive repressors are thought to function by competing with transcriptional activators for DNA binding, or by binding and displacing coactivators required for transcriptional activation. They typically do not have an intrinsic repressing activity or a distinguished repression domain. By contrast, active repressors exhibit intrinsic repression activity that targets chromatin organization. They function via modifying histone deacetylation, or altering histone methylation and inducing heterochromatin formation (Thiel et al., 2004).

Cys2/His2-type (C2H2) zinc finger proteins that contain the ERF-associated amphiphilic repression (EAR) domain are thought to play an important role in regulating the defense response of Arabidopsis to abiotic stress conditions (Ohta et al., 2001; Kazan, 2006). Key members of this group include Zat12 (At5g59820) and Zat10/AZF (At1g27730). Zat12 was initially identified as a light stress-response protein (Iida et al,
It was found to be involved in the defense response of plants to cold and oxidative stress, and was shown to be required for the expression of the defense enzyme cytosolic ascorbate peroxidase 1 (APX1) during oxidative stress (Rizhsky et al., 2004; Vogel et al., 2005). Constitutive expression of Zat12 results in the enhanced expression of a defense regulon that includes different transcripts involved in plant acclimation to high light and osmotic stress (Davletova et al., 2005b). In accordance, transgenic plants constitutively expressing Zat12 are more tolerant to high light, osmotic and oxidative stresses, and knockout plants lacking Zat12 are more sensitive to osmotic stress and salinity (Rizhsky et al., 2004; Davletova et al., 2005b). Zat10 was initially identified as a salt- drought- and cold-response protein (Sakamoto et al., 2000). It was shown to contain a functional EAR motif and to suppress the transcription of different reporter and defense genes (Ohta et al., 2001; Lee et al., 2002; Sakamoto et al., 2004). Constitutive expression of Zat10 was found to result in growth suppression and enhanced tolerance of plants to drought stress, osmotic stress and salinity (Sakamoto et al., 2004; Mittler et al., 2006). Interestingly, Zat10 loss-of-function lines are also more tolerant to osmotic and salinity stress, suggesting that Zat10 plays a dual role in modulating the defense response of plants to abiotic stresses (Mittler et al., 2006).

Although different Zat proteins were shown to suppress the transcription of reporter and defense genes (Ohta et al., 2001; Lee et al., 2002; Sakamoto et al., 2004), it is not clear whether the EAR-motif of these proteins is involved in this function (Kazan, 2006). In addition, it is not clear whether the enhanced tolerance of transgenic plants expressing different Zat proteins to abiotic stresses is mediated by the EAR-motif, and whether or not growth suppression observed in some of these plants is also a result of
transcriptional repression by the EAR-motif. Here we report that transgenic Arabidopsis plants expressing the C2H2 zinc-finger protein Zat7 (At3g46090) are more tolerant to salinity stress. Transgenic plants expressing Zat7 are suppressed in their growth. A deletion or a mutation of the EAR-motif of Zat7 abolishes salinity tolerance without affecting growth suppression. These results demonstrate that the EAR-motif of Zat7 is directly involved in enhancing the tolerance of transgenic plants to salinity stress. In contrast, the EAR-motif appears not be involved in suppressing the growth of transgenic plants. Further analysis of Zat7 using RNAi lines suggests that Zat7 functions to suppress a repressor of defense responses. A yeast two-hybrid analysis identified putative interactors of the EAR-domain. Our findings demonstrate that the EAR domain of Zat7 plays a key role in the defense response of Arabidopsis to abiotic stress.

Materials and Methods

Generation of 35S::Zat7-RNAi and 35S::Zat7, Zat7Δ and Zat7m plants

For the Zat7-RNAi3’ construct, a 115 bp fragment corresponding to 38 bp of Zat7 (At3g46090) coding sequence and 77bp of 3’UTR was PCR amplified from genomic DNA with ZAT7IR-1 (5’-CTCGAGGGATCCGGAAGTTGGAGTTGGGAAGA-3’) and ZAT7IR-2 (5’-GGTACCATCGATAATATTCACATCGATCGGTA-3’) primers and cloned into pCRScript (Stratagene). For the Zat7-RNAi5’ construct, a 120 bp fragment corresponding to coding sequence at the 5’ end of Zat7 was PCR amplified from genomic DNA with ZAT7IR-3 (5’-CTCGAGGGATCCATGGTTGCGAGAAGTGAGGA-3’) and ZAT7IR-4 (5’-GGTACCATCGATGAAAACGGTGTTGCGAGAAGTGGGAAG-3’) primers and cloned into pCRScript (Stratagene). After verification of the sequence, each of these Zat7
fragments was then subcloned into pHannibal in the sense and antisense directions (Wesley et al., 2001). The entire inverted repeat construct including the 35S promoter was digested from pHannibal and subcloned into pART27 as a NotI fragment. The 35S::ZAT7-3’IR/pART27 and 35S::ZAT7-5’IR/pART27 plasmids were transformed into Agrobacterium ASE by electroporation.

For the 35S::Zat7 construct, the Zat7 cDNA was amplified from genomic DNA using ZAT7-3 (5’-CCTAGAAGTCACTAAGTCGATTCAAAATGGTT-3’) and ZAT7-2 (5’-ATTGTATCAAAAATAATATTCCACAT-3’) and cloned into pCRScript (Stratagene). After verification of the sequence, the Zat7 cDNA was subcloned as an XhoI/NotI fragment into 35SpBARN (LeClere and Bartel, 2001). For the 35S::Zat7Δ construct, Zat7 was amplified from genomic DNA using ZAT7-3 and ZAT7-7 (5’-TTAACAAGCCACTCTCTTCCAC-3’) and cloned into pCRScript. After verification of the sequence, Zat7Δ was subcloned as an XhoI/NotI fragment into 35SpBARN. The resulting plasmid was transformed into Agrobacterium strain GV3101. 35S::Zat7m was made using overlap extension PCR. In the first round of PCR, two partially overlapping pieces of Zat7 were amplified using ZAT7-3 and ZAT7-7 (5’-TTAACAAGCCACTCTCTTCCAC-3’) and cloned into pCRScript. After verification of the sequence, Zat7Δ was subcloned as an XhoI/NotI fragment into 35SpBARN. The resulting plasmid was transformed into Agrobacterium strain EHA105.
Arabidopsis Ler plants were transformed using the floral dip method and transformants were selected on Gamborg’s B5 medium supplemented with the herbicide basta. Plants were grown in soil at 21-22°C, constant light, 100 µmol m⁻² sec⁻¹ and watered with 1X Peters 20-20-20 solution. Transgenic plants were tested by RNA blot analysis as previously described (Rizhsky et al., 2004; Davletova et al., 2005b). Knockout Apx1 plants were obtained and analyzed as previously described (Pnueli et al., 2003).

**Nucleic acid sequence analysis and bioinformatics**

RNA was isolated and analyzed by RNA blots as previously described (Davletova et al., 2005a). Analysis of microarray data available from [https://www.genevestigator.ethz.ch](https://www.genevestigator.ethz.ch) (Zimmermann et al., 2004), was performed as previously described (Miller and Mittler, 2006). Positively interacting prey clones from yeast two-hybrid screens were sequenced and the insert sequences were analyzed using the MacVector®/AssemblyLIGN™ sequence analysis programs (Accelrys, San Diego, CA). BLAST searches were performed at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). InterPro scan searches were performed using the InterPro Scan server ([http://www.ebi.ac.uk/interpro/scan.html](http://www.ebi.ac.uk/interpro/scan.html)) (Mulder et al., 2003). The WoLF protein subcellular localization prediction (PSORT) software ([http://wolfpsort.seq.cbrc.jp/](http://wolfpsort.seq.cbrc.jp/)) was used to predict putative protein subcellular localization (Horton et al., 2006).

**Stress Assays**

For the analysis of stress-tolerance, seeds of wild type and three independent 35S::Zat7, Zat7m, Zat7Δ, Zat7-RNAi3’ and Zat7-RNAi5’ lines were surface-sterilized
with bleach and placed in rows on 1% agar plates (0.5 X MS medium), containing different concentrations of NaCl or sorbitol as previously described (Rizhsky et al., 2004; Davletova et al., 2005b). Plates were placed at 4°C for 48 hours and maintained vertically in a growth chamber for 5 days (21-22°C, constant light, 100 µmol m⁻² sec⁻¹). Root length was scored at 3, 4 and 5 days after vernalization. Four- or five-day-old seedlings grown on 0.5 X MS agar plates were also subjected to heat (38°C) or cold stress (4°C) for different times, allowed to recover for 24 hours, and analyzed (Davletova et al., 2005b).

For soil stress experiments, 7-day-old seedlings of wild type and three independent 35S::Zat7, Zat7m, Zat7Δ lines were transferred to vermiculite, watered for 5 days with 0.1-0.5X Peters 20-20-20 solution and subjected to salinity stress by watering with 0, 50, 75, 100 or 150 mM NaCl prepared in 0.1-0.5X Peters 20-20-20 solution for 20 days. Plants were then photographed and sampled for RNA blot analysis. All experiments were performed with 3 to 5 technical replications, each containing 15-30 seedlings or plants per line, and repeated at least 3 times. Statistical analysis was performed as described in (Suzuki et al., 2005).

**Yeast two-hybrid analysis**

**Bait construction:** The Full-length ZAT7 and the last 25 amino acids were cloned into the pENTR vector with Gateway recombination sites. A Gateway cassette was introduced into the yeast two-hybrid vector pXDGATCY86 containing cycloheximide sensitive gene (CYH5) (Ding et al., 2004). Recombination between pENTR vectors and destination vectors were performed according to Invitrogen’s instructions (Invitrogen, Carlsbad, CA). After confirming that baits are in-frame with the DNA binding domain,
constructs were transformed into MaV204K yeast strain (Ito et al., 2000) (MATα, leu2-3,112; trp1-901; his3 Δ200; ade2-101; cyh2R; can1R; gal4 Δ; gal80 Δ; GAL1::lacZ; HIS3UASGAL1::HIS3@LYS2; SPAL10::URA3) as described in the Clontech Yeast Protocols Handbook. Baits were checked for autoreactivation of the reporter genes by growth on synthetic dropout (SD) -His/-Trp supplemented with 0, 1, 5, 7, 10 and 20 mM 3-AT (3-amino-1,2,4-triazole). Construction of the yeast prey library: The Matchmaker™ Library Construction and Screening Kit (Clontech, Mountain View, CA, USA) was used to construct a prey library in the pGADT7-Rec vector according to manufacturer’s instructions. Total RNA isolated from 14 day-old Arabidopsis Col-0 seedlings exposed to various treatments including NaCl (150 mM), Sorbitol (440 mM), 4°C, 42°C, high light (>300 μE/m²/sec), darkness, salicylic acid (100 μM), ABA (100 μM), Benzylaminopurine (100 μM), Methyl Jasmonate (100 μM), Indole acetic acid (100 μM), Gibberellic acid (100 μM), Paraquat (10 μM), wounding (with a needle), 2,4 D (100 μM), Brassinolide (100 μM), hydrogen peroxide (20 mM), desiccation (RT, 60% RH) and cell suspension (7 days old cultures), control and water deficit stressed roots, flowers, and siliques. Tissue was collected 30 min, 60 min, 90 min and 120 min after the start of each treatment. The cDNA ‘prey’ library was introduced into AH109 yeast cells (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ, MEL1) by in vivo recombination. Yeast two-hybrid screening: Bait strains were mated with the prey library according to manufacturer’s recommendations, followed by selection of interacting partners on solid SD media lacking Ade, His, Trp and Leu supplemented with 10 mM 3-AT. Positive and negative controls for interaction and
mating were performed following manufacturer’s recommendations. Positive interactions grew on solid SD media were selected three times on SD –Ade/-His/-Trp/-Leu supplemented with 10mM 3-AT. Bait plasmid was cured from positive clones that passed three rounds of selection by adding 1µg/ml of cycloheximide and grown overnight with shaking. Curing of bait plasmids was confirmed by PCR followed by plasmid preparation from cured clones containing only prey plasmids. Plasmids were then amplified from yeast using Rolling Circle Amplification (RCA) with the TempliPhi 100 Amplification Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) according to the manufacturer’s recommendations. The RCA products were used to transform yeast strains containing specific baits to verify interaction (Ding et al., 2003). Clones containing only prey were tested for autoactivated by growing them on SD- Leu/His and 10 mM 3-AT for 4 days. Preys capable of autoactivation of the reporter gene were excluded and verified clones were sequenced. Validation and β-Galactosidase Assays: Yeast stains with WRKY or HASTY genes as preys were mated one-on-one in parallel against the yeast expressing the full-length Zat7 bait and the last 25aa of Zat7 as bait as well as mating between the empty prey vector as a negative controls. AH109 strain harboring the pGADT7-RecT vector was mated with the MaV204K strain harboring the pGBK7-53 vector as a positive interaction control. Matting was performed in 1:1 ratios between each bait and prey pairs on 2X YPAD media for 24 hours followed by plating on SD media lacks Ade, His, Trp, and Leu supplemented with 10 mM 3-AT. Growth assays were performed in positive interaction pairs as well as empty bait vector grown on SD medium lacking Ade, His, Trp and Leu plus 10 mM 3-AT for 16 hours. OD_{600} was measured for 3 independent 100 µl culture and then assayed for β-galactosidase activity
using the Yeast β-galactosidase Assay kit (Cat # 75768; Pierce Biotechnology, Inc., Rockford, IL, U.S.A) following the manufacturer’s instructions. OD\textsubscript{600} and OD\textsubscript{420} were measured using the Perkin-Elmer Victor 3\textsuperscript{v} multiplate reader. The β-galactosidase activity was calculated using the equation: β-galactosidase activity = 1000X \( A_{420} / T \times V \times OD_{600} \) where (T) = time (in minutes) of incubation and (V) = volume of cells (ml) used in the assay.

RESULTS

Expression of Zat7 in Arabidopsis and phenotype of transgenic plants with constitutive expression of Zat7

Transcripts encoding at least 4 different members of the Zat protein family show broad response to biotic and abiotic stresses (Zat6, 10, 11 and 12; Mittler et al., 2006). In contrast, the expression of Zat7 appears to be more specific, with enhanced expression mainly in roots during salinity stress (Suppl. Fig. 1A, generated from transcriptome profiling data available at Zimmermann et al., 2004). In contrast to the early and transient expression of Zat12 during stress (Davletova et al., 2005a; Davletova et al., 2005b), the expression of Zat7 in Arabidopsis roots subjected to salinity stress is enhanced at 6, 12 and 24 hours, suggesting that Zat7 functions relatively late during salinity stress (Suppl. Fig. 1B). Expression of Zat7 was enhanced at concentrations as low as 50 mM NaCl (Fig. 1A). Expression of Zat7 was previously reported to be enhanced in Arabidopsis plants subjected to heat stress (Suzuki et al., 2005), as well as in knockout-\textit{Apx1} plants grown under controlled growth conditions (Pnueli et al., 2003; Davletova et al., 2005a),
suggesting that Zat7 expression could also be associated with heat or hydrogen peroxide stress.

In a previous report we expressed Zat7 in *Arabidopsis thaliana* cv. Columbia and found that expression of Zat7 resulted in growth suppression of transgenic plants (Rizhsky et al., 2004). Different studies on the expression of Zat12 in transgenic plants produced conflicting reports on the suppression of growth induced by this protein in transgenic plants (Rizhsky et al., 2004; Davletova et al., 2005b; Vogel et al., 2005). The discrepancies in phenotypes were linked to Zat12 expression level in the different plants, as well as to the use of different cultivars (Rizhsky et al., 2004; Vogel et al., 2005). To test whether Zat7 expression would also induce growth suppression in *Arabidopsis thaliana* cv Landsberg erecta (Ler), used in this study, we generated transgenic Ler lines with different levels of Zat7 expression. As shown in Fig. 1B, high level of Zat7 expression resulted in growth suppression. Constitutive expression of Zat7, therefore, could cause growth suppression in Columbia as well as in Landsberg erecta cultivars. Constitutive expression of different Zat proteins was shown to result in the enhanced expression of transcripts encoding different regulatory and defense proteins in transgenic plants (Davletova et al., 2005b; Vogel et al., 2005; Mittler et al., 2006). The enhanced expression of Zat7 in response to salinity stress (Fig. 1A and Suppl. Fig. 1), prompted us to test whether Zat7 expression in transgenic plants, grown under controlled conditions, is also associated with the elevated expression of different defense transcripts involved in the response of plants to salinity stress. As shown in Fig. 1C, the expression of different transcripts associated with salinity tolerance in plants (Apse et al., 1999; Blumwald,
2003) was elevated in transgenic plants expressing Zat7, grown under controlled conditions.

**Mutational analysis of the EAR-motif of Zat7**

To perform functional characterization of the EAR-motif of Zat7 we generated transgenic lines expressing two different variants of Zat7: a deletion of the C-terminal 25 aa region that contains the EAR-motif (Zat7Δ), and a site-specific mutagenesis converting the LDLDL EAR-motif at position 144-148 to LAAAL at the same position (144-148; Zat7m). As shown in Fig. 2, deletion or mutation of the EAR-domain did not alter the growth suppression phenotype of Zat7. This result indicated that the EAR-motif of Zat7 is not involved in suppression of plant growth in transgenic plants. In addition, it also suggested that a deletion or a mutation of the EAR-motif does not result in a significant decrease in Zat7 stability because plants with similar expression levels of Zat7, Zat7Δ or Zat7m showed a similar degree of growth suppression (Fig. 2).

**Salinity tolerance of transgenic plants expressing Zat7, Zat7Δ or Zat7m**

To test whether the EAR-motif of Zat7 is involved in the defense response of Arabidopsis to salinity stress, we subjected wild type plants (WT) and transgenic plants expressing Zat7 (Zat7), or Zat7 with altered EAR-motif (Zat7Δ or Zat7m) to salinity stress. As shown in Fig. 3A, seedlings of transgenic plants expressing Zat7 were more tolerant to salinity stress than seedlings of wild type plants. In contrast, seedlings of transgenic plants expressing Zat7m or Zat7Δ were more susceptible to salinity stress than wild type plants. As shown in Figs 3B and 3C, similar results were found with plants
grown in soil. Thus, compared to wild type plants, transgenic plants expressing Zat7 were more tolerant, and transgenic plants expressing Zat7m or Zat7Δ were more susceptible to salinity stress.

The enhanced sensitivity of transgenic plants expressing Zat7m or Zat7Δ to salinity stress could indicate that Zat7 protein, which lacks a functional EAR-motif (i.e., Zat7m or Zat7Δ), functions in transgenic plants as dominant-negative suppressor of the endogenous Zat7 protein. Thus, it could compete with the endogenous Zat7 protein for protein, RNA or DNA interactions, but it lacks the capability to activate defenses, thus causing more susceptibility.

Enhanced sensitivity of Zat7-RNAi lines to salinity stress

The results obtained with transgenic plants expressing Zat7m and Zat7Δ subjected to salinity stress (Fig. 3) suggested that suppression of Zat7 function in plants will result in enhanced sensitivity to salinity stress. To further test this possibility we generated RNAi lines to Zat7. Two different sets of lines were generated: Zat7-RNAi3’, in which the RNA repeat was directed at the 3’ part of the cDNA, and Zat7-RNAi5’, in which the RNA repeat was directed at the 5’ part of the cDNA (Fig. 4A). RNA blot analysis of RNAi lines revealed that despite the use of a 35S promoter, no accumulation of Zat7 RNA was observed in plants grown under controlled conditions (Fig. 4A). Expression of Zat7 was suppressed to 10-20% that of wild type in Zat7-RNAi3’ lines during stress, and expression of Zat7 was suppressed to 5% that of wild type in Zat7-RNAi5’ during stress (not shown). Expression of Zat8 (the closest homolog of Zat7) was unchanged in Zat7-
RNAi3’ lines, but was 70% that of wild type in Zat7-RNAi5’ (not shown). No growth suppression was observed in 5’ or 3’ Zat7-RNAi lines (Fig. 4A).

As shown in Fig. 4B and 4C, seedlings of 3’ or 5’ RNAi lines for Zat7 were more sensitive to salinity stress. This sensitivity was mainly observed in 3-5 day-old seedlings subjected to 100 mM NaCl. The results shown in Figs. 3 and 4 suggest that suppression of Zat7 function in plants could result in enhanced susceptibility to salinity stress.

**Identifying putative interactors of Zat7**

Our mutational analysis of the EAR-motif of Zat7 suggested that this domain plays a key role in the defense response of Arabidopsis to salinity stress (Fig. 3). This observation prompted us to test what other proteins in Arabidopsis interact with Zat7 and its EAR-domain. To this end we performed a yeast two-hybrid analysis of Zat7 using, as two independent baits, the full-length clone of Zat7, as well as a clone containing the last 25 aa of Zat7 that includes the EAR-motif. As prey we used a library constructed from Arabidopsis seedlings subjected to different abiotic stresses. As shown in Table 1 and Suppl. Table 1, several different proteins were found to interact in this system with both the full-length clone and the last 25 aa of Zat7. Taking into consideration parameters such as predicted subcellular localization, transcript expression in roots during salinity stress, and proteins that typically interact with the bait in this system (i.e., yeast two-hybrid artifacts), nine proteins were identified as potentially interacting with the EAR-domain (Table 1). All interactors were validated by directed interaction assays (see example in Fig. 5A), and β-galactosidase reporter activity (see example is Suppl. Fig. 2). Of the nine interactors, a protein involved in miRNA transport (HASTY; Park et al., 2005),
WRKY70 (Li et al., 2006), and a protein kinase appear to be possible candidates involved in signal transduction events associated with Zat7 function (Table 1, Fig. 5A). In contrast to the interaction of Zat7 with WRKY70 and HASTY (Table 1, Fig. 5A), Zat7Δ or Zat7m did not interact with WRKY70 or HASTY in a directed yeast two-hybrid interaction assay (not shown). Interestingly, constitutive expression of Zat7 resulted in enhanced expression of WRKY70 (Fig. 1C).

WRKY70, Zat7 and HASTY were reported to be co-expressed in knockout Apx1 (KO-Apx1) plants grown under controlled conditions (Table 1; Pnueli et al., 2003; Davletova et al., 2005a). These findings could suggest that a pathway leading to enhanced salinity tolerance is activated in KO-Apx1 plants and that this pathway involves WRKY70, Zat7 and HASTY. To test whether the co-expression of these transcripts in KO-Apx1 plants is associated with enhanced tolerance to salinity stress, we subjected KO-Apx1 and wild type plants to the same salinity stress assays shown in Fig. 3. Surprisingly, as shown in Fig. 5B, KO-Apx1 plants were significantly more tolerant to salinity stress than wild type plants.

**Tolerance of transgenic plants that constitutively express Zat7, Zat7Δ or Zat7m to cold and osmotic stress**

The EAR-domain is found in several different C2H2 zinc finger proteins that are thought to be involved in regulating the response of plants to abiotic stress conditions (Kazan et al., 2006). However, the specificity and the degree of cooperation between different Zat proteins such as Zat10, Zat12 and Zat7 are unclear at present. The availability of transgenic plants that constitutively express Zat7, Zat7Δ or Zat7m
prompted us to test how these lines would behave in response to abiotic stresses other than salinity, and whether or not the Zat7Δ or Zat7m lines would suppress the tolerance of plants to these stresses (similar to their effect on the tolerance of plants to salinity stress; Fig. 3). To this end we tested the tolerance of transgenic plants that constitutively express Zat7, Zat7Δ or Zat7m to osmotic, cold and heat stresses.

As shown in Fig. 6A, transgenic plants that constitutively express Zat7 were more tolerant to cold stress. Transgenic plants that constitutively express Zat7Δ or Zat7m were not, however, more susceptible to cold. In contrast, transgenic plants that constitutively express Zat7 were more susceptible to osmotic stress (Fig. 6B). Although transgenic plants that constitutively express Zat7Δ or Zat7m were also more susceptible to osmotic stress, they appear to be less susceptible to this stress than transgenic plants that constitutively express Zat7 (Fig. 6B). The response of transgenic plants that constitutively express Zat7, Zat7Δ or Zat7m to heat stress was not significantly different from that of wild type (not shown). The results presented in Figs. 3 and 6, as well as those reported for Zat12 by Davletova et al., (Davletova et al., 2005b), provide an initial glimpse into the complex nature of the C2H2-EAR-domain zinc finger network and its role in regulating abiotic stress responses.

Discussion

Zinc-finger proteins that contain the EAR transcriptional repressor domain are thought to play a key role in regulating the defense response of Arabidopsis to abiotic stress. Their expression level is elevated during different abiotic stresses, and they were shown to suppress transcription of different endogenous and/or reporter genes (Sakamoto
et al., 2000; Lee et al., 2002; Rizhsky et al., 2004; Sakamoto et al., 2004; Davletova et al., 2005b; Vogel et al., 2005; Kazan, 2006). Nonetheless, the role the EAR-domain of these proteins play in the defense response of plant to abiotic stress is unclear (Kazan, 2006; Mittler et al., 2006). Here we show that mutation or deletion of the EAR-domain of Zat7 renders this protein incapable of enhancing plant tolerance to abiotic stress (Fig. 3). Moreover, constitutive expression of Zat7 with a mutation in the EAR domain causes plants to become more sensitive to stress (Fig. 3). These results strongly suggest that the EAR-domain of Zat7 plays a key role in the defense response of Arabidopsis to salinity stress.

Constitutive expression of different C2H2-EAR-motif-containing, proteins, including Zat12, Zat10 and Zat7, was shown to enhance the tolerance of transgenic plants to abiotic stresses (Rizhsky et al., 2004; Sakamoto et al., 2004; Davletova et al., 2005b; Vogel et al., 2005; Mittler et al., 2006). Based on previous findings that the EAR-domain functions as a transcriptional repressor (Ohta et al., 2001; Lee et al., 2002; Kazan, 2006), and that its function is essential for enhancing plant tolerance to abiotic stress in transgenic plants (Figs. 3 and 6A), it is possible that the enhanced tolerance of transgenic plants expressing different EAR-containing Zat proteins is a result of these proteins suppressing a repressor of defense responses (Fig 7). Thus, when a Zat protein is constitutively expressed in transgenic plants it suppresses a repressor, removes its inhibition of defense responses, thereby causing the activation of plant defenses (Figs. 1C, 7), as well as the enhancement of plant tolerance to stress (Figs. 3 and 6A). Based on this model, loss-of-function mutations of Zat proteins should result in a decreased tolerance to stress because the repressor of defense responses is not removed. This
prediction was confirmed for Zat12 (Davletova et al., 2005b) and Zat7 (Fig. 4). The extent of defense response activation caused by the constitutive expression of a Zat protein in Arabidopsis could be estimated from microarray studies of Zat12 that identified 42 different defense and regulatory transcripts elevated in response to the constitutive expression of this protein (Davletova et al., 2005b; see also Vogel et al., 2005). In the case of Zat7, several transcripts encoding defense and regulatory transcripts, involved in plant tolerance to salinity stress (Apse et al., 1999; Blumwald, 2003), are elevated in Zat7 expressing plants (Figs. 1C and 7).

The finding that constitutive expression of Zat7 proteins with a mutated EAR-domain caused plants to become more susceptible to abiotic stress could be viewed as additional evidence for the model proposed in Fig. 7. Thus, EAR-less Zat7 proteins could physically interact with the repressor of defense responses, but could not cause its suppression. However, by interacting with this repressor they prevent the endogenous Zat7 protein from performing its function, thereby generating a dominant-negative effect that prevents the removal of the suppressor (Fig. 3).

Constitutive expression of different Zat proteins was found to cause growth suppression in transgenic plants (Rizhsky et al., 2004; Sakamoto et al., 2004; Vogel et al., 2005). Although it is tempting to speculate that the growth suppression of transgenic plants is a result of the transcriptional repressing activity of these Zat proteins, our findings show that growth suppression in transgenic plants that constitutively express Zat7 is independent of the EAR-domain. This finding is important for the interpretation of another aspect of our results, namely the enhanced tolerance of transgenic plants to abiotic stress. Enhanced tolerance of transgenic plants to abiotic stresses has been
suggested, in some cases, to be the outcome of growth suppression, because plants with suppressed growth are less sensitive to stress (Mittler et al., 2001; Blumwald, 2003; Vinocur and Altman, 2005). However, our results show that the suppressed growth of transgenic plants expressing Zat7 does not correlate with enhanced tolerance (Figs. 2 and 3). What could be the cause of growth suppression in Zat7 over-expressing plants? One possibility is that the constitutively expressed Zat protein interacts with different endogenous Zat proteins involved in regulating plant growth and development and disrupts their function. It is possible that the function of different C2H2-zinc-finger proteins is coordinated within the context of a global regulatory network in plants (Englbrecht et al., 2004), and that constitutive expression of a particular Zat protein could potentially interfere with this network, especially because zinc-finger proteins tend to interact with each other or with other proteins. The differential tolerance of Zat7-expressing plants to cold and osmotic stress could serve as possible evidence for a complex mode of interaction between different zinc-finger proteins (Fig. 6). Cold and osmotic stresses are accompanied by elevated expression of Zat6, Zat10 and Zat12 (Mittler et al., 2006). Constitutive expression of Zat7 enhances plant tolerance to cold stress, yet it also causes plants to become more susceptible to osmotic stress (Fig. 6). Thus, a complex mode of interaction may exist between different defense pathways associated with, or regulated by, different zinc-finger proteins.

The tendency of zinc-finger proteins to interact with different cellular proteins (as well as with RNA and DNA), has made our analysis of protein-protein interactions complex. Thus, a large number of potential yeast two-hybrid artifacts is expected to be identified in a screen using a zinc-finger protein such as Zat7. Nevertheless, our analysis
(Table 1) identified several interesting proteins that could be linked to Zat7 function, including WRKY70, a protein kinase and a protein involved in miRNA transport (HASTY). Zat7, WRKY 70 and HASTY are constitutively co-expressed in knockout plants lacking APX1 (Pnueli et al., 2003; Davletova et al., 2005a). Interestingly, compared to wild type plants, KO-Apx1 plants, that express Zat7, WRKY70 and HASTY, are more tolerant to salinity stress (Fig. 5B). WRKY 70 was recently shown to function as a convergence point for jasmonic and salicylic acid mediated signals in Arabidopsis and is likely to play an important role in abiotic stress tolerance (Li et al., 2004; Li et al., 2006). Micro-RNAs were recently shown to be important for regulating defense responses to abiotic stress (Sunkar et al., 2006). The co-expression of Zat7, WRKY 70 and HASTY in KO-Apx1 plants, their potential interactions (Table 1), and the enhanced tolerance of KO-Apx1 plants to salinity stress (Fig. 5B), could suggest that these proteins participate in mediating different stress-response signals related to salinity stress. The different proteins indicated in Table 1 could serve as a basis for future studies on the function of Zat7 and the EAR-domain in Arabidopsis.
Table and Figure Legends

**Table 1.** Proteins that interact with Zat7 and/or the last 25 aa of Zat7 in a yeast two-hybrid screen. The proteins presented in the table were selected from the complete list of interactors (Suppl. Table 1) based on their predicted subcellular localization and expression. Locus identifiers are given on left followed by clone description, BLAST E-value for clone identification, predicted localization based on clone annotation in MIPS, TAIR and PSORT, and expression in roots of Arabidopsis subjected to salinity stress as obtained from [https://www.genevestigator.ethz.ch](https://www.genevestigator.ethz.ch) (Zimmermann et al., 2004), or in knockout plants deficient in cytosolic ascorbate peroxidase 1 grown under controlled conditions (KO-\textit{Apx1};16, 17). Abbreviations: NF, not found; NC, no change in expression; up, expression enhanced by 2-fold or more.

**Figure 1.** Expression of Zat7 in Arabidopsis and phenotype of transgenic plants with constitutive expression of Zat7. A. RNA blot showing enhanced expression of Zat7 in Arabidopsis plants subjected to salinity stress. B. Suppression of growth in transgenic plants expressing Zat7. Top, DNA construct used for Zat7 expression in transgenic plants. Middle, photograph of control and transgenic plants with different expression levels of Zat7 (2, 8 and 37 represent independent lines expressing Zat7). Bottom, RNA gel blots showing the expression level of Zat7 in the different transgenic plants shown above. C. RNA blots showing enhanced expression of transcripts encoding different defense and regulatory proteins in transgenic plants expressing Zat7, grown under
controlled conditions. Plant transformation, growth, stress treatments and analysis by RNA blots were performed as described in Materials and Methods.

**Figure 2.** Growth suppression in transgenic plants with constitutive expression of Zat7, Zat7Δ or Zat7m. A. DNA construct used to generate the Zat7Δ lines. B. DNA construct used to generate the Zat7m lines. C. Photograph of control and transgenic plants expressing Zat7, Zat7Δ or Zat7m. D. RNA gel blot of RNA obtained from the plants shown in C. Plant transformation, growth and analysis by RNA blots were performed as described in Materials and Methods.

**Figure 3.** Differential tolerance of transgenic plants with constitutive expression of Zat7, Zat7Δ or Zat7m to salinity stress. A. Tolerance of plants to salinity stress measured in seedlings subjected to different concentrations of NaCl. B. Tolerance of plants to salinity stress observed in soil-grown plants subjected to different concentrations of NaCl. C. Quantification of % survival for soil-grown plants subjected to 150 mM NaCl. Stress assays and statistical analysis were performed as described in Materials and Methods. **, t-test significant at p < 0.01.

**Figure 4.** Characterization of RNAi lines for Zat7. A. Construction of RNAi lines. Top, DNA constructs used to generate the Zat7-RNAi3’ and 5’ lines. Middle, photograph of Wild type (WT), Zat7-RNAi3’ (RNAi3’) and a 35S::Zat7 lines [Zat7(2)]. Bottom, RNA gel blot of RNA obtained from the plants shown above. B and C. Tolerance of two
independent Zat7-RNAi5’ (B), and two independent Zat7-RNAi3’ (C) lines to salinity stress. Plant transformation, analysis by RNA blots and stress assays were performed as described in Materials and Methods. **, t-test significant at p < 0.01; *, t-test significant at p < 0.05.

**Figure 5.** Interaction of WRKY70 with Zat7 and tolerance of knockout Apx1 (KO-Apx1) plants to salinity stress. A. Picture of a directed interaction assay showing that WRKY70 interacts with both the full-length Zat7 (Zat7), as well as the 25 aa fragment of Zat7 (25 aa) that contains the EAR-domain. B. Graph showing that knockout plants deficient in Apx1 (KO-Apx1) are more tolerant to salinity stress than wild type plants (WT). KO-Apx1 plants were previously shown to constitutively co-express Zat7, WRKY70 and HASTY (Pnueli et al., 2003; Davletova et al., 2005a). Yeast mating and stress assays were performed as described in Materials and Methods. **, t-test significant at p < 0.01.

**Figure 6.** Differential tolerance of transgenic plants with constitutive expression of Zat7, Zat7Δ or Zat7m to cold and osmotic stress. A. Differential tolerance of Zat7, Zat7Δ or Zat7m to cold stress. B. Differential tolerance of Zat7, Zat7Δ or Zat7m to osmotic stress. Stress assays and statistical analysis were performed as described in Materials and Methods. **, t-test significant at p < 0.01.

**Figure 7.** A model showing the putative function of Zat7 during salinity stress in Arabidopsis. The EAR-motif of Zat7 is shown to suppress the activity of a suppressor
that is a negative regulator of defense response activation during salinity stress. Accumulation of Zat7 during stress will therefore result in the suppression of the suppressor and the acquisition of enhanced tolerance to salinity stress. Suppression of growth by constitutive expression of Zat7 is shown to be mediated by a different domain of Zat7 (unknown at present).

**Supplementary Figure 1.** Expression of Zat7 in Arabidopsis. **A.** Expression of Zat7 in leaves and roots in response to biotic and abiotic stresses. Expression is presented as fold change and is the average of two independent measurements. **B.** Expression of Zat7 in leaves and roots in response to salinity stress. Expression is presented as fold change and is the average of two independent measurements. Expression of Zat7 was obtained from [https://www.genevestigator.ethz.ch](https://www.genevestigator.ethz.ch) (18) as described in Materials and Methods.

**Supplementary Figure 2.** Fresh weight of 4-week-old wild type and transgenic plants expressing Zat7 and Zat7 mutants. Results are average and SE of 35 different plants per line. Please see Materials and Methods for details on growth conditions.

**Supplementary Figure 3.** Validation of yeast two-hybrid interactions for WRKY70 and HASTY by β-Galactosidase Assays. Please see Materials and Methods for details.
**Supplementary Figure 4.** Directed yeast two-hybrid assays demonstrating that WRKY70 does not interact with Zat7m or Zat7 Δ. Please see Materials and Methods for details on yeast two-hybrid assays.

**Supplementary Figure 5.** Directed yeast two-hybrid assays demonstrating that HASTY does not interact with Zat7m or Zat7 Δ. Please see Materials and Methods for details on yeast two-hybrid assays.
Figure 1

A

NaCl (mM)

0 50 100 150

Zat7

rRNA

B

CaMV35S  Zat7  NOS ter

WT  37  8  2

C

WT  Zat7

Zat7

rRNA

WRKY70

AOX1

NHX1

Cor78

APX1

rRNA
Figure 2

A △  CaMV35S Zat7 NOS ter

B m  CaMV35S Zat7 NOS ter

L_{144}DLDDL_{148} \rightarrow L_{144}AAAL_{148}

C

WT  Zat7  Zat7\triangle  Zat7m

D

WT  Zat7  Zat7\triangle  Zat7m

Zat7

rRNA
Figure 3

A

B

C
Figure 4

A

5' Zat7 3'

Zat7-RNAi5'

CaMV35S OSC ter

CaMV35S OSC ter Zat7-RNAi3'

WT RNAi3' Zat7(2)

WT RNAi3' Zat7(2)

Zat7

rRNA

B

120

% Control (Root Length)

0 50 100 150 200

NaCl (mM)

C

140

% Control (Root Length)

0 50 100 150 200

NaCl (mM)
Figure 5

A

25aa+WRKY70  WRKY70
25aa
25aa+Empty vector
Zat7+WRKY70  Zat7
Zat7+Empty vector
Positive control

B

Root Growth (% Control)

NaCl (mM)

0 50 100 150 200

W
KO-

**

**

25aa
25aa+Empty vector
Zat7+Empty vector
Zat7+WRKY70  Zat7

Positive control
Figure 6

A

B

- WT
- 35S Zat7
- 35S Zat7-Δ
- 35S Zat7-m

**Root Length (% of Control)**

**Cold Stress (Days)**

0 1 2 3

0 20 40 60 80 100 120

**Root Length (% of Control)**

**Sorbitol (mM)**

0 50 100 200 300

0 20 40 60 80 100 120
Figure 7

NaCl Stress → Zat7 → EAR-motif Repressor → Defense Responses: WRKY70, AOX1, Cor78, NHX1 → Tolerance

Unknown domain → ? → Growth Suppression
Supplementary Figure 1

A

B

Field Expression

Leaf

Root

Cold

Heat shock

Drought

Salt

Oxidative

Wound

Osmotic

Genetic

UV-B

Field Expression

30 min

1hr

3hr

6hr

12hr

24hr
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
Supplementary Table 1

Supplementary Table 1. Proteins that interact with Znt7 in a yeast two-hybrid screen

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Clones that interact with last 25aa only

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Clones that interact with full-length only

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NF = Not Found
NC = No Change
up = expression increased 2 fold or more
down = expression decreased 2 fold or more
References


function as transcription repressors under drought, cold, and high-salinity stress conditions. Plant Physiol. 136, 2734-2746.


Acknowledgment

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Chapter 3

Characterization of zat10/zat12

Double-Knockout Arabidopsis Plants
Abstract

Both Zat10 and Zat12 are C2H2-type zinc-finger proteins that play key roles in the abiotic stress response network of Arabidopsis. Recent studies showed that both proteins could be involved in the cold stress response of plants and could act in a coordinated manner. To investigate the relationship between Zat10 and Zat12 during cold stress in Arabidopsis, we produced a zat10/zat12 double knockout. Double and single knockouts as well as WT were subjected to cold stress. The zat10/zat12 plants showed enhanced sensitivity to cold treatment whereas, wt, zat10 and zat12 plants did not. Our results suggest that there are at least two different cold stress response pathways; one involves Zat10, and the other involves Zat12. These pathways are linked to each other and could function in a complementary manner.
Introduction

C2H2-type zinc finger proteins play important roles in many metabolic pathways, as well as in stress response and defense activation in plants. Recent studies emphasized the importance of C2H2-type zinc finger proteins with a putative repression activity to the defense and stress response network of plants. Most of these proteins are thought to acquire their repression activity via their ERF-associated amphiphilic repression (EAR) domain. Key members of this group include Zat10/STZ and Zat12. Both proteins play a key role in the response of plants to different abiotic stresses. Transcriptome profiling analysis has shown that the steady-state transcript level of these zinc finger proteins is elevated during different stress conditions including cold stress (Mittler et al., 2006). Other studies have also indicated that both proteins could be involved in the cold stress response of Arabidopsis (Chinnusamy et al., 2007).

Transcriptome analysis of Arabidopsis in response to cold revealed that 302 genes were up-regulated and 212 genes were down-regulated in response to low temperature (Vogel et al., 2005). Genes highly regulated were assigned to two main regulons: CBF (C-repeat binding factor) and a regulon controlled by Zat12 (Vogel et al., 2005). Constitutive expression of CBF3 (C-repeat binding factor, also known as dehydration-responsive element-binding protein 1A or DREB1A) that participates in cold acclimation resulted in enhanced expression of Zat10 (Maruyama et al., 2004). CBF3 is one member of the CBF regulon that regulates expression of COR (Cold Responsive) genes in Arabidopsis (Chinnusamy et al., 2007). Enhanced expression of CBF regulon and COR genes increased freezing tolerance of transgenic Arabidopsis plants that constitutively express ICE1 (Inducer of CBF Expression 1) (Chinnusamy et al., 2007). Moreover,
mutation in ICE1 lead to a decrease in CBF3 transcript level that eventually resulted in a decrease in Zat10 transcript level in response to cold stress (Chinnusamy et al., 2007). Zat10 can bind to the RD29A promoter and repress its transcription suggesting that Zat10 could regulate RD29A transcription during stress (Lee et al., 2002). RD29A is a COR gene that is regulated by the CBF regulon. This data suggested that Zat10 might act downstream of the CBF regulon and might regulate a subset of COR genes. Interestingly, these studies have also suggested that Zat12 acts upstream of Zat10 by negatively regulating the CBF regulon (Vogel et al., 2005; Chinnusamy et al., 2007). These data, therefore, suggest that Zat10 and Zat12 play a coordinated role in the response of Arabidopsis to cold stress.

To study the relationship between Zat10 and Zat12 during the cold stress response of Arabidopsis, we obtained zat10/zat12 line and subjected it to cold stress. Here we report that zat10/zat12 plants showed enhanced sensitivity to cold stress, whereas both individual zat10 and zat12 plants were undistinguishable from WT plants in their response to cold. Additionally, zat10/zat12 plants showed enhanced accumulation of hydrogen peroxide when compared with wild-type and single knockout plants in response to cold.

Materials and Methods

Generation of zat10, zat12 and zat10/zat12 plants

Zat10 and Zat12 knockout lines were obtained as previously described in Davletova et al., (2005) and Mittler et al., (2006). Knockout lines were crossed to each other to obtain a line that is a knockout for both Zat10 and Zat12. Seeds from the first cross were
selfed and T2 generations were screened for the zat10/zat12 homozygous line. For Zat10 and Zat12 PCR genotyping the following primers were used: Zat10-F (5′-TCGAGAGACAAGAAATCCTC-3′), Zat10-R (5′-CGAAATCTTATCGTCTAAGT-3′), Zat12-F (5′-AAAGCAAAACCATAGGTTTTTG-3′), Zat12-R (5′-CCTTCTTCATCAATCCAGACG -3′). To investigate growth phenotypes, seedlings of wild-type, zat10, zat12 and zat10/zat12 plants were grown at 21-22°C, constant light, 100 µmol m⁻² sec⁻¹ for three weeks.

**Cold Stress Assay**

For the analysis of cold stress-response, seeds of wild type, zat10, zat12 and zat10/zat12 plants were surface sterilized with bleach and put in rows on 0.8% agar plates containing 0.5X MS medium (Davletova et al., 2005b). For vernalization, seeds were kept in 4°C for 2 days. Then, plates were placed vertically in a growth chamber for 3 days (21-22°C, constant light, 100 µmol m⁻² sec⁻¹). Plates were then kept at 4°C vertically for different times, allowed to recover for 24 hrs, and analyzed (Davletova et al., 2005b). All experiments were repeated 3 times with 3 replicates for each time. Each replicate contained 10-20 seedlings per line. To determine the statistical significance a student’s t test was used.

To analyze hydrogen peroxide accumulation in seedlings subjected to cold stress, 4-day-old seedlings were kept at 4°C for one day and treated with 0.2 µM Amplex® Red (Molecular Probes, Inc., Eugene, OR) for 30 minutes. Seedlings were then imaged with a Kodak 2000MM image station, 535nm for excitation, 600 nm for emission. Generated images were than analyzed by ImageJ 1.41o (Wayne Rasband, NIH, USA).
**Results**

**Enhanced sensitivity of zat10/zat12 plants to cold stress**

Previous studies have suggested that Zat10 and Zat12 act in a coordinated manner in the response to cold stress in Arabidopsis (Figure 1) (Chinnusamy et al., 2007). To investigate the relationship between Zat10 and Zat12 during cold stress, we obtained zat10/zat12 plants by crossing zat10 (SALK_054092) and zat12 (SALK_037357) plants (Davletova et al., 2005b, Mittler et al., 2006). As seen in Figure 2, when grown under controlled growth conditions (21-22°C, constant light, 100 µmol m⁻² sec⁻¹) zat10/zat12 plants were indistinguishable from wild-type, zat10 and zat12 plants. zat10/zat12 seedlings were kept at 4°C for 3 days and grown vertically for 3 days at 21-22°C, constant light, 100 µmol m⁻² sec⁻¹. Plates were then placed at 4°C vertically for 2 or 3 days, allowed to recover for 24 hours, and analyzed for root growth. Root growth of wild-type, zat10 and zat12 was not found to be significantly affected by cold treatment when compared with control group kept at 21-22°C (Figure 3). In contrast, zat10/zat12 plants showed enhanced sensitivity in response to cold (Figure 3).

Previous studies indicated that Zat12 plays a role in ROS signaling (Davletova et al., 2005). Constitutive expression of Zat10 resulted in enhanced expression of ROS-related genes suggesting that Zat10 is also involved in ROS metabolism (Mittler et al., 2006). To test whether the enhanced sensitivity of zat10/zat12 plants was accompanied by ROS accumulation, we measured hydrogen peroxide accumulation in wild-type, single knockout and double knockout lines. Four-day old seedlings of wild-type, zat10, zat12 and zat10/zat12 plants were kept at 4°C for one day. Then, they were treated with Amplex® Red and analyzed. zat10/zat12 plants showed increased accumulation of...
hydrogen peroxide whereas single knockout plants did not show enhanced accumulation of hydrogen peroxide when compared with wild-type plants in response to cold (Figure 4).

**Discussion**

Cold stress is one of the major abiotic stresses that negatively affects plant growth and development (Chinnusamy et al., 2007). Recent studies have suggested that many pathways are activated in response to cold stress in plants at the transcriptional, post-transcriptional and post-translational level (Chinnusamy et al., 2007). Transcriptional level regulation of the cold response was assigned to two main regulons; CBF and Zat12 that regulate COR genes (Vogel et al., 2005). Interestingly, constitutive expression of Zat12 dampened CBF1, CBF2 and CBF3 expression during cold acclimation and increased freezing tolerance of Arabidopsis (Vogel et al., 2005). Increased expression of CBF2, CBF3 and COR genes as a result of over-expression of ICE1 gene also increased freezing tolerance of Arabidopsis (Chinnusamy et al., 2007). These data suggested that Zat12 acts upstream of CBF regulon as a negative regulator (Figure 1).

Zat10 is regulated by CBF3 during cold acclimation (Chinnusamy et al., 2007). Increased expression of CBF3 resulted in enhanced expression of Zat10, whereas decreased expression of CBF3 resulted in decreased expression of Zat10 (Chinnusamy et al., 2007). Additionally, in a transient expression assay Zat10 repressed expression of RD29A, one of the COR genes regulated by the CBF regulon, suggesting that Zat10 might regulate a subset of COR genes downstream of the CBF regulon. Both Zat10 and Zat12 are up-regulated in response to similar abiotic stresses including cold suggesting
that Zat10 and Zat12 act in a coordinated manner and might be members of the same cold response pathway.

Our data demonstrated that, individual *zat10* and *zat12* plants did not show enhanced sensitivity in response to cold, whereas *zat10/zat12* plants showed enhanced sensitivity. These data suggest that there are at least two main pathways that regulate the cold stress response of Arabidopsis at the transcriptional level; one includes CBFs and Zat10 and the other includes Zat12. These two pathways are linked to each other, but could function at individual levels. When both pathways are defective, plants became more sensitive to cold. Even though plants became more sensitive to cold stress, the lack of Zat10 and Zat12 was not fatal suggesting that additional pathways function in parallel to Zat10 and Zat12 during cold acclimation in Arabidopsis, or that a backup pathway is activated in response to cold when both pathways were defective. To obtain additional information about the involvement of Zat10 and Zat12 proteins in the cold stress response, the relationship between Zat10, Zat12 and the CBF regulon must be investigated.

Previous studies indicated that Zat12 is involved in ROS metabolism (Davletova et al., 2005). Constitutive expression of Zat10 resulted in enhanced expression of ROS-related transcript indicating that Zat10 might also be involved in ROS metabolism (Mittler et al., 2006). Our data showed that *zat10/zat12* plants accumulated significantly more hydrogen peroxide when compared with wild-type, *zat10* and *zat12* in response to cold (Figure 4). This accumulation could be a result of an impaired ROS metabolism. Further investigation of ROS transcript accumulation in single and double knockout plants is needed to validate these data.
Our research indicates that the cold stress response is a complex network involving many different and possibly redundant pathways. Although, zinc-finger proteins play an important role in this network, there are still many undiscovered parts of the cold stress response network that need to be investigated, possibly including genes of unknown function.
Figure Legends

**Figure 1.** A diagram showing the suggested relationship between Zat10 and Zat12 during cold response in *Arabidopsis*. (Diagram is based on Chinnusamy et al., 2007, Figure 1.)

**Figure 2.** Phenotype of *zat10/zat12* plants grown under the controlled growth conditions.

**Figure 3.** Differential cold stress response of WT, *zat10*, *zat12* and *zat10/zat12* plants.

**, t-test significant at p < 0.01; *, t-test significant at p < 0.05.**

**Figure 4.** Hydrogen peroxide accumulation in WT, *zat10*, *zat12* and *zat10/zat12* seedlings in response to cold stress. *, t-test significant at p < 0.05.
Figure 1

Low temperature

Kinases

ICE1

ZAT12

LOS2

CBF regulon

ZAT10

COR genes

Cold acclimation
Figure 2

WT  zat10  zat12  zat10/zat12
Figure 3

Figure 3 shows the root elongation (% control) under different conditions of cold stress (Days). The graph compares WT, zat10, zat12, and zat10/zat12 genotypes. The x-axis represents cold stress in days (0, 2, 3), and the y-axis represents root elongation (% control) from 0 to 140. The data points indicate a decrease in root elongation with increasing cold stress for all genotypes.
Figure 4

[Graph showing percentage control over cold stress (Days) with different genotypes: WT, zat10, zat12, and zat10/zat12.]

- **WT**
- **zat10**
- **zat12**
- **zat10/zat12**
References


Chapter 4

Enhanced tolerance to oxidative stress in transgenic Arabidopsis thaliana plants expressing proteins of unknown function

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**Abstract**

Over a quarter of all plant genes encode proteins of unknown function which can be further classified as Proteins with Obscure Features (POFs), that lack currently defined motifs or domains, or Proteins with Define Features (PDFs), that contain at least one previously defined domain or motif. Although empirical data in the form of transcriptome and proteome profiling suggest that many of these proteins play important roles in plants, their functional characterization remains one of the main challenges in modern biology. To begin the functional annotation of proteins with unknown function, which are involved in the oxidative stress response of *Arabidopsis thaliana*, we generated transgenic Arabidopsis plants that constitutively expressed 23 different POFs (of which 4 were specific to Arabidopsis), and 18 different PDFs. All previously found to be expressed in response to oxidative stress in Arabidopsis. Transgenic plants were tested for their tolerance to oxidative stress imposed by paraquat or t-butyl hydroperoxide, or subjected to osmotic, salinity, cold and heat stresses. More than 70% of all expressed proteins conferred tolerance to oxidative stress. In contrast, over 90% of the expressed proteins did not confer enhanced tolerance to the other abiotic stresses tested, and about 50% rendered plants more susceptible to osmotic or salinity stress. Two Arabidopsis-specific POFs, and an Arabidopsis and Brassica-specific protein of unknown function, conferred enhanced tolerance to oxidative stress. Our findings suggest that tolerance to
oxidative stress involves mechanisms and pathways that are unknown at present, including some that are specific to Arabidopsis or the Brassicaceae.
**Introduction**

On average, 20-40% of all eukaryotic genomes sequenced to date contain genes that encode for proteins of unknown function (Gollery et al., 2006). We recently used several different bioinformatics approaches to annotate genes of unknown function in Arabidopsis (Gollery et al., 2006, 2007; Horan et al., 2008). One such approach used a hidden Markov model protein family (HMMPFAM) search to identify all proteins that contain no previously defined domains or motifs. These unknown proteins were termed Proteins with Obscure Features (POFs), and were distinguished from Proteins with Define Features (PDFs), that contained at least one previously defined domain or motif (Gollery et al., 2006, 2007). In a comparison among ten different eukaryotic proteomes, including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Arabidopsis thaliana, Oryza sativa, Drosophila melanogaster, Anopheles gambiae, Caenorhabditis elegans, Mus musculus, Rattus norvegicus, and Homo sapiens*, POFs were found to be similar to PDFs in their relative contribution to biological function, as indicated by their transcript expression, participation in protein-protein interactions and association with mutant phenotype (Gollery et al., 2006). Surprisingly, 60% of the POFs identified within the different proteomes were, on average, species specific, compared to only 7.5% of the PDFs (Gollery et al., 2006). POFs were also found to contain more disordered structure, and were shorter and more hydrophilic than PDFs (Gollery et al., 2006). A comparison among the Arabidopsis, rice and poplar (*Populus trichocarpa*) proteomes identified over 2,000 POFs that were specific to Arabidopsis, and revealed that POFs were mainly represented as singletons within the different plant proteomes (Gollery et al., 2007).
The identification of over 5,000 proteins of unknown function in Arabidopsis suggests that many of the known pathways and networks, currently being studied in Arabidopsis, include additional genes and proteins that have an unknown function (Gollery et al., 2007; Horan et al., 2008). Some of these can be identified using correlation studies, which are based on transcriptome profiling analyses (Horan et al., 2008). In addition to the proteins with unknown function which might participate in known pathways and networks, many POFs and PDFs in plants can serve unknown or possibly novel functions involved in basic or specialized processes, and might comprise new and undiscovered pathways (Gollery et al., 2006, 2007). The identification of genes with unknown function, such as POFs, which are unique to Arabidopsis might suggest that some of these proteins are associated with, or involved in, processes that are unique to Arabidopsis or the Brassicaceae (Gollery et al., 2007). An additional possibility, however, is that some POFs, fold and function much like some of the known proteins, but do not share any sequence similarity to them (Gollery et al., 2006; Siew and Fischer, 2004). Additionally, some POFs and PDFs might represent misannotated proteins or genes (Gollery et al., 2006). The functional characterization of genes with unknown function might provide an insight into the role of unknown proteins in different organisms, and has become a major goal in modern biological research (Fischer and Eisenberg, 1999; Chothia et al., 2003; Siew and Fischer, 2003; Alonso et al., 2003; Roberts, 2004; Gollery et al., 2006, 2007).

To begin the functional characterization of proteins with unknown function in Arabidopsis, we identified 41 different proteins of unknown function that respond to endogenous oxidative stress in Arabidopsis (Davletova et al., 2005), and constitutively
expressed them in transgenic plants. We specifically chose oxidative stress as our source for genes of unknown function because this stress is considered to be common among many different aerobic organisms and many of the known pathways and genes involved in the response of different organisms to oxidative stress have overlapping functions and/or structural similarities (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1999; Mittler et al., 2004; Halliwell, 2006; Vandenbroucke et al., 2008). We found that more than 70% of the expressed unknown proteins conferred tolerance to oxidative stress. In contrast, the majority of expressed unknowns (over 90%) did not confer tolerance to the other stresses tested, and about 50% of the expressed unknown proteins rendered plants more susceptible to osmotic or salinity stress. Two Arabidopsis-specific POFs and an Arabidopsis and Brassica-specific protein of unknown function, which contained a zinc finger domain, conferred enhanced tolerance to oxidative stress when expressed in transgenic plants. Our findings suggest that tolerance to oxidative stress in Arabidopsis involves different proteins, pathways and mechanisms, which are unknown at present, including some that are specific to Arabidopsis or the Brassicaceae.

Materials and Methods

Plant Growth, Transformation and Molecular Analysis

*Arabidopsis thaliana* cv Columbia plants were grown under controlled conditions: 21°C, 100 µmol m$^{-2}$ s$^{-1}$ (Suzuki et al, 2005), and monitored for growth and flowering time as described by Miller et al., (2007). RNA and protein were isolated and analyzed by gel blot analysis (Rizhsky et al, 2004a; Davletova et al, 2005a, 2005b). cDNA probes corresponding to the following Arabidopsis genes were used for the RNA gel blots
shown in Figure 7: APX1, AT1G07890; Cat2, AT4G35090; FSD1, AT4G25100. Antibodies for APX1, tylAPX, s/mAPX and RBCL were obtained as described by Miller et al., (2007). Antibodies to CSD2 were obtained from Agrisera (Vannas, Sweden), and antibodies to GFP were obtained from Clontech (Mountain View, CA). pUNI clones (Yamada et al., 2003) were obtained from the ABRC, PCR cloned into pGEM-T (Promega, Madison, WI) using gene specific primers (Supplementary Table 2), and sequenced. The resulting plasmids were then digested and cloned in frame to GFP (C-terminal fusion) into a modified pGreen vector (Suzuki et al., 2008). GFP, or GFP fused in-frame to the C-terminal of the different unknown proteins, were expressed in plants under the control of the 35S CaMV promoter (Hellens et al., 2000; Suzuki et al., 2008). Transgenic plants were generated using the floral dip method (Zhang et al., 2006), and homozygous lines were selected using hygromycin resistance and RNA and protein blots. In addition, plants were visualized for GFP using a Nikon Eclipse E400 epifluorescence microscope (Nikon Corp, Tokyo) or an Olympus IX 81 FV 1000 confocal microscope (Olympus, Tokyo) as described by Suzuki et al., (2008). Analysis of microarray data available from https://www.genevestigator.ethz.ch (Zimmermann et al., 2004) was normalized and performed as previously described (Miller and Mittler, 2006).

**Stress Assays**

For the analysis of stress-tolerance, seeds of wild type, and two independent homozygous transgenic lines for 35S::GFP or 35S::POFs-GFP were surface-sterilized with bleach and placed in rows on 1% agar plates (0.5 X MS medium), containing different concentrations of paraquat, t-butyl hydroperoxide, NaCl, Sorbitol or ABA.
(Sigma-Aldrich, St. Louis, MO), as described by Davletova et al., (2005b), Mittler et al., (2006), Ciftci-Yilmaz et al., (2007), and Miller et al., (2007). Each row of seeds (25-30 seedlings) placed on a plate was divided into two parts: control seeds and seeds of transgenic plants expressing the different proteins of unknown function. Thus, the different seeds were placed side-by-side on the same plate. Plates were maintained vertically in a growth chamber (21-22°C, constant light, 100 µmol m⁻² sec⁻¹) and % germination and root length were scored 5 days after seed plating. Four- or five-day-old seedlings grown on 0.5 X MS agar plates were also subjected to heat stress (38°C; 24 hours), or cold stress (10°C; 48 hours) and scored for % germination and root length as described by Davletova et al., (2005b), Mittler et al., (2006), Ciftci-Yilmaz et al., (2007), and Miller et al., (2007). All experiments were repeated at least 3 different times, each with at least 3 different technical repeats. Results are shown as mean and standard error bars. Statistical analysis was performed as described in Suzuki et al., (2008). Significant difference between each construct (35S::POFs-GFP) and the 35S::GFP control was assigned only when both lines tested for each of the constructs were t-test significant at p < 0.05. To image hydrogen peroxide accumulation in seedlings subjected to oxidative stress 5-day-old seedlings grown in the presence of paraquat or t-butyl hydroperoxide were treated with 0.2 µM Amplex® Red (Molecular Probes, Inc., Eugene, OR) for 1 hour and imaged with a Kodak 2000MM image station (Davletova et al., 2005b).

**Characterization of proteins with unknown function in yeast cells**

pUni clones were digested with EcoRI and NotI, cloned into pESC-His vector (Stratagene, La Jolla, CA) under the GAL10 promoter, and transformed into wild-type
BY4743 ([4741/4742] MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+met15Δ0/+ ura3Δ0/ ura3Δ0, American Type Culture Collection # 201390) and Yap1Δ mutant (MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+met15Δ0/+ ura3Δ0/ura3Δ0 ΔYAP1, American Type Culture Collection # 4030569) yeast strains. Empty plasmids were also transformed into the yeast strains as controls. Transformants were selected on solid minimal media that contained yeast nitrogen base (Difco, Lawrence, KS) and –His Dropout supplement (Clontech, Mountain View, CA) (per 20g/L of galactose) (SG-His). Transformants grown overnight in liquid SG-His media were subjected to stress on solid SG-His media supplied with either 2 mM methyl viologen (Acros Organics, City, NJ) or 0.25 mM t-butyl hydroperoxide (Sigma-Aldrich, St. Louis, MO) as serial dilutions (Tiên Nguyên-nhu and Knoops, 2003), or subjected to the same oxidative stress treatment in liquid SG-His media (Davies et al., 1995).

Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes Inc., Invitrogen, Carlsbad, CA) was used for the detection of \( \text{H}_2\text{O}_2 \) in the growth media of yeast. Yeast were grown overnight in SG-His liquid media up to mid-log phase, and 50 µl of the growth media was used to detect hydrogen peroxide according to the manufacturer’s instructions. For each sample four replicates were used and 50 µl of working solution (100 µM Oxired probe (MBL International, Woburn, MA), 0.2U/ml Horseradish Peroxidase, Type II (Sigma-Aldrich; St. Louis, MO) supplied with 0.05 M Sodium Phosphate, pH 7.4 were added to each sample. Reactions were incubated at room temperature as recommended and fluorescence was measured with a VICTOR³ V™ Multilabel Counter (Model 1420, PerkinElmer, Turku, FINLAND) using excitation at 531 and emission at 595.
**Methyl Viologen (Paraquat)** (Halliwell and Gutteridge, 1999)

Paraquat is a bipyridyl herbicide that can cross the membrane easily. Once it enters chloroplasts in plants it accepts electron from proteins associated with photosystem I and forms bipyridyl radicals. These can be re-oxidized by interacting with O$_2$ which in turn forms O$_2^{-}$ in a redox cycle. Much of O$_2^{-}$ is converted into H$_2$O$_2$ which can cause the inactivation of Calvin cycle enzymes, thus resulting in the inhibition of CO$_2$ fixation.

**tert-Butyl Hydroperoxide** (Halliwell and Gutteridge, 1999)

*tert*-butyl hydroperoxide (tB-OOH) is an artificial organic hydroperoxide. It interacts with all cellular membranes and results in the accumulation of alkoxyl radicals that initiate lipid peroxidation. Lipid peroxidation takes place at two steps; initiation and propagation.

**Initiation:** Initiation of lipid peroxidation starts with attack of any species that has enough reactivity to remove a hydrogen atom from a methylene group.

\[-\text{CH}_2- + \text{OH}^\cdot \rightarrow -\text{C}^\cdot\text{H}^\cdot + \text{H}_2\text{O}\]

**Propagation:** Abstraction of a hydrogen atom from a methyl group will leave the carbon in methyl group with an unpaired electron. Under aerobic conditions this carbon radical combines with O$_2$ which in turn produces peroxyl radical.

\[\text{R}^\cdot + \text{O}_2 \rightarrow -\text{ROO}^\cdot\]

Peroxyl radicals can remove H from another lipid molecule. Then, newly formed carbon radical can react with O$_2$ to form another peroxyl radical known as chain reaction of lipid peroxidation.
Results

Selection of POFs and PDFs and generation of transgenic plants

In a previous study we conducted a detailed GeneChip® (ATH1) time-course analysis comparing wild type plants to knockout plants lacking the key H₂O₂ scavenging enzyme cytosolic ascorbate peroxidase 1 (Apx1), subjected to a moderate treatment of light stress (Davletova et al., 2005). This treatment resulted in the endogenous accumulation of H₂O₂ and oxidized proteins in the knockout plants compared to the wild types during light stress (Davletova et al., 2005). Of the 3,915 transcripts that were found to be significantly altered in their expression in knockout-\textit{Apx1} plants, compared to wild types, during this treatment, 119 were designated as unknowns by the GeneChip® (ATH1) annotation (Davletova et al., 2005). Fifty of these were selected based on their mRNA abundance in the knockout plants (exceeding a two-fold increase threshold), the availability of pUni clones (Yamada et al., 2003), and the lack of internal restriction sites used for cloning into the binary vectors. Of the 50 clones selected 41 clones were successfully used in the generation of transgenic plants. As shown in Table 1, 23 of these proteins were designated as POFs and 18 as PDFs (unknowns that contained a previously defined domain or motif).

All pUni clones obtained from the Arabidopsis Biological Resource Center (Yamada et al., 2003), were PCR cloned into pGEM-T vectors and sequenced. They were then cloned into a modified pGreen binary vector (Hellens et al., 2000; Suzuki et al., 2008), as an in-frame fusion protein upstream to GFP and expressed in transgenic plants under the control of the CaMV 35S promoter (\textit{35S::POF-GFP}; C-terminal fusion). At least fifteen independent transgenic lines were initially obtained for each unknown
protein. These were scored based on the presence of the selectable marker (hygromycin), GFP imaging, and transcript and protein expression. Two homozygous independent lines with similar expression level of the \textit{POF-GFP} transcript/protein were then selected for each gene for further analysis.

To examine the relationship of the different unknown proteins to other organisms, their predicted amino acid sequences were compared with the National Center for Biotechnology Information (NCBI) non-redundant, as well as EST, databases translated in all reading frames, and a BLAST E-value cut-off of $10^{-6}$ was used to determine sequence homology with other organisms (http://www.ncbi.nlm.nih.gov/; Gollery et al., 2006, 2007; Table 1). The proteins were then classified as Arabidopsis-specific (i.e., homology was found only to Arabidopsis), plant-specific (i.e., homology was found to at least one other plant; with the exception of AT5G18040 and AT2G22080 that had homology only to Arabidopsis and \textit{Brassica napus}, and were designated as such), or found to have homologs in other plant or other organisms including algae, microbes and/or animals (Table 1). Altogether, we identified four Arabidopsis-specific, two Arabidopsis and \textit{Brassica}-specific, and 25 plant-specific proteins. Interestingly, despite the relative similarity in transcript expression in response to oxidative stress between plants and animals (Vandenbroucke et al., 2008), only three of the unknown proteins identified and selected for analysis in Arabidopsis had homologs in animals (AT1G27330, AT2G15560 and AT2G24150; Table 1). It should be noted, however, that the domain and homology identification for the proteins included in this study is likely to change with time as more plant sequences and HMMPFAM models will be deposited in public databases (Gollery et al., 2006, 2007).
Oxidative and abiotic stress assays

To examine the stress tolerance of the different transgenic lines expressing proteins of unknown function, seedlings of transgenic plants were subjected to different oxidative and abiotic stresses as described previously (Davletova et al., 2005b; Mittler et al., 2006; Ciftci-Yilmaz et al., 2007; Miller et al., 2007). As controls we used seedlings of transgenic plants that expressed GFP under the control of the CaMV 35S promoter (35S::GFP) and seedlings of wild type plants.

As shown in Figure 1 and Supplementary Table 1, more than 70% of the lines expressing proteins of unknown function were found to be more tolerant to oxidative stress imposed by paraquat or t-butyl hydroperoxide (35 or 29 out of 41 respectively). In contrast, as shown in Figure 2 and Supplementary Table 1, the majority of expressed unknowns did not confer tolerance to osmotic or salinity stresses (over 92%; 38 and 38 out of 41 respectively), and about 50% of the lines expressing protein of unknown function were found to be more sensitive to osmotic or salinity stresses (21 or 27 out of 41 respectively). An inverse correlation between tolerance to oxidative stress imposed by paraquat and salinity was identified in 46% of the lines (a total of 19 different lines; Figures 1B, 2B, and Supplementary Table 1). This finding was in agreement with our previous observation made with knockout-Apx1 plants that showed an inverse correlation between tolerance to salinity and oxidative stress (Ciftci-Yilmaz et al., 2007).

As shown in Figure 3, and below for Arabidopsis and/or Arabidopsis and Brassica-specific proteins, the tolerance of selected lines was tested against a range of paraquat and t-butyl hydroperoxide concentrations to confirm the results shown in Figure 1 (obtained with one concentration of paraquat and t-butyl hydroperoxide). As shown in...
Supplementary Figure 1, and compared to the results obtained with oxidative, salinity and osmotic stresses (Figures 1 and 2, and Supplementary Table 1), tolerance to cold or heat stress was not dramatically enhanced in the transgenic lines. With the exception of AT5G19875 and AT1G11210, no significant effects were also observed in the germination of the different lines in the presence or absence of 0.5 µM ABA (Supplementary Figure 2 and Supplementary Table 1).

As shown in Figure 1C, the expression level of the different unknown proteins between the different constructs did not correlate with the resistance phenotype (i.e., some constructs with a low expression level in both lines tested showed enhanced tolerance to oxidative stress compared to other lines with a high level of expression in both lines tested). This result suggested that the mechanism of action of the different unknown genes is different and that for some genes even a low expression level is sufficient to result in high tolerance to oxidative stress.

**Analysis of Arabidopsis-specific POFs**

To further characterize the four Arabidopsis-specific POFs identified in this study (Table 1), we examined their relative mRNA abundance in response to different abiotic stresses, as monitored by microarray (Affymetrix GeneChip®) experiments and deposited in public databases (Zimmermann et al., 2004; Miller and Mittler, 2006). As shown in Figure 4, AT2G41650 was mainly expressed in roots in response to oxidative stress, AT1G64360 was mainly expressed in roots in response to osmotic stress, AT1G21520 was mainly expressed in roots and leaves in response to salt stress, AT2G50290 was mainly expressed in roots in response to heat stress, and AT2G22080 (an Arabidopsis
and Brassica-specific protein used as a control) was mainly expressed in leaves in response to cold stress. At least based on their transcript expression patterns (Figure 4; Davletova et al., 2005a), the different Arabidopsis-specific POFs appear to have a putative function in Arabidopsis during stress.

As shown in Figure 5, constitutive expression of four of the five proteins selected for further analysis (AT1G64360, AT1G21520, AT2G50290 and AT2G22080), resulted in an accelerated flowering time phenotype when grown under short day conditions. An altered flowering time phenotype was previously observed for certain oxidative stress mutants grown under controlled growth conditions (Pnulei et al., 2003; Rizhsky et al., 2003; Miller et al., 2007).

As shown in Figure 6, constitutive expression of two of the Arabidopsis-specific POFs (AT1G21520, AT1G50290) and the Arabidopsis and Brassica-specific protein with unknown function, which contained a zinc finger domain (AT2G22080), conferred enhanced tolerance to oxidative stress imposed by a range of different concentrations of paraquat, or t-butyl hydroperoxide. To test whether the enhanced tolerance of transgenic plants expressing these proteins was associated with a general decrease in the level of reactive oxygen species, we used Amplex® Red to measure hydrogen peroxide in five-day-old seedlings of transgenic plants expressing GFP or AT1G21520, AT1G50290 and AT2G22080 fused to GFP, grown on agar plates in the presence or absence of 0.1 µM paraquat. As shown in Figure 7A, transgenic seedlings expressing AT1G21520, AT1G50290 or AT2G22080 accumulated less hydrogen peroxide compared to control plants when grown on agar plates that contained paraquat. In contrast, no differences were observed between the levels of hydrogen peroxide in control seedlings or seedlings
of transgenic plants expressing AT1G21520, AT1G50290 or AT2G22080 grown on agar plates in the absence of paraquat (Supplementary Figure 3).

To test whether the enhanced tolerance of transgenic plants expressing AT1G21520, AT1G50290 or AT2G22080 to oxidative stress was associated with enhanced expression of known reactive oxygen scavenging enzymes, we conducted RNA and protein gel blot analysis on these transgenic plants grown under controlled growth conditions. As shown in Figures 7B and 7C, this analysis revealed that expression of the key reactive oxygen scavenging enzymes Apx1, thylakoid APX (tylAPX), stromal mitochondrial APX (s/mAPX), Catalase 2 (Cat2), copper-zinc superoxide dismutase 2 (CSD2), or iron superoxide dismutase (FSD1) in transgenic plants grown under controlled growth conditions was not elevated, or did not correlate with enhanced tolerance to oxidative stress (Figure 6) or accumulation of hydrogen peroxide (Figure 7A and Supplementary Figure 3). Because elevated expression of Apx1, tylAPX, s/mAPX, Cat2, CSD2, or FSD1 could be used as a measure for endogenous oxidative stress in plants (Mittler et al., 2004), these results might also indicate that, at least with respect to the classical markers for internal oxidative stress, the transgenic plants tested did not suffer from an internal oxidative stress. As shown in Figure 8, AT2G22080 appeared to be localized to nuclei, AT1G50290 appeared to be localized to the cytosol, but excluded from nuclei, and AT1G21520 appeared to be localized to the endoplasmic reticulum (ER) and perhaps other cellular membrane systems.
Expression of AT1G50290 in yeast

To determine whether constitutive expression of some Arabidopsis proteins with unknown function will confer enhanced tolerance against oxidative stress to other organisms, we transformed and tested the oxidative stress tolerance of yeast expressing the following proteins: AT2G41650, AT1G72060, and AT3G10020 (proteins that did not confer enhanced tolerance to oxidative stress in transgenic plants; Figure 1), and AT5G19875, AT5G59080, AT5G43750, AT5G18040, AT3G51610, AT4G12000, AT1G52200, AT1G78410, AT1G21520, AT1G80130, AT1G73120, AT1G50170, AT2G04795, AT1G27330, At1g50290, AT2G19310, AT2G44240, and AT2G40000 (proteins that did confer enhanced tolerance to oxidative stress in transgenic plants; Figure 1). Two different yeast strains were used for these assays: wild type yeast (BY4743), and a yeast mutant lacking the transcription factor Yap1 that is essential for tolerance to oxidative stress (in the BY4743 background; termed Yap1Δ; Kuge and Jones, 1994). Transformed and untransformed yeast were subjected to oxidative stress imposed by paraquat or t-butyl hydorperoxide as described by Tiên Nguyên-nhu and Knoops, (2003) and Davies et al., (1995). In contrast to the results obtained with plants (Figure 1), none of the genes expressed in yeast was able to enhance the tolerance of transgenic yeast (Yap1Δ) to oxidative stress (Supplementary Figure 4). These results might suggest that, at least with respect to their functionality in yeast, the majority of proteins identified in this study did not have a general anti-oxidative function.

One of the proteins tested, AT1G50290, an Arabidopsis-specific POF, was found to have a negative impact on the oxidative stress tolerance of yeast. As shown in Figure 9A and 9B, expression of AT1G50290 in yeast rendered yeast (wild type or Yap1Δ) more
susceptible to oxidative stress imposed by paraquat or t-butyl hydroperoxide. Expression of AT1G50290 in yeast (wild type or Yap1Δ) was also found to have a deleterious effect on growth in the absence of oxidative stress (Figure 9B). As shown in Figure 9C, expression of AT1G50290 in wild type yeast resulted in enhanced accumulation of hydrogen peroxide in the growth media of cells grown under controlled growth conditions. In contrast to these findings, expression of AT1G50290 did not result in accumulation of H$_2$O$_2$ in transgenic plants grown under controlled growth conditions (Supplementary Figure 3).

**Discussion**

Reactive oxygen species are produced in cells in response to many different abiotic or biotic conditions, and their uncontrolled accumulation can lead to oxidative stress (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1999; Kovtun et al., 2000; Mittler 2002; Apel and Hirt, 2004; Foyer and Noctor, 2005; Asada, 2006; Halliwell, 2006; Van Breusegem and Dat, 2006). Enhanced tolerance to oxidative stress, brought about by constitutive expression of a single protein (Figures 1, 3 and 6), might result from a number of different mechanisms: 1) The expressed protein might have a direct scavenging activity that can detoxify certain species of reactive oxygen; 2) The expressed protein might be part of a cellular network, or signal transduction pathway that protects the cell from damage caused by oxidative stress or is involved in the detoxification of reactive oxygen, and its constitutive expression enhances the activity of this network or pathway; 3) The expressed protein might alter plant metabolism causing the accumulation of reactive oxygen and indirectly activating the cells’ scavenging,
protection, and/or repair mechanisms against oxidative stress similar to some transgene-induced lesion mimics (Mittler and Rizhsky, 2000).

Previous studies have shown a direct correlation between enhanced tolerance to oxidative stress and enhanced tolerance to different abiotic stresses, leading to the assumption that enhancing the ability of plants to scavenge reactive oxygen would also enhance the ability of plants to tolerate other stresses (e.g., Roxas et al., 1997; Deak et al., 1999; Kortun et al., 2000; Rivero et al., 2007). Interestingly, in our hands the majority of transgenic plants expressing proteins of unknown function and showing enhanced tolerance to oxidative stress (Figure 1) did not show enhanced tolerance to osmotic, salinity, heat, or cold stresses (Figure 2, Supplementary Figure 1, and Supplementary Table 1). This finding could suggest that the expressed unknown proteins do not have a general reactive oxygen scavenging activity that would have made them more tolerant to other stresses (Roxas et al., 1997; Deak et al., 1999; Kortun et al., 2000; Rivero et al., 2007). A direct scavenging activity would have also made at least some of the yeast cells expressing the unknown proteins more tolerant to oxidative stress. However, none of the eighteen different proteins with unknown function which enhanced the tolerance of transgenic plants to oxidative stress enhanced the tolerance of yeast cells to oxidative stress suggesting that these proteins do not function to directly scavenge ROS such as superoxide radicals, or lipid peroxides.

The finding that the expressed proteins with unknown function did not confer enhanced tolerance to other abiotic stresses might also suggest that they did not alter plant metabolism in a way that will indirectly trigger the cells’ scavenging, protection, and/or repair mechanisms against general stress, or oxidative stress (Roxas et al., 1997;
Deak et al., 1999; Kovtun et al., 2000; Mittler and Rizhsky, 2000). Moreover, with the exception of AT1G50290 (Figure 9), none of the unknown proteins tested in yeast exhibited deleterious effects on growth, or caused yeast to have an altered susceptibility to oxidative stress (Supplementary Figure 4). This finding supports the possibility that constitutive expression of proteins with unknown function in plants did not have an indirect effect on cellular metabolism causing a generalized stress tolerance response (Mittler and Rizhsky, 2000).

At least two different findings, therefore, point to a high specificity in the function of the expressed proteins with unknown function: 1) Enhanced tolerance to oxidative stress caused by their constitutive expression did not cause a general cellular effect that made plants more tolerant to other stresses (Figures 1, 2 and Supplementary Figure 1); 2) An inverted correlation was found for at least 19 of them between tolerance to oxidative stress imposed by paraquat and tolerance to salinity (Figures 1, 2 and Supplementary Table 1). We previously found that knockout plants lacking Apx1 were more susceptible to oxidative stress, but more tolerant to salinity stress (Ciftci-Yilmaz et al., 2008). The finding that constitutive expression of 19 proteins with unknown function, that were identified in knockout-\textit{Apx1} plants as responsive to internal oxidative stress (Davletova et al., 2005a), caused plants to become more tolerant to oxidative stress, but more susceptible to salinity or osmotic stress could suggest that these proteins have very specific functions that are tied to the pathway(s) activated in knockout-\textit{Apx1} plants. The differences observed between the tolerance of the different lines to paraquat, which functions by enhancing the cellular rate of superoxide radical production, and tolerance to \textit{t}-butyl hydroperoxide, which function by inducing lipid peroxidation (Figures 1a and 1b),
could also suggest that the different unknown genes have specific functions within the ROS gene network (Mittler et al., 2004).

Because the majority of expressed proteins with unknown function appear to have a specific oxidative stress tolerance phenotype when expressed in transgenic plants (Figures 1, 2 and Supplementary Figures 1 and 2), they could be involved in different networks that protect cells from specific aspects of oxidative stress. This hypothesis is based on the finding that they do not enhance tolerance to other abiotic stresses, many of them cause plants to become more susceptible to osmotic or salinity stresses, and many of them do not protect yeast against oxidative stress. These findings are in agreement with our previous hypothesis that genes of unknown function are highly specific to different organisms and function as part of specific cellular networks (Gollery et al., 2006, 2007). Our findings, therefore, demonstrate that the study of proteins with unknown function could unravel new and specialized functions that could be phylogenetically specific.

Our characterization of Arabidopsis- and Arabidopsis and Brassica-specific proteins of unknown function revealed that three of these proteins enhanced the tolerance of plants to oxidative stress (Figure 6). The constitutive expression of these proteins did not enhance the production of hydrogen peroxide in plants grown under controlled growth conditions (Supplementary Figure 3), did not result in the enhanced expression of known reactive oxygen scavenging mechanisms (Figures 7B and 7C), and did not cause plants to be more tolerant to salinity or osmotic stresses (Figure 2), suggesting that at least in plants they did not cause the enhanced production of reactive oxygen, thereby triggering a general oxidative stress response. Because of their high specificity to reactive
oxygen stress, it is possible that these proteins function as part of an Arabidopsis- or Arabidopsis and Brassica-specific network that is involved in cellular repair and/or protection against oxidative stress. It appears, therefore, that Arabidopsis and/or Brassica plants could contain specific pathways that are unknown at present, involved in the protection of cells against oxidative stress. These pathways could function in different cellular compartments (Figure 8) and could interact with different developmental pathways (Figure 5). The possible existence of repair and/or protection pathways against oxidative stress which are specific for Arabidopsis or the Brassicaceae is very interesting because oxidative stress is considered to be a general type of stress common to many different organisms (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1999; Mittler et al., 2004; Halliwell, 2006; Vandenbroucke et al., 2008). Our study, therefore, highlight the need to characterize phylogenetic specific proteins of unknown function in different organisms because these could shed light on new and possibly novel pathways involved in different aspects of plant metabolism.

The approach of using microarray expression data as a tool to predict function for proteins of unknown function (Horan et al., 2008) appears to be a good approach, because over 70% of the proteins tested in this study showed an oxidative stress response phenotype (Figures 1 and 2). We are in the process of generating a database that includes the abiotic stress-phenotypic characterization of over 1,000 knockout mutants (Alonso et al., 2003) for proteins of unknown function (Gollery et al., 2007; work in progress) with the goal of identifying additional pathways and mechanisms involving proteins of unknown function in Arabidopsis.
Acknowledgments

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**Figure Legends**

**Figure 1.** Tolerance of transgenic Arabidopsis seedlings expressing proteins of unknown function to oxidative stress. **A.** Root growth assays showing tolerance to t-butyl hydroperoxide in seedlings of transgenic plants. **B.** Root growth assays showing tolerance to paraquat in seedlings of transgenic plants. **C.** A composite protein gel blot showing the expression level of the different fusion proteins using GFP antibody. Molecular size of the different proteins is indicated in Supplementary Table 1. Two independent lines were tested for each construct. Abbreviations: 35S::GFP, control transgenic seedlings expressing GFP; WT, control wild type seedlings; AT2G41650 to AT2G40000, transgenic seedlings expressing different proteins of unknown function under the control of the CaMV 35S promoter and fused in frame to the N-terminal of GFP. *(Generated by Song Luhua)*

**Figure 2.** Tolerance of transgenic Arabidopsis seedlings expressing proteins of unknown function to osmotic and salinity stresses. **A.** Root growth assays showing tolerance to osmotic stress in seedlings of transgenic plants. **B.** Root growth assays showing tolerance to salinity in seedlings of transgenic plants. Two independent lines were tested for each construct. Abbreviations: 35S::GFP, control transgenic seedlings expressing GFP; WT, control wild type seedlings; AT2G41650 to AT2G40000, transgenic seedlings expressing different proteins of unknown function under the control of the CaMV 35S promoter and fused in frame to the N-terminal of GFP. *(Generated by Song Luhua)*
**Figure 3.** Tolerance of transgenic Arabidopsis seedlings expressing proteins of unknown function to oxidative stress. **A.** Root growth assays showing enhanced tolerance to a range of t-butyl hydroperoxide concentrations in seedlings of transgenic plants. **B.** Root growth assays showing enhanced tolerance to a range of paraquat concentrations in seedlings of transgenic plants. Two independent lines were tested for each construct. *(Generated by Song Luhua)*

**Figure 4.** Expression of four Arabidopsis-specific POFs, and an Arabidopsis and Brassica-specific protein of unknown function, in response to different abiotic treatments in leaves (A) and roots (B) of wild type plants. Microarray expression data was obtained from Genevestigator (Zimmermann et al., 2004), and is presented as fold expression compared to control untreated (Miller and Mittler, 2006). *(Generated by Song Luhua)*

**Figure 5.** Early flowering phenotype in transgenic plants expressing Arabidopsis-specific POFs and an Arabidopsis and Brassica-specific protein of unknown function. Measurements of leaf number at time of flowering under short and long day conditions are shown in transgenic plants expressing Arabidopsis specific POFs. *(Generated by Song Luhua)*

**Figure 6.** Tolerance of transgenic Arabidopsis seedlings expressing Arabidopsis-specific POFs, or an Arabidopsis and Brassica-specific protein of unknown function, to oxidative stress. **A.** Root growth assays showing enhanced tolerance to a range of t-butyl hydroperoxide concentrations in seedlings of transgenic plants. **B.** Root growth assays
showing enhanced tolerance to a range of paraquat concentrations in seedlings of transgenic plants. Two independent lines were tested for each construct. *(Generated by Song Luhua)*

**Figure 7.** Accumulation of hydrogen peroxide and protein and transcript expression in transgenic Arabidopsis seedlings expressing Arabidopsis-specific POFs, or an Arabidopsis and Brassica-specific protein of unknown. A. Accumulation of hydrogen peroxide in roots of 5-day-old seedlings grown in the presence of 0.1 µM paraquat. B. RNA gel blot analysis showing the expression of transcripts encoding the reactive oxygen scavenging enzymes ascorbate peroxidase 1 (APX1), catalase 2 (CAT2) and iron superoxide dismutase 1 (FSD1) in transgenic plants grown under controlled growth conditions. Ribosomal RNA was used to control for RNA loading. C. Protein gel blot analysis showing the expression of the reactive oxygen scavenging enzymes thylakoid APX (tylAPX), stromal/mitochondrial APX (s/mAPX), chloroplastic CuZnSOD2 (CSD2) and GFP fusion proteins (GFP) in transgenic plants grown under controlled growth conditions. Rubisco large subunit (RBCL) was used to control for protein loading. Size and loading factor for the GFP fusion proteins in C is: 35S::GFP, 46 kD, 1X loading; AT1G21520, 53kD 1X loading; AT1G50290, 61.7 kD, 20X (concentrated); AT2G22080, 65 kD, 1X loading. *(Generated by Song Luhua)*

**Figure 8.** Imaging of GFP in root tips of transgenic seedlings expressing Arabidopsis-specific POFs, or an Arabidopsis and Brassica-specific protein of unknown function, which conferred tolerance to oxidative stress, grown under controlled growth conditions.
The different unknown proteins or POFs were expressed in plants under the control of the CaMV 35S promoter and fused in frame to the N-terminal of GFP. **(Generated by Song Luhua)**

**Figure 9.** Enhanced sensitivity to oxidative stress in yeast cells expressing AT1G50290. **A.** Plate growth assays of wild type yeast, Yap1Δ, and Yap1Δ transformed with AT1G50290, showing enhanced sensitivity to oxidative stress induced by paraquat and t-butyl hydroperoxide in Yap1Δ cells transformed with AT1G50290. **B.** Liquid media growth assays of wild type yeast, wild type yeast transformed with AT1G50290 (Top), and Yap1Δ, and Yap1Δ transformed with AT1G50290 (bottom), showing suppressed growth and enhanced sensitivity to oxidative stress induced by paraquat and t-butyl hydroperoxide in yeast cells transformed with AT1G50290. **C.** Accumulation of H₂O₂ in the growth media of wild type yeast cells transformed with AT1G50290. H₂O₂ was determined in the media of log phase cells as described in Methods.

**Table 1.** A summary of the different proteins with unknown function expressed and characterized in transgenic plants. The locus, pUni and predicted length is given on left for each clone. Homology to other organisms determined by a BLAST cut off value of 10⁻⁶ and annotation based on an HMMPFAM search are given on the right. **(Generated by Song Luhua)**

**Supplementary Figure 1.** Effect of **A.** Heat (38°C, 24 hr), or **B.** Cold (4°C, 48 hr) on root elongation of transgenic plants expressing genes of unknown function. All seeds
germinated at the same time. Please see Materials and Methods for experimental details.

*(Generated by Song Luhua)*

**Supplementary Figure 2.** Effect of ABA (0.5 μM) on root elongation of transgenic plants expressing genes of unknown function. All seeds germinated at the same time. Please see Materials and Methods for experimental details. *(Generated by Song Luhua)*

**Supplementary Figure 3.** Accumulation of hydrogen peroxide in roots of 5-day-old seedlings grown on 0.5XMS agar plates under controlled growth conditions. *(Generated by Song Luhua)*

**Supplementary Figure 4.** Yeast growth assays performed with wild type (WT) Yap1 mutants and Yap1 Mutants transformed with different Arabidopsis genes grown on plates containing paraquat or t-butyl hydroperoxide.
Figure 1

A

T-butyl hydroperoxide (0.1 mM)

Root length (% of control)

B

Paraquat (0.1 µM)

Root length (% of control)

C
Figure 2

A

Sorbitol (250 mM)

B

NaCl (100 mM)

Root length (% of control)
Figure 3

A

Graph showing root length (% of control) against T-Butyl Hydroperoxide (mM) for different conditions.

B

Graph showing root length (% of control) against Paraquat (µM) for different conditions.
Figure 4

A

Leaf

B

Root

Fold expression

Oxidative stress
Salt stress
Osmotic stress
Cold stress
ABA treatment
Heat stress
AT2G41650
AT1G64360
AT1G21520
AT1G50290
AT2G22080

Leaf

Root

Fold expression

Oxidative stress
Salt stress
Osmotic stress
Cold stress
ABA treatment
Heat stress
AT2G41650
AT1G64360
AT1G21520
AT1G50290
AT2G22080
Figure 5

Leaf number at flowering

- Short day
- Long day

Species:
- col-wt
- 35S::GFP
- AT2G41650
- AT1G64360
- AT1G21520
- AT1G50290
- AT2G22080
Figure 6

A

B

Root length (% of control)

T-Butyl hydroperoxide (mM)

Paraquat (µM)

35S::GFP

AT2G41650

AT1G64360

AT1G21520

AT1G50290

AT2G22080
Figure 7

A

Bar graph showing the percentage of PCR amplification for different samples.

B

Western blot images showing the expression levels of various proteins.

C

Western blot images showing the expression levels of various proteins.
Figure 8

35S::GFP

AT2G22080

AT1G50290

AT1G21520

AT1G50290

AT1G21520
Figure 9

A

Control

Paraquat (2 mM)

T-Butyl Hydroperoxide (0.25 mM)

B

WT (BY4743)

Yap1Δ

AT1G50290 (Yap1Δ)

WT (BY4743)

Yap1Δ

AT1G50290 (Yap1Δ)

WT (BY4743)

Yap1Δ

AT1G50290 (Yap1Δ)

C

H2O2 (µM)/OD600

WT

WT-AT1G50290

H2O2 (µM)/OD600

WT

WT-AT1G50290
### Supplementary Table 1

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*No change, More tolerant, Less tolerant*
**Supplementary Table 2**

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Supplementary Figure 1

A

B
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4

WT
Yap1Δ
AT2G41650 Control
AT5G59080
AT5G43750

0.25 mM t-BOOH

2mM Paraquat
WT
Yap1Δ
AT1G27330
AT2G32210
AT1G63980

Control

0.25 mM t-BOOH

2mM Paraquat

WT
Yap1Δ
AT1G27330
AT2G32210
AT1G63980
References


Roxas VP, Smith RK Jr, Allen ER, Allen RD (1997) Overexpression of glutathione S-
transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Nat Biotechnol. 15: 988-991


Chapter 5

Concluding Remarks
Plants are sessile organisms and have developed sophisticated stress response and defense networks to cope with environmental stresses such as drought and cold (Kreps et al., 2002). In spite of such complex networks, environmental stresses are still the major cause for crop-loss world-wide (Boyer, 1982). Environmental stresses reduce yield of most major crop plants by more than 50% worldwide (Bray et al., 2002). These kind of big loses threaten the world food supply. Moreover, environmental stresses cause significant economical losses. According to the USDA, the U.S. loses about $5.1 billion only because of cold stress every year. Understanding the stress and defense response network of plants will help us to genetically modify and select different cultivars to overcome environmental stresses. Recent studies have suggested that EAR motif containing C2H2 type zinc finger proteins play major roles in plant defense and stress response mechanisms by transcriptional repression (Kazan, 2006) or activation of different stress response related genes.

Our data demonstrated that the EAR motif, a putative repressor domain, plays an important role in the response of plants to abiotic stresses (Ciftci-Yilmaz et al., 2007). Moreover, constitutive expression of full-length EAR motif containing Zat7 resulted in enhanced tolerance of Arabidopsis to salt stress (Ciftci-Yilmaz et al., 2007). When the EAR motif was mutated or truncated, the enhanced tolerance to salinity was abolished (Ciftci-Yilmaz et al., 2007). Even though the EAR motif is required for the salinity response and can interact with other stress-related proteins in a yeast-two hybrid system, there are still many questions that need to be answered about the EAR motif. What will be the outcome if only the last 25 aa of Zat7 which contains the EAR motif overexpressed in Arabidopsis? Overexpressing Zat10, which contains an EAR motif,
resulted in enhanced tolerance to salinity (Mittler et al., 2006), whereas overexpressing Zat12, which also contains EAR motif, did not result in enhanced tolerance to salinity (Rizhsky et al., 2004). On the other hand, Zat12 knockout line showed enhanced sensitivity to salt stress (Rizhsky et al., 2004). All data together suggest that there are probably other domain(s) of Zat7 required for enhanced tolerance to salt stress. Does the EAR motif play role in protein-protein interaction in plant? A pull-down assay with proteins with either their EAR motif mutated or truncated might shed light on this question. Another approach will be using bimolecular fluorescence complementation with the above mutated and truncated proteins or with candidate proteins whose EAR motif containing proteins interact in other assays such as yeast two hybrid and pull-down. More importantly, is the EAR motif also crucial for the stress response role of other zinc finger proteins? Obtaining EAR motif mutated or truncated versions of other stress responsive zinc finger proteins such as Zat10 and Zat12 and subjecting them to stress might answer this question. Previous studies suggested that zinc-finger proteins mainly function as DNA-binding protein and some of the C2H2-EAR-zinc finger proteins might act as transcription factors such as Zat12 (Ciftci-Yilmaz et al., 2008). The role of the EAR domain of zinc finger proteins in DNA binding could be investigated by “Systematic Evolution of Ligands by Exponential Enrichment” or electro mobility shift assay by using full length, as well as the EAR domain truncated zinc finger proteins.

Some EAR motif-containing zinc finger proteins were reported to cause growth suppression when constitutively expressed (Mittler et al., 2006, Ciftci-Yilmaz et al., 2007). Our study showed that the EAR motif is not involved in this phenotype, nevertheless the domain that causes growth suppression is still unknown today. It might
be possible to overcome growth suppression by expressing proteins with stress-specific or tissue-specific promoters.

Recent studies have pointed out that C2H2-type zinc finger proteins might interact with each other and/or act in a combinatorial manner and/or act in redundant pathways. For example, during the oxidative stress response Zat12 is required for the expression of Zat7 (Rizhsky et al., 2004). On the other hand, Zat12 itself is insufficient for the expression of Zat7, demonstrating that other factors that are currently unknown are required. Preliminary data suggested that Zat12 could also act upstream of Zat10 during the cold stress response (Chinnusamy et al., 2007). Our data indicated that Zat10 and Zat12 might play a role in redundant or parallel pathways instead of the same pathway. There are many other C2H2-type zinc finger proteins responsive to similar abiotic stresses (Ciftci-Yilmaz et al., 2008). Their mode of action still needs to be investigated. The Zat family could be further investigated by obtaining double and/or triple knockout lines of the proteins that are responsive to similar stresses such as Zat12 and Zat7 and subjecting these lines to abiotic stresses. Localization and temporal expression of Zat family proteins in response to different abiotic stresses needs to be investigated to have a better understanding of how the different family members function. Interaction between Zat family members responsive to similar stresses could also be investigated by directed yeast-two hybrid and/or BiFunctional Complementation assay. Another way to systematically analyze Zat family could be either knocking out or overexpressing the family members one by one and screening the expression pattern of the rest of the family during the abiotic stress response.
Even though previous studies clearly demonstrated involvement of the EAR domain of the zinc finger proteins in abiotic stress response (Ciftci-Yilmaz et al., 2007), the roles of the zinc finger domains in stress response are still unclear. Involvement of zinc finger domains in stress response could be investigated by obtaining lines that express stress responsive zinc finger proteins such as Zat12 with a mutated zinc finger domains and subjecting them to abiotic stress.

The stress response network of Arabidopsis is highly complex. The involvement of zinc-finger proteins in this complicated network takes place in many different ways and most aspects of this involvement are still unknown. Exploring the mode of action of zinc-finger proteins and their domains will help us alter development of plants, obtain plants with enhanced tolerance to different abiotic stress conditions. Present data showed that simply overexpressing a protein cannot be a solution because it can cause some harmful side effects such as growth suppression, sensitivity to stresses, and reduced yield quality and quantity.
References


