

University of Nevada, Reno

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

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy in Biochemistry

by

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THE GRADUATE SCHOOL

We recommend that the dissertation
prepared under our supervision by

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be accepted in partial fulfillment of the
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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 shows 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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Table of Contents

Chapter 1: Introduction.....	1
I. The zinc finger network of plants.....	2
II. Proteins of unknown functions.....	29
References.....	31
Chapter 2: The EAR-motif of the C2H2 zinc-finger protein <i>Zat7</i> plays a key role in the defense response of <i>Arabidopsis</i> to salinity stress.....	45
Abstract.....	46
Introduction.....	47
Materials and methods.....	49
Results.....	55
Discussion.....	61
Figure legends.....	66
References.....	84
Chapter 3: Characterization of <i>zat10/zat12</i> Double-Knockout <i>Arabidopsis</i> Plants....	92
Abstract.....	93
Introduction.....	94
Materials and methods.....	95
Results.....	97
Discussion.....	98
Figure legends.....	101
References.....	106
Chapter 4: Enhanced tolerance to oxidative stress in transgenic <i>Arabidopsis thaliana</i> plants expressing proteins of unknown function.....	108
Abstract.....	109
Introduction.....	111
Materials and methods.....	113
Results.....	118
Discussion.....	125
Figure legends.....	131
References.....	162
Chapter 5: Concluding Remarks.....	169
References.....	174

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 $CX_{2-4}CX_3FX_5LX_2HX_{3-5}H$ (Please see example in Figure 1) (Pabo et al., 2001). Each finger forms two β strands and one α 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 (Englbrecht 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 into 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 denotes 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 et 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). Cytokinin 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 gynoeceium 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 (Dinnyeny 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 *Zat 7* 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/FDLNL/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 results 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-X₃₋₄-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 *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

(Figure 2) (Sakamoto et al., 2000; Sakamoto et al., 2004). 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 finger 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)

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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**

```

At5g04340 (Zat6)      LLLPPLPTPIYKCSVCDKAFSSYQALGGHKASHRKSFSLSLTQSAGGDELSTSSAITTSGIS-
At1g27730 (Zat10)    PPPAVEKLSYKCSVCDKTFSSYQALGGHKASHRKNLSQTLSSGGDDHSTSSATTTSAVT-
At5g43170 (AZF3)     SVTVAEKPSYKCGVICYKTFSSYQALGGHKASHR-----SLYGGGENDKSTPSTAV-----
At3g19580 (AZF2)     PPPEKNLPHYKCNVCEKAFPSYQALGGHKASHRIKPPTVISTTADDSTAPTISIVAGEK-
At3g49930 (Zat13)    SPLSDHQKDYKCSVCGKSFPSYQALGGHKTSHRKPVSVVDVNNNSNGTVTNNGNISNG----
At5g67450 (AZF1)     ASPSDHR-DYKCTVCGKSFSSYQALGGHKTSHRKPTNTSITSGNQELSNNSHNSGVSVI
At3g10470 (Zat15)    GGRAGYVYVYQCKTCDRTFSPFQALGGHRASHKKPKAAMGLHSNHDHKKSNYD-DAVSLH
At5g04390             SGGKAGYVYVYQCKTCDRTFSPFQALGGHRASHKKPKAAS-FYSNLDLKKNTYANDAVSLV
At2g28200 (Zat5)     ----SSFVYECKTCNRFTSSFQALGGHRASHKKPRTSTEEKTRLPLTPQPKSS--ASEEG
At5g03510 (Zat14)    SLGLGLDGVYQCKTCDKSFHSFQALGGHRASHKKPKLGASVFKCCEKKTASAS--TVETV
At3g46090 (Zat7)     --CGGDERVFRCKTCLKEFSSFQALGGHRASHKKLINSNDPSSLGSLSNK--K-----
At3g46080 (Zat8)     ---GGEKRVFRCKTCLKEFSSFQALGGHRASHKKLINSDDPSLLGSLSNK--K-----
At3g46070 (Zat16)    ----RKKRVFRCKTCEKDFDSFQALGGHRASHKLTNSDDKSLPGSPKKPKT-----
At5g59820 (Zat12)    --GGDQKRVTCTCLKQFHSFQALGGHRASHKKPNND--ALSSGLMCK-----
At2g28710 (Zat17)    -----KSRVFACTCNKEFSPFQALGGHRASHRRSAALEGHAPPSPKRVK-----
At2g37430 (Zat11)    ESHT--SNQFECKTCNRKFSFQALGGHRASHKKPKLTVEQKDVKHLSDNY-----
At3g53600 (Zat18)    GSKTNHNNHFECKTCNRKFDSPFQALGGHRASHKKPKLIVDQEQVKHRN-----
At1g02040            VKKQKTAQVVFQCKACKVFTSHQALGGHRASHKKVKGCFASQDKEEEEEEYKEDDDND

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B.**Second zinc finger domain**

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At5g04340 (Zat6)      -----GGGGSVKSHVCSICHKSFATGQALGGHKRCHYEGKNGG-
At1g27730 (Zat10)    -----TGSG---KSHVCTICNKSFPSTGQALGGHKRCHYEGNNN--
At5g43170 (AZF3)     -----KSHVCSVCGKSFATGQALGGHKRCHYD-----
At3g19580 (AZF2)     -----HPAASGKIHECSICHKVFPPTGQALGGHKRCHYEGNLGGG
At3g49930 (Zat13)    -----LVGQSGKTHNCSICFKSFPSGQALGGHKRCHYDGGN---
At5g67450 (AZF1)     NVT-----VNTGNGVVSQSGKIHTCSICFKSFASGQALGGHKRCHYDGGN--
At3g10470 (Zat15)    LNNVLTTPNNSNHRSLVVYKGSNNKVHECGICGAEFTSGQALGGHMRRHRGAVVAAA
At5g04390             HT---TTTVFKNNSRSLVVYKASKNKVHECGICGAEFTSGQALGGHMRRHRGAVVPA
At2g28200 (Zat5)     QNSHFVSGSALASQASNIIN---KANKVHECSI CGSEFTSGQALGGHMRRHRTAVTTIS
At5g03510 (Zat14)    EAG----AVGSFSLSLQVTSDDGSKKPEKTHECSICKAEFSSGQALGGHMRRHRLTINAN
At3g46090 (Zat7)     -----TK--TSHPCPCICGVKFPMGQALGGHMRRHRNEKVS--
At3g46080 (Zat8)     -----TKTATSHPCPCICGVKFPMGQALGGHMRRHRSEKASP-
At3g46070 (Zat16)    -----TTTTAHTCPCICGLEFPMGQALGGHMRRHRNEKEREK
At5g59820 (Zat12)    -----VKTSSHPCPCICGVKFPMGQALGGHMRRHRNESGAAG
At2g28710 (Zat17)    -----PVKHECPCICGAEFVAVGQALGGHMRRHRGGSGGGG
At2g37430 (Zat11)    -----KGNHFHKCSICSFSGTGTGQALGGHMRRHRSSMTVEP
At3g53600 (Zat18)    -----KENDMHKCTICDQMFGTGQALGGHMRRHRRTSMITEQ
At1g02040            EDE-----DEEEDDEEDKSTAHIAARKRSNAHECTICHRVFSGQALGGHKRCHWLTPSNYL

```

C.

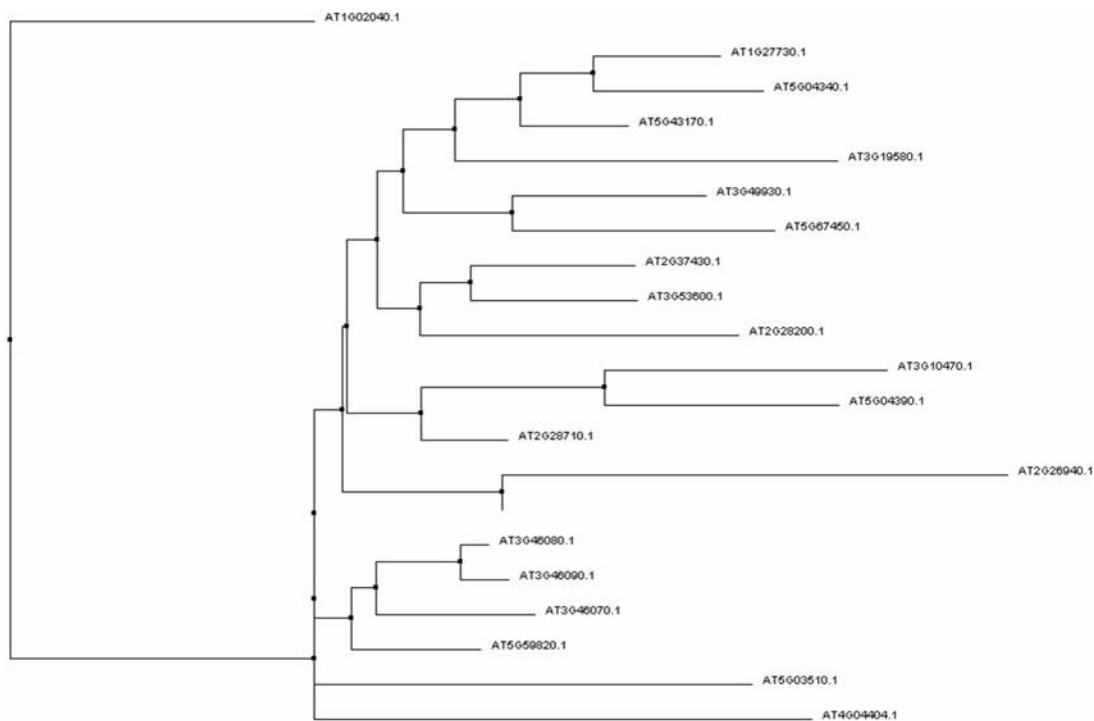
EAR Motif

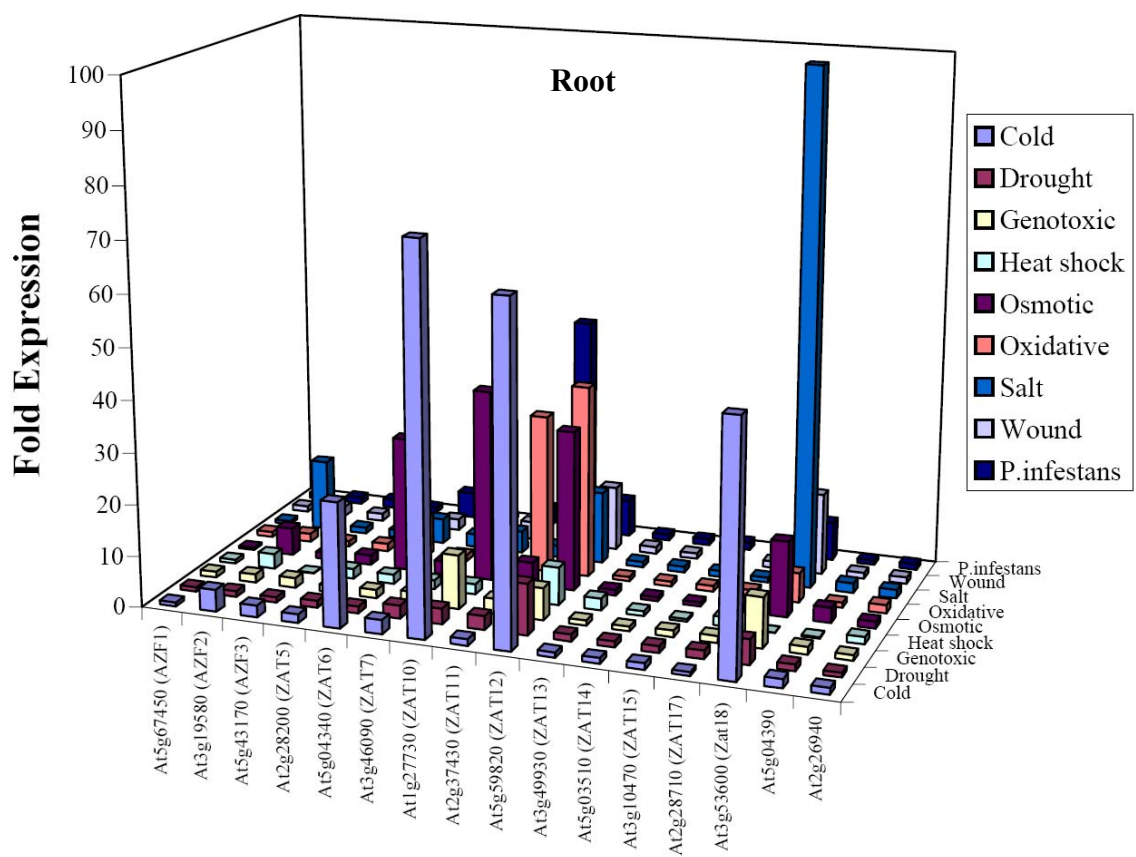
```

At5g04340 (Zat6)      ---GVSSSVSNSEEDVGSTSHVSSGHRGFDLNIPPIPEF-----
At1g27730 (Zat10)    ---INTSSVSNSEAGAGSTSHVSSSHRGFDLNIPPIPEF-----
At5g43170 (AZF3)     -----GVSNSEGVGSTSHVSSSHRGFDLNIIPVQGF-----
At3g19580 (AZF2)     -GGGSKSISHSGSVSSTVSEERSHRGFIDLNLPALPEL-----
At3g49930 (Zat13)    -----GNSNGDNSHKFDLNLPADQVSDETIGKSQL-----
At5g67450 (AZF1)     -----GFDLNLPADQVSVTTS-----
At3g10470 (Zat15)    AASTATVSVAAIPATANTALSLSLSPMSFDQMSEGP IQAPVKRARSVVSLDLDLNLPA---
At5g04390            VIAP-TVTVATAAANTELSSLSSMSFDQISDGHQDHLAMPAKKKARTVVSLDLDLNLPA--
At2g28200 (Zat5)     PVAA-TAEVSR--NSTEEIEINIGRSMEQQRKYLPLDLNLPA-----
At5g03510 (Zat14)    ----ATSAIKTAISSSSHHHHEESIRPKNFLQLDLNLPA-----
At3g46090 (Zat7)     --GSLVTRSFLPETTTVTALKKFSSGKRVACLDLDSME-----
At3g46080 (Zat8)     ----GTLVTRSFLPETTTVTTLKSSSGKRVACLDLDSME-----
At3g46070 (Zat16)    ASNVLVTHSFMPEPTTTVTTLKSSSGKRVACLDLTSVE-----
At5g59820 (Zat12)    --GALVTRALLPEP-TVTTLKSSSGKRVACLDLDSLMVD-----
At2g28710 (Zat17)    -----GRSLAPATAPVTMKKSGGGNGKRVLCLDLNLTPLE-----
At2g37430 (Zat11)    -----SFISPMIPSPMPVLKRCGSSKRI LSLDLNLTPLENDLEYIF
At3g53600 (Zat18)    SIVPSVVYSRPVFNRCSSSKEILDNLNLTPLENDLVLIF-----
At1g02040            RMTSLHDHHHSVGRPQLDQPSLDLNLACQEYSVDPTAMSVGMIE-----

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D.





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 Yap1 Δ 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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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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Running Title: The EAR-Motif and Abiotic Stress**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,

2000). 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*, *Zat7* Δ and *Zat7*m 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'-GGTACCATCGATGAAAACACGTTTCATCTCCCC-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'-CCTAGAAGTCACTAAGTCGATTCAAATGGTT-3') and ZAT7-2 (5'-ATTGTATCAAATAATATTCACAT-3') and cloned into pCRScript (Stratagene). After verification of the sequence, the *Zat7* cDNA was subcloned as a *XhoI/NotI* fragment into 35SpBARN (LeClere and Bartel, 2001). For the 35S::*Zat7* Δ construct, *Zat7* was amplified from genomic DNA using ZAT7-3 and ZAT7-7 (5'-TTAACAAGCCACTCTCTTCCCAC-3') and cloned into pCRScript. After verification of the sequence, *Zat7* Δ was subcloned as a *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 ZAT7-9 (5'-ATCTAAGGCCGCAGCCAAACAAGCCACTCTCTTCC-3') for one piece and ZAT7-8 (5'-TGTTTGGCTGCGGCCTTAGATTCGATGGAGAGTTT-3') and ZAT7-2 for the other piece. A second round of PCR was carried out with these two PCR products and the outer primers (ZAT7-3 and ZAT7-2). After verification of the sequence, *Zat7m* was subcloned as a *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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ 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> (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>) (Mulder et al., 2003). The WoLF protein subcellular localization prediction (PSORT) software (<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*, *Zat7* Δ , *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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$). 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, Zat7 Δ 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 (CYH^S) (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) (*MAT* α , *leu2-3,112*; *trp1-901*; *his3* Δ 200; *ade2-101*; *cyh2^R*; *can1^R*; *gal4* Δ ; *gal80* Δ ; *GAL1::lacZ*; *HIS3UASGAL1::HIS3@LYS2*; *SPAL10::URA3*) as described in the Clontech Yeast Protocols Handbook. Baits were checked for autoactivation 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 $\mu\text{E}/\text{m}^2/\text{sec}$), 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 (*MAT* α , *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4* Δ , *gal80* Δ , *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 pGBKT7-53 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. OD₆₀₀ 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_{600} and OD_{420} were measured using the Perkin-Elmer Victor 3v multiplate reader. The β -galactosidase activity was calculated using the equation: β -galactosidase activity = $1000 \times A_{420} / T \times V \times OD_{600}$ 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-*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 (*Zat7* Δ), and a site-specific mutagenesis converting the LDLDL EAR-motif at position 144-148 to LAAAL at the same position (144-148; *Zat7*m). 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*, *Zat7* Δ or *Zat7*m showed a similar degree of growth suppression (Fig. 2).

Salinity tolerance of transgenic plants expressing *Zat7*, *Zat7* Δ or *Zat7*m

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 (*Zat7* Δ or *Zat7*m) 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 *Zat7*m or *Zat7* Δ 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), *Zat7* Δ 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*, *Zat7* Δ 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*, *Zat7* Δ 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> (Zimmermann et al., 2004), or in knockout plants deficient in cytosolic ascorbate peroxidase 1 grown under controlled conditions (KO-*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*, *Zat7* Δ or *Zat7**m* to cold and osmotic stress. A. Differential tolerance of *Zat7*, *Zat7* Δ or *Zat7**m* to cold and osmotic stress. B. Differential tolerance of *Zat7*, *Zat7* Δ or *Zat7**m* to cold stress. C. Differential tolerance of *Zat7*, *Zat7* Δ or *Zat7**m* 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> (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

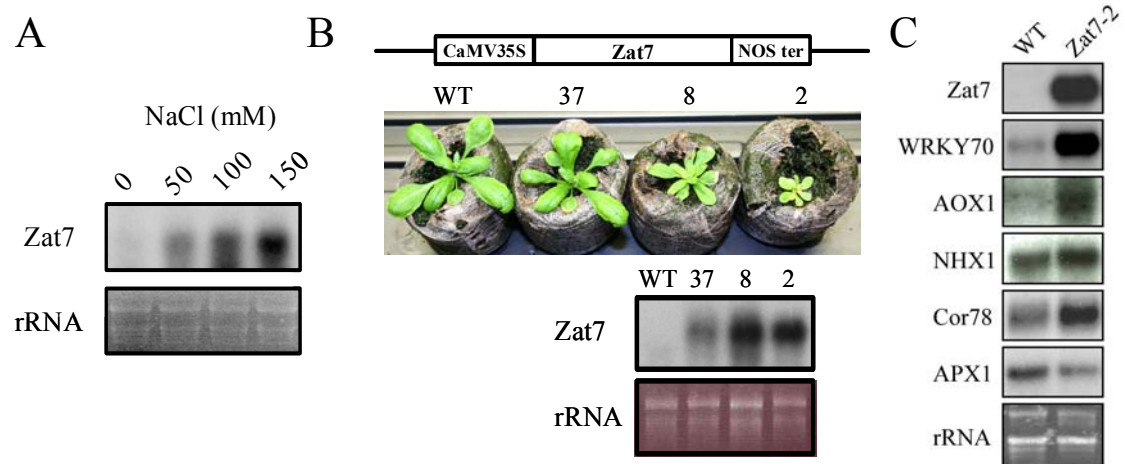


Figure 2

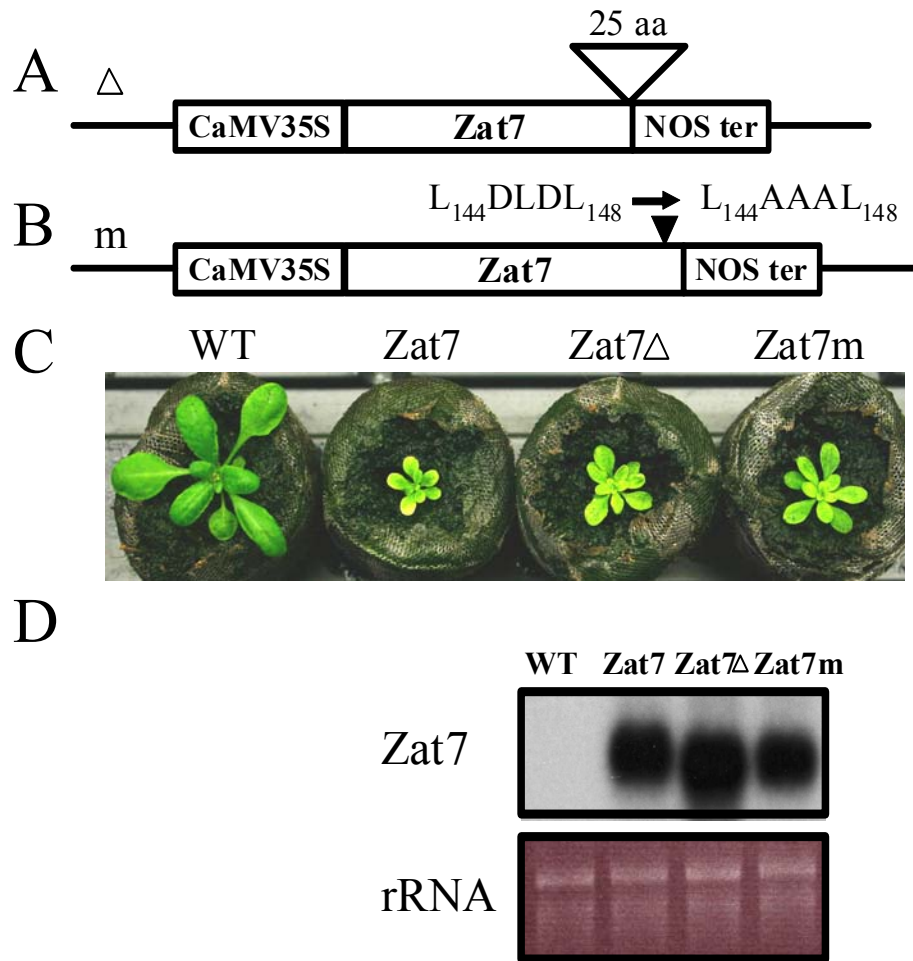


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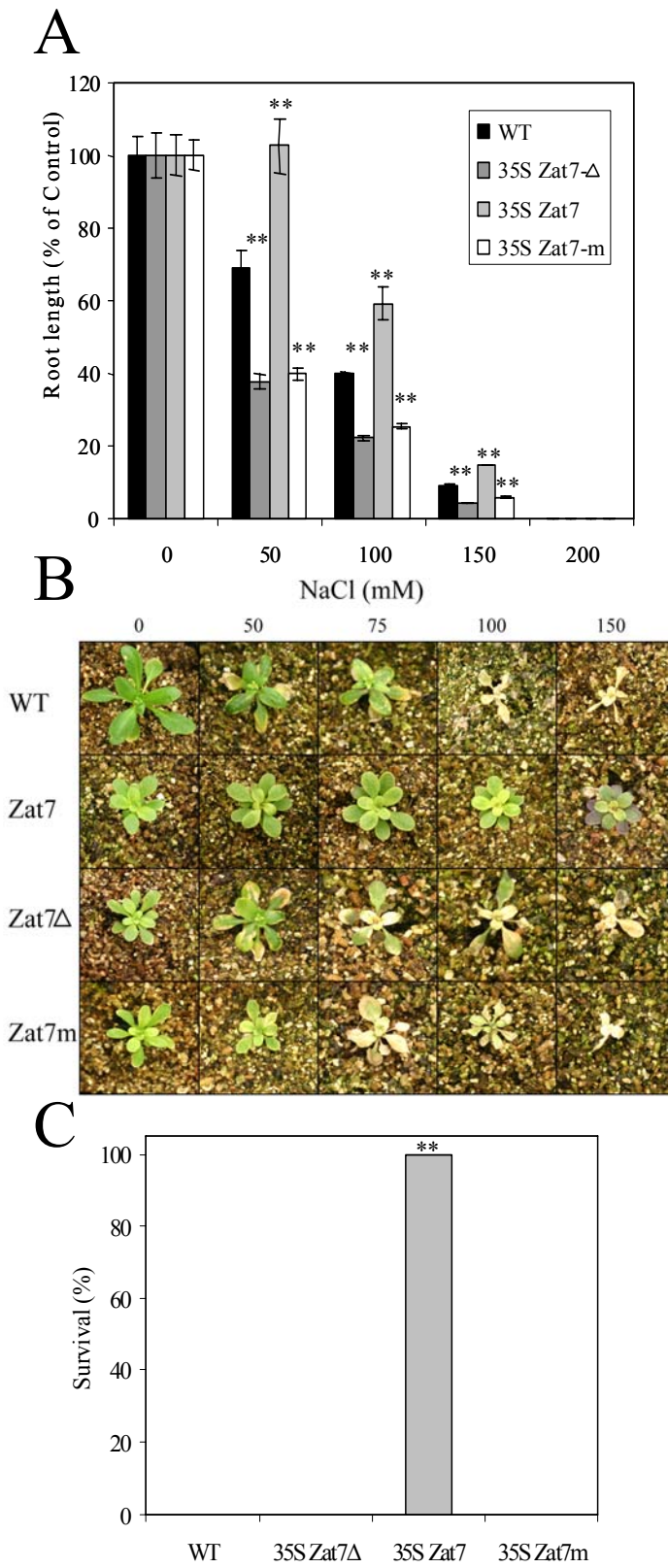
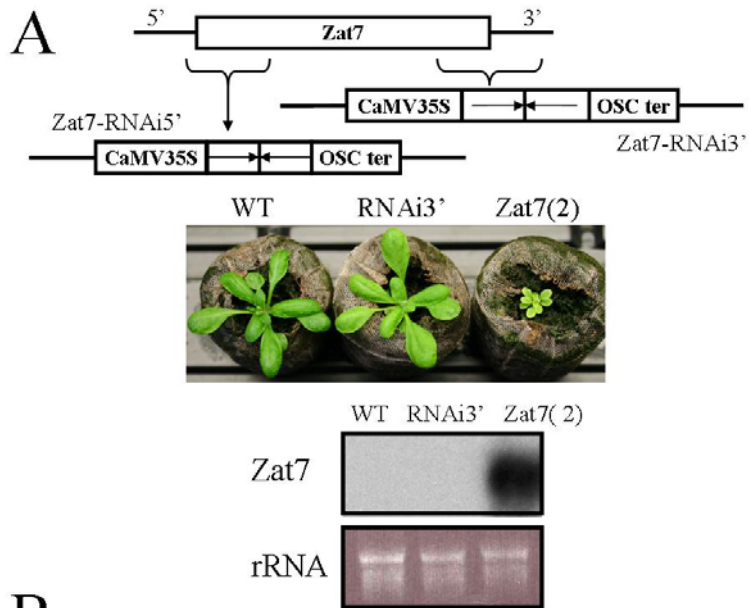
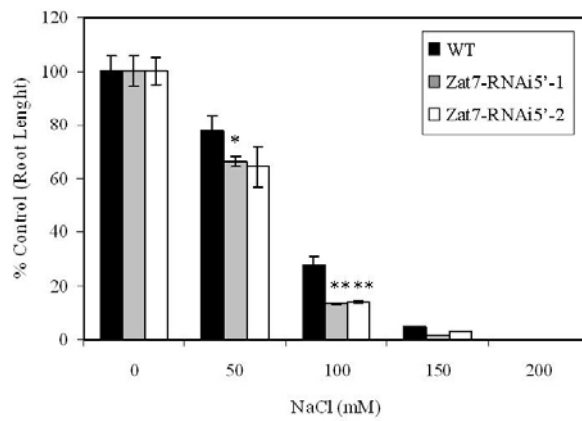


Figure 4



B



C

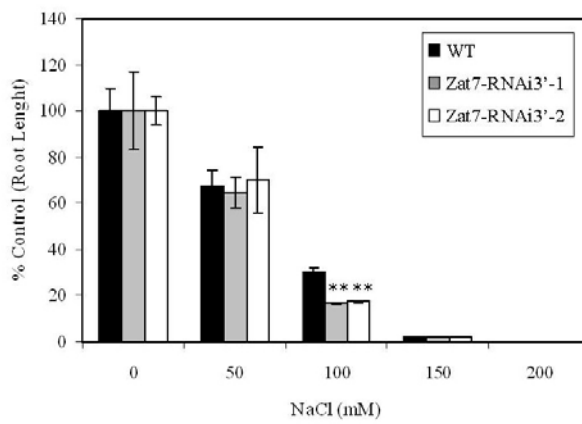


Figure 5

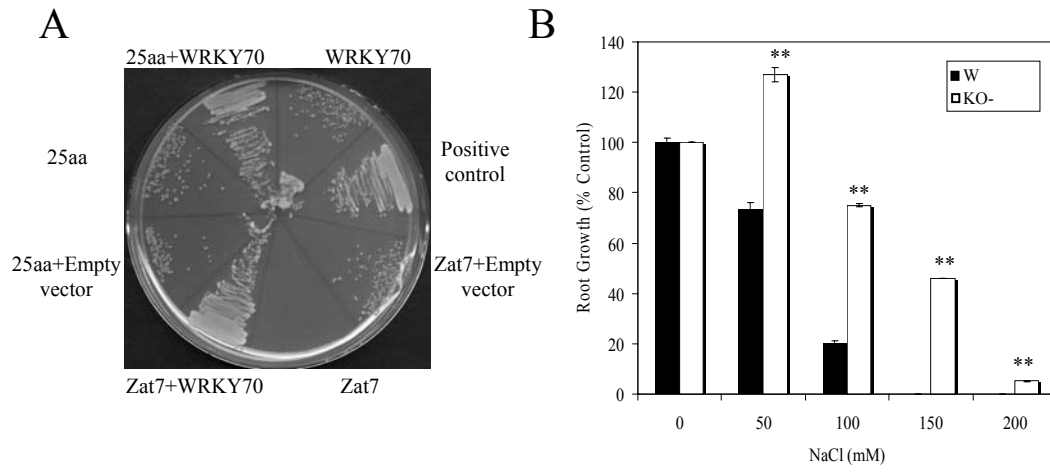


Figure 6

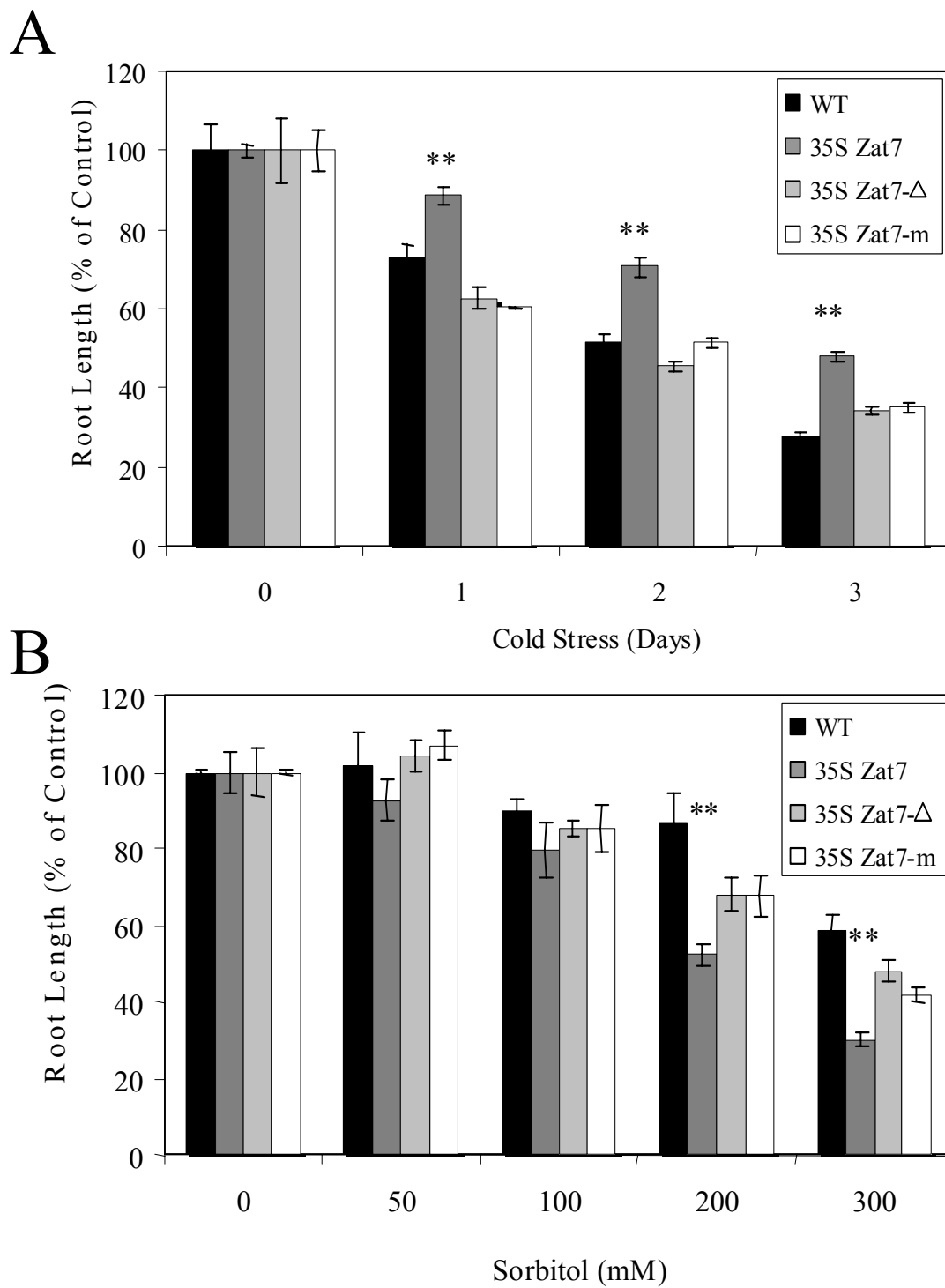
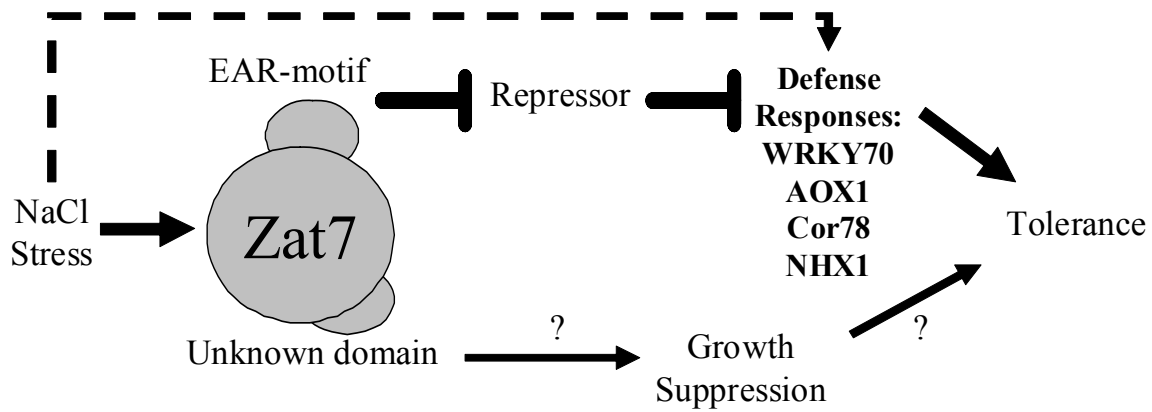
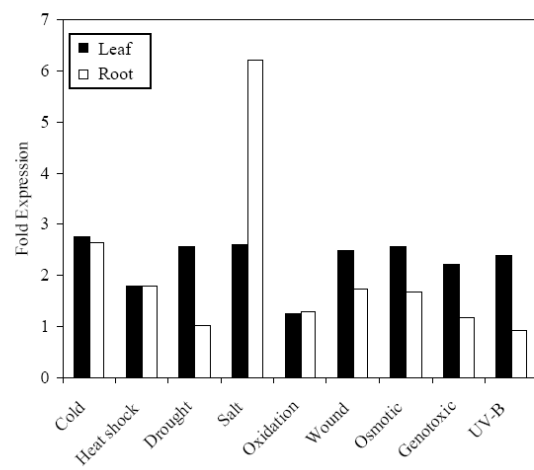


Figure 7

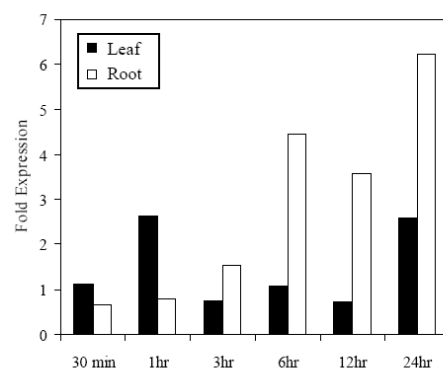


Supplementary Figure 1

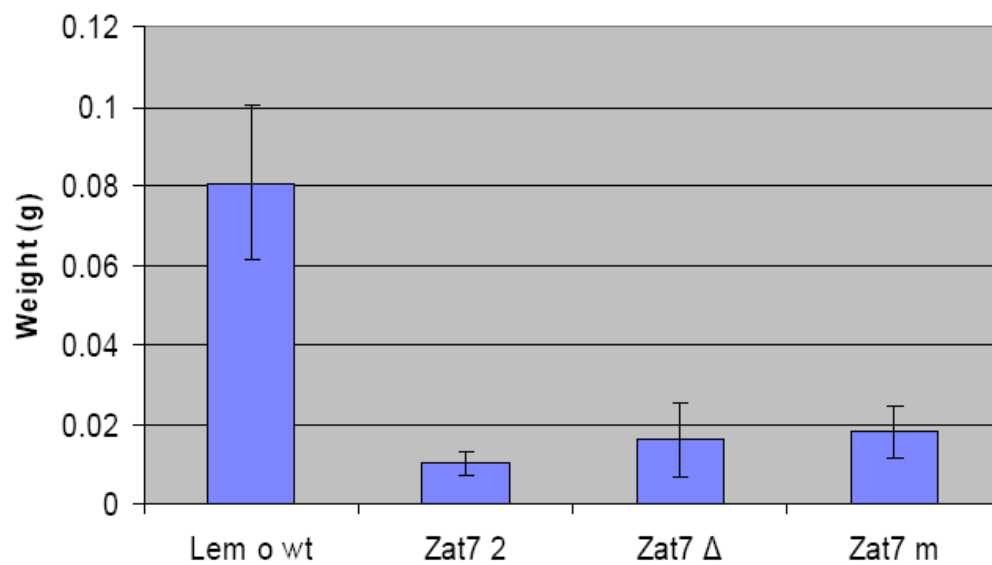
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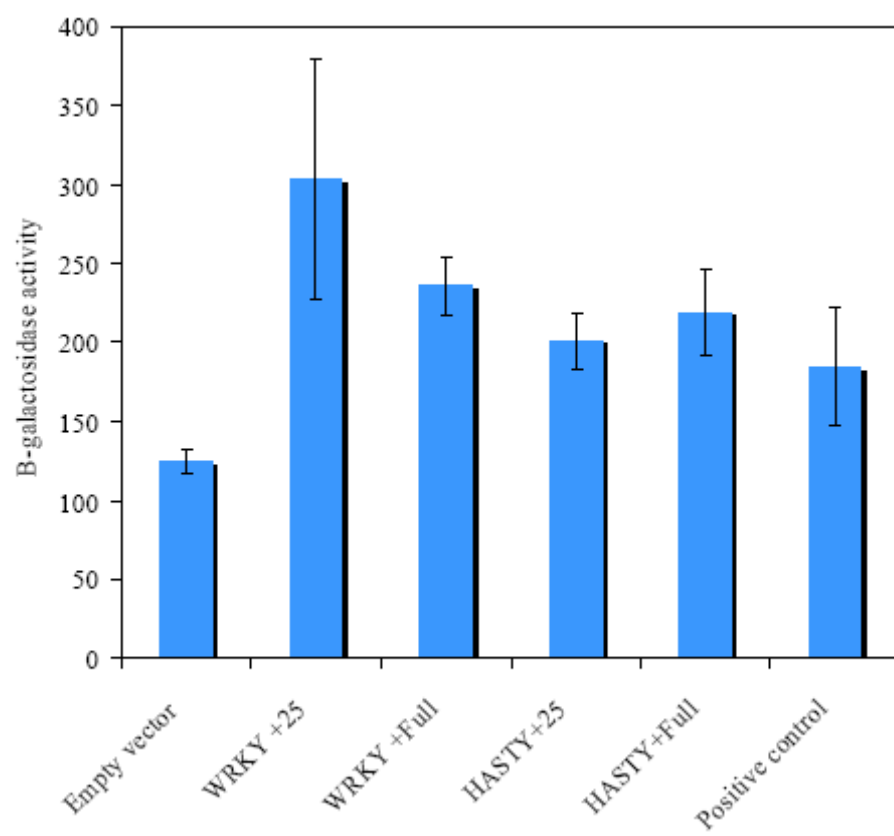
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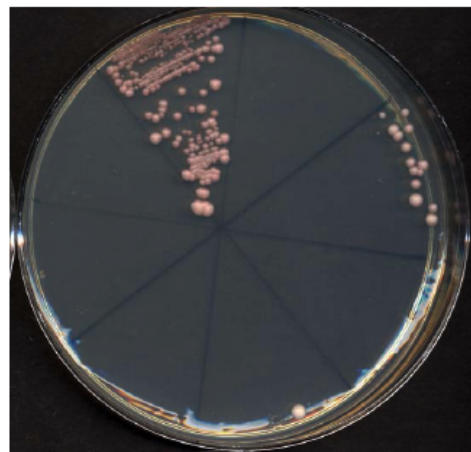
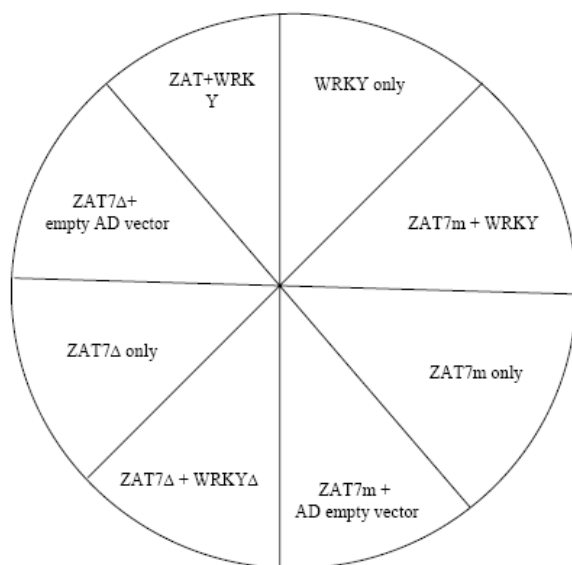
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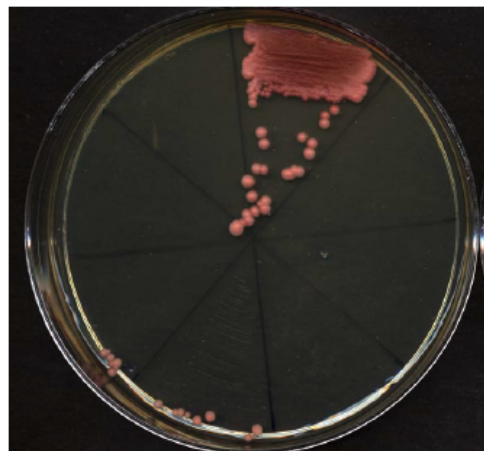
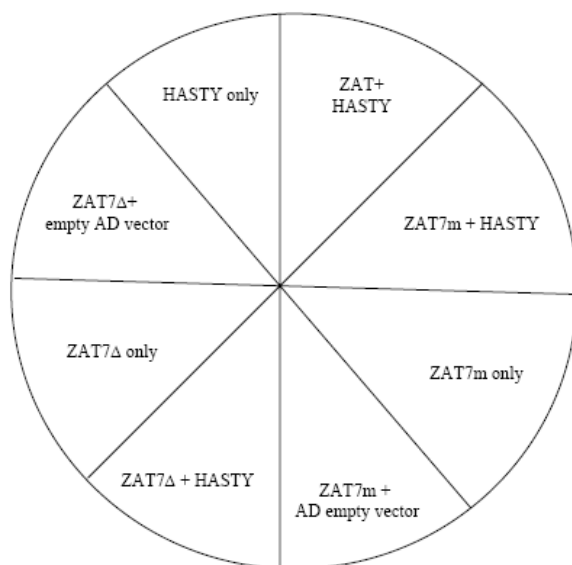
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Table 1

Supplementary Table 1. Proteins that interact with Zat7 in a yeast two-hybrid screen

AGI	Description	E-value	Redundancy	Localization	Exp. (root, salinity)
<i>Clones that interact with full-length and last 25aa</i>					
At3g56400	WRKY70	3.00E-24	1	nucleus	up
At3g05040	member of importin/exportin family, involved in miRNA transport.	3.00E-66	1	nuclear envelope	NC
At5g07140	protein kinase family contains eukaryotic protein kinase domain	2.00E-20	2	unknown	NF
At2g25670	expressed protein	8.00E-09	2	unknown	NC
At5g06060	short-chain dehydrogenase/reductase family protein	7.00E-42	2	unknown	NC
At1g24764	MAP70 protein family	4.00E-64	2	microtubule	NC
At5g23660	nodulin MtN3 family protein	2.00E-11	2	endomembrane	NC
At5g19120	expressed protein, low similarity to extracellular dermal glycoprotein	3.00E-19	3	endomembrane	down
At1g02300	cathepsin B-like cysteine protease	2.00E-14	2	endomembrane	down
At5g02800	protein kinase family contains protein kinase domain	9.00E-40	4	chl	NC
At5g54770	thiazole biosynthetic enzyme, chloroplast (ARA6)	4.00E-41	3	chl, mit	down
At2g13360	alanine-glyoxylate aminotransferase	1.00E-12	2	per	down
At5g13070	MSF1-like family protein	4.00E-73	2	mit	NC
At1g53850	20S proteasome alpha subunit E1	6.00E-90	2	Unknown	NC
At4g27090	60S ribosomal protein L14	5.00E-13	2	cytosolic	NC
At5g14780	formate dehydrogenase	2.00E-44	2	mit	up
<i>Clones that interact with last 25aa only</i>					
At2g22780	malate dehydrogenase	5.00E-54	1	unknown	up
At3g16190	isochorismatase hydrolase family	3.00E-13	1	unknown	NC
At5g49000	Kelch repeat containing F-box protein	8.00E-05	1	unknown	NC
At4g22190	expressed protein	3.00E-71	1	endomembrane	NC
At5g26880	tRNA/rRNA methyltransferase	4.00E-27	1	mit	down
At2g04795	expressed protein	5.00E-18	1	mit	down
At1g79730	hydroxyproline-rich glycoprotein family protein	3.00E-13	1	chl	NC
At3g14420	glycolate oxidase	7.00E-24	1	per	NC
At2g47400	chloroplast protein CP12	6.00E-25	1	chl	down
At1g29910	photosystem II type I chlorophyll a /b binding protein	2.00E-04	1	chl	up
At2G44065	Ribosomal protein L2	8.00E-27	1	mit, ribosome	NC
<i>Clones that interact with full-length only</i>					
At1g24822	hypothetical protein	8.00E-05	1	unknown	NC
At4g25170	expressed protein	2.00E-40	1	mit	NC
At5g46420	16S rRNA processing protein	8.00E-67	1	chl	up
At2g05520	glycine-rich protein (GRP) identical to glycine-rich protein; atGRP	1.00E-06	1	endomembrane	up
At4g29400	hypothetical protein	7.00E-96	1	chl	NF
At4g00570	malate oxidoreductase	3.00E-07	1	mit	NC
At2g02050	NADH-ubiquinone oxidoreductase B18 subunit	1.00E-53	1	mit	NC
At2g47400	CP12 domain-containing protein	9.00E-26	1	chl	down
At2g06050	12-oxophytodienoate reductase	8.00E-87	1	chl	up
At5g51070	ATP-dependent Clp protease (ClpD)	6.00E-27	1	chl	up
At4g02890	polyubiquitin (UBQ14)	1.00E-63	1	unknown	NC
At1g60160	potassium transporter	9.00E-49	1	membrane	NC
At1g19920	sulfate adenyltransferase	5.00E-69	1	chl	NC
At4g27130	translation initiation factor SUI1	1.00E-14	1	unknown	NC
At3g16640	translationally controlled tumor protein -related	1.00E-05	1	cyto	NC
At3g20060	ubiquitin-conjugating enzyme	1.00E-55	1	cyto, nucleus	NC
At1g29930	light-harvesting chlorophyll a/b binding protein	1.00E-07	1	chl	up
At5g38430	ribulose biphosphate carboxylase	2.00E-32	1	chl	NF
At1g67090	ribulose-biphosphate carboxylase	4.00E-37	1	chl	down

NF = Not Found

NC = No Change

up = expression increased 2 fold or more

down = expression decreased 2 fold or more

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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'-AAAGCAAACCATAGGTTTTTGG-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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ 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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$). 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 then 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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) *zat10/zat12* plants were undistinguishable 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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. 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

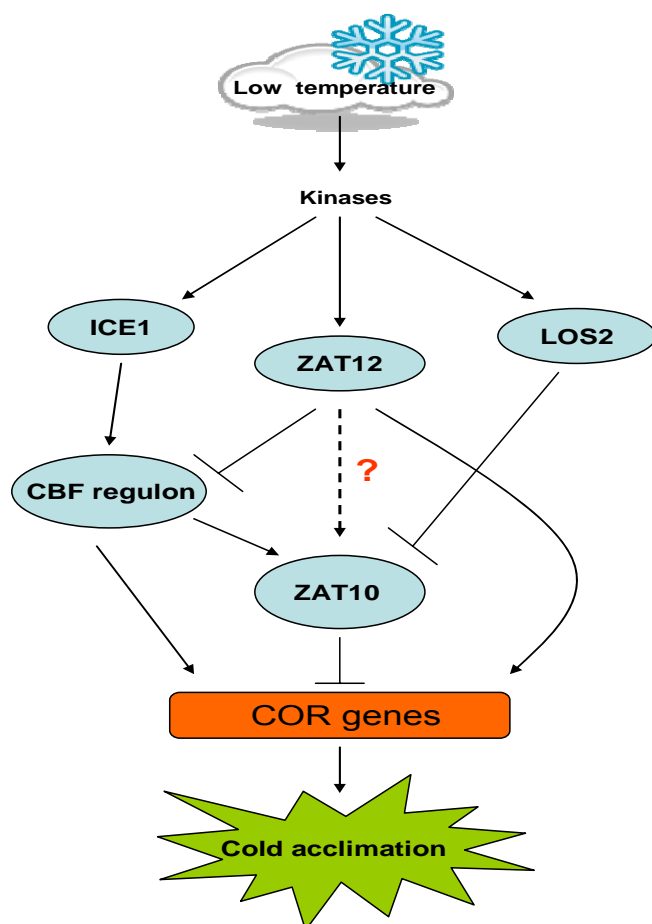


Figure 2



Figure 3

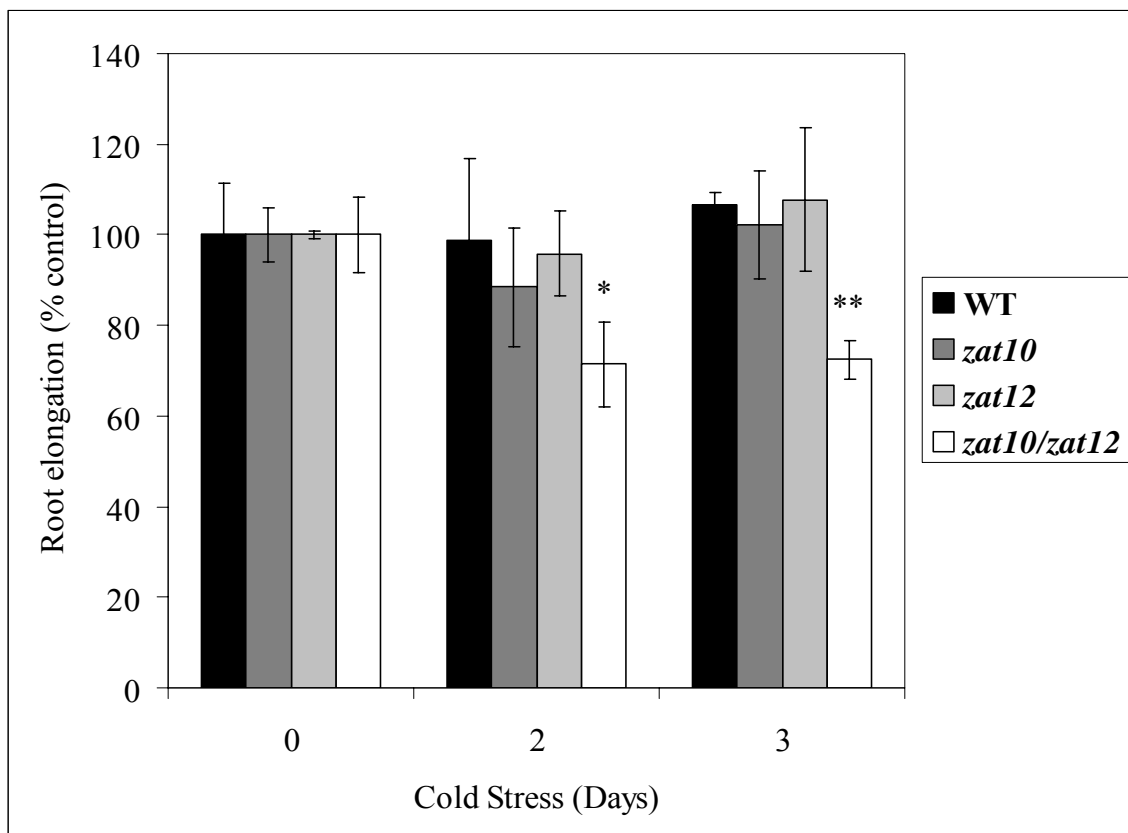
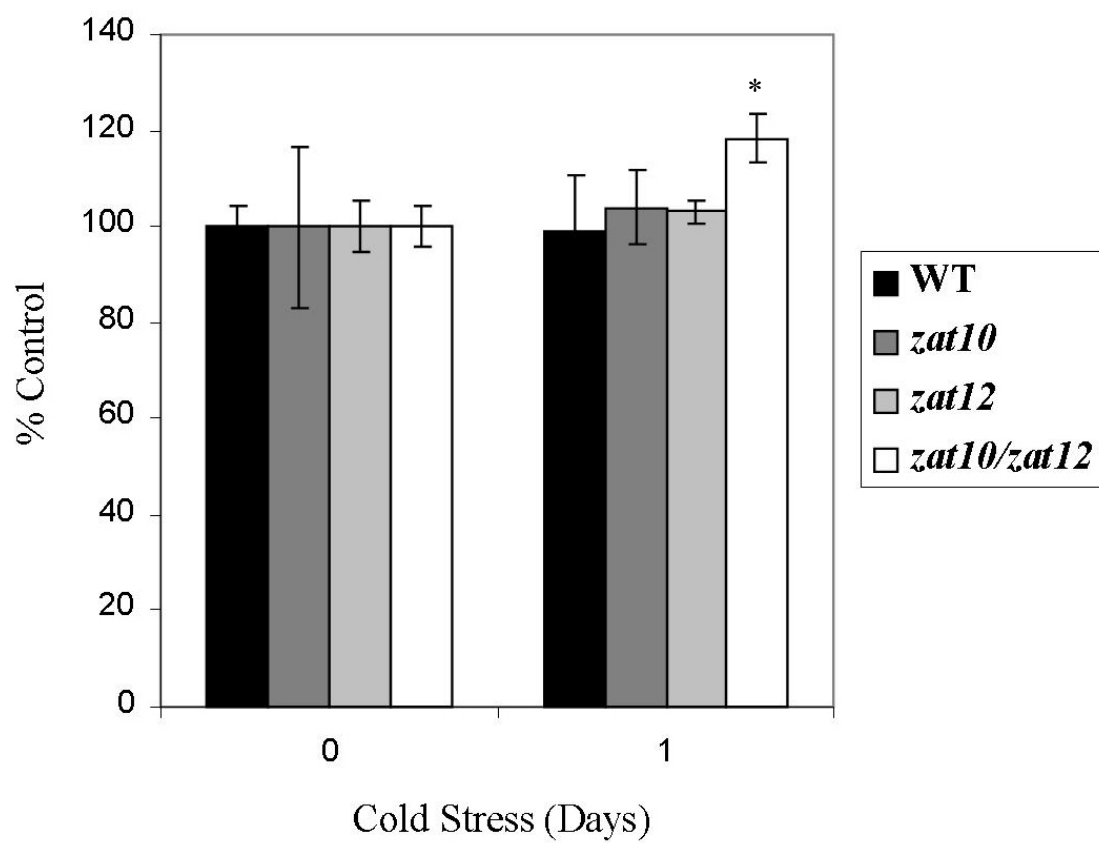


Figure 4



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Chapter 4

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

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Keywords: Arabidopsis, unknown proteins, obscure features, abiotic stress, oxidative stress.

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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (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 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) 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/MAT α his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/ +met15 Δ 0/+ ura3 Δ 0/ ura3 Δ 0, American Type Culture Collection # 201390) and Yap1 Δ mutant (MATa/MAT α his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/+met15 Δ 0/+ ura3 Δ 0/ura3 Δ 0 Δ YAP1, 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 Nguyễn-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 H₂O₂ in the growth media of yeast. Yeast were grown overnight in SG-His liquid media up to mid-log phase, and 50 μ l 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 μ l of working solution (100 μ M 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 VTM Multilabel Counter (Model 1420, PerkinElmer, Turku, FINLAND) using excitation at 531 and emission at 595.

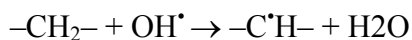
Methyl Viologen (Paraquat) (Halliwell and Gutteridge, 1999)

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 O₂ which in turn forms O₂^{•-} in a redox cycle. Much of O₂^{•-} is converted into H₂O₂ which can cause the inactivation of Calvin cycle enzymes, thus resulting in the inhibition of CO₂ fixation.

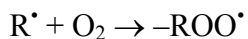
***tert*-Butyl Hydroperoxide** (Halliwell and Gutteridge, 1999)

tert-butyl hydroperoxide (tB-OOH) is an artificial organic hydroperoxide. It interacts with all cellular membranes and results in the accumulation of alkoxy 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.



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 O₂ which in turn produces peroxy radical.



Peroxy radicals can remove H from another lipid molecule. Then, newly formed carbon radical can react with O₂ to form another peroxy 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-*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 (*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 *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 *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 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 μ M 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 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 μ M 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 Yap1 Δ ; Kuge and Jones, 1994). Transformed and untransformed yeast were subjected to oxidative stress imposed by paraquat or t-butyl hydroperoxide as described by Tiên Nguyễn-nhu and Knoop, (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 (Yap1 Δ) 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 Yap1 Δ) more

susceptible to oxidative stress imposed by paraquat or t-butyl hydroperoxide. Expression of AT1G50290 in yeast (wild type or Yap1 Δ) 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 H₂O₂ 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; Kovtun 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; Kovtun 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-*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-*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 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; Vandembroucke et al., 2008). Our study, therefore, highlights 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.

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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 μ M 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, Yap1 Δ , and Yap1 Δ transformed with AT1G50290, showing enhanced sensitivity to oxidative stress induced by paraquat and t-butyl hydroperoxide in Yap1 Δ cells transformed with AT1G50290. **B.** Liquid media growth assays of wild type yeast, wild type yeast transformed with AT1G50290 (Top), and Yap1 Δ , and Yap1 Δ 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^oC, 24 hr), or **B.** Cold (4^oC, 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 μ M) 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

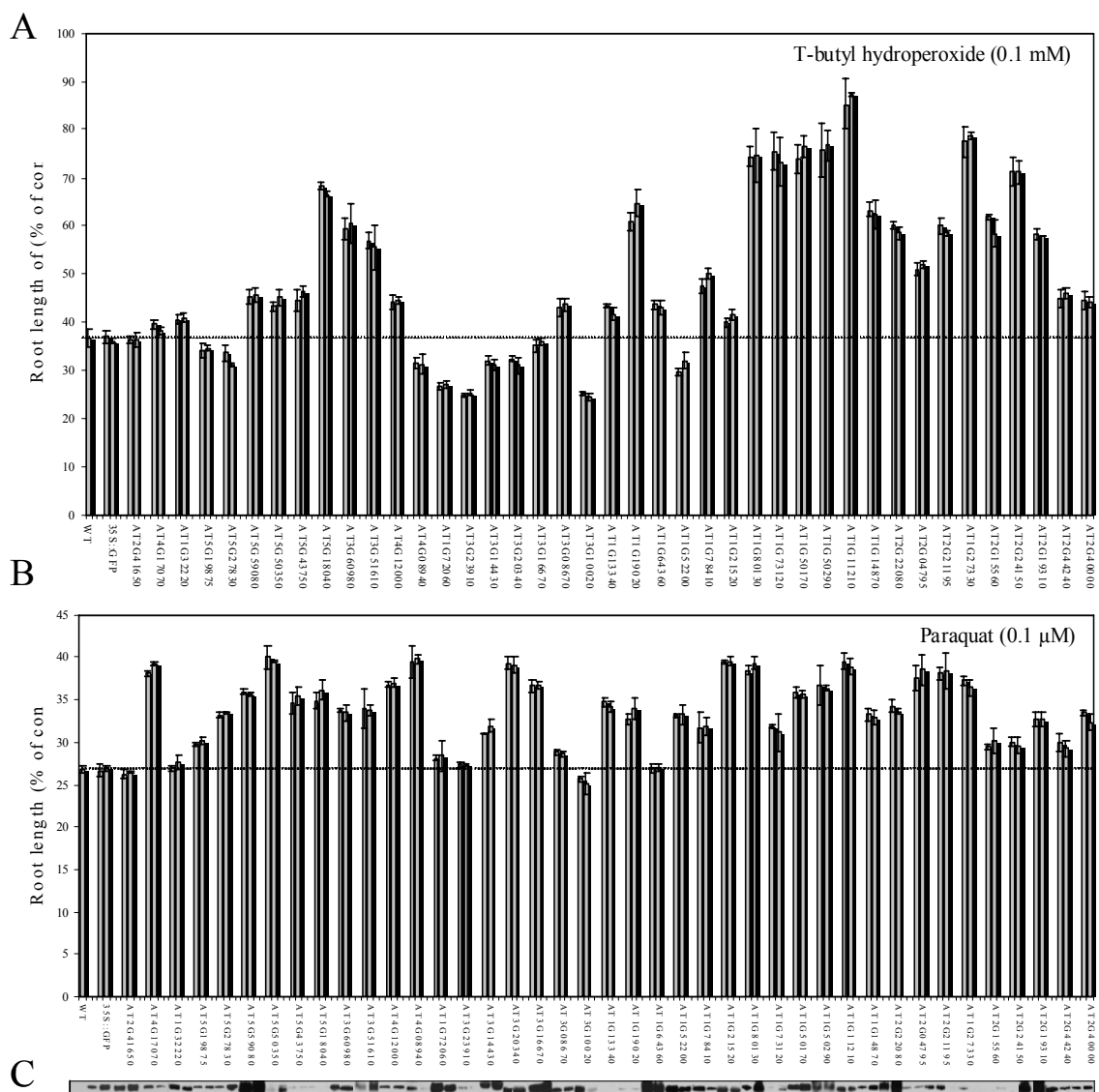


Figure 2

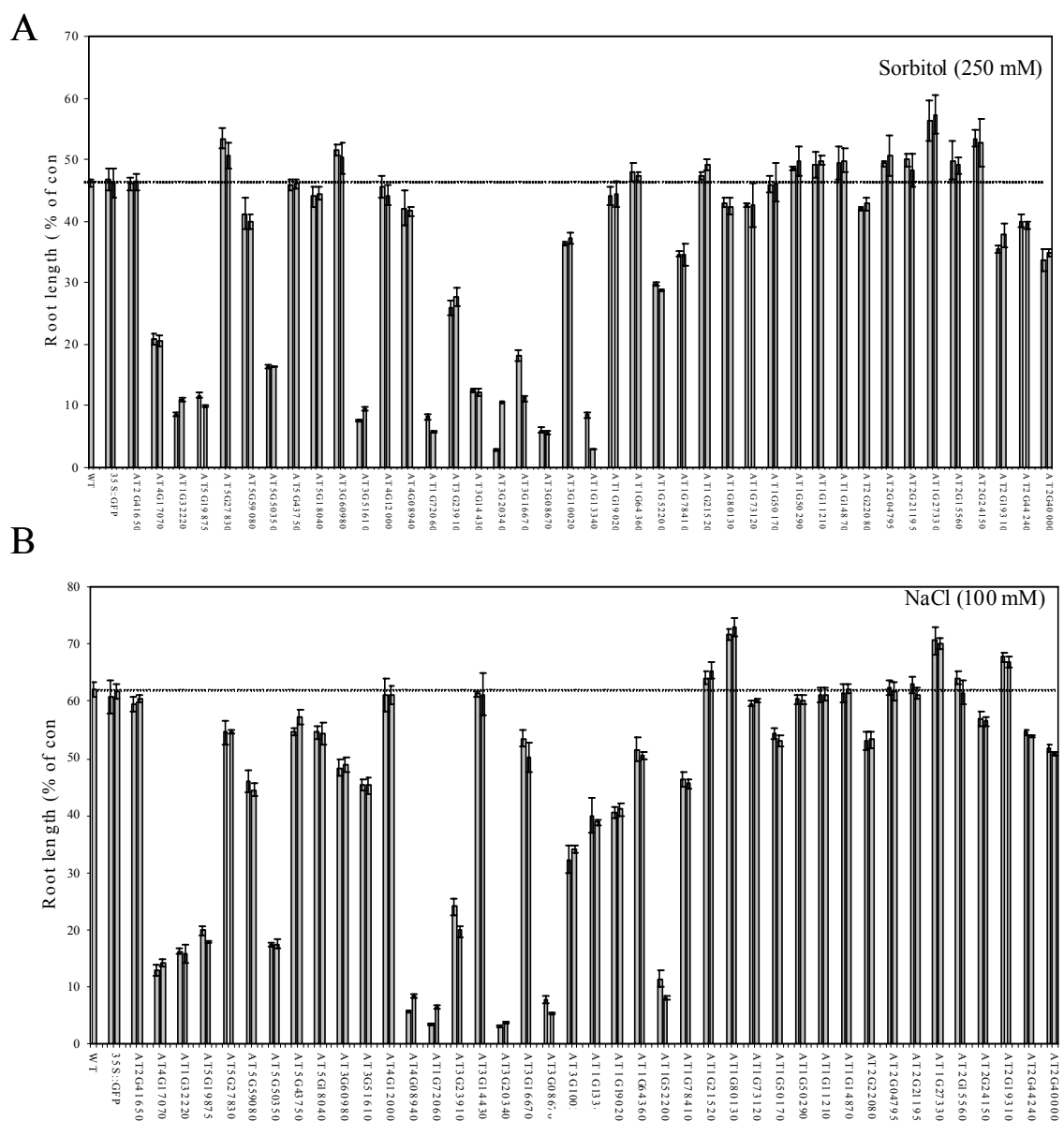


Figure 3

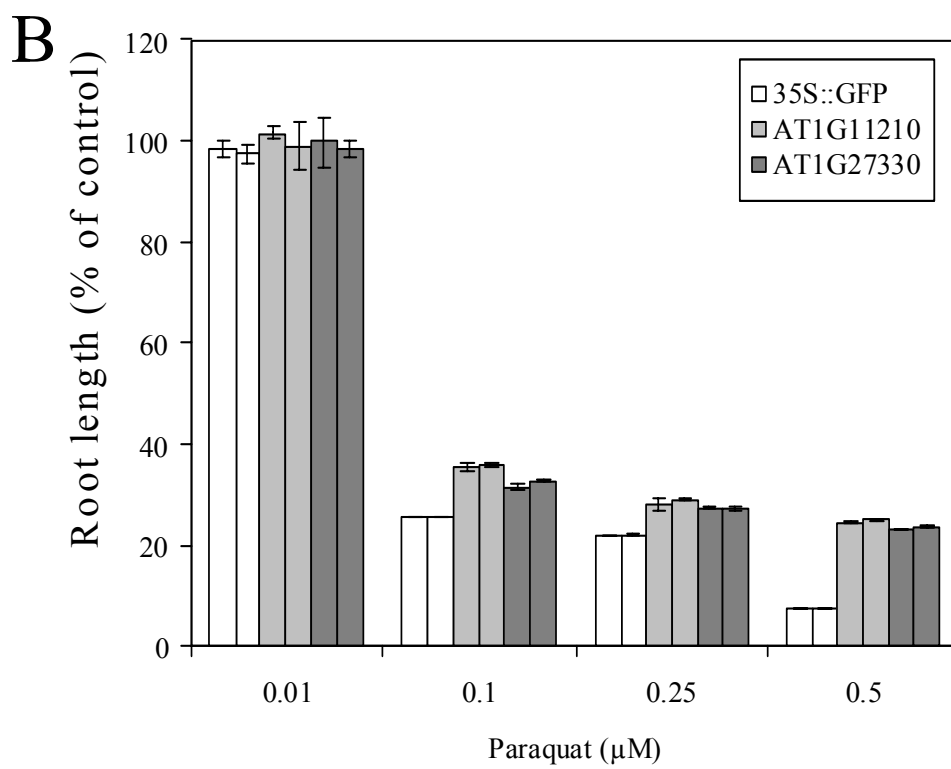
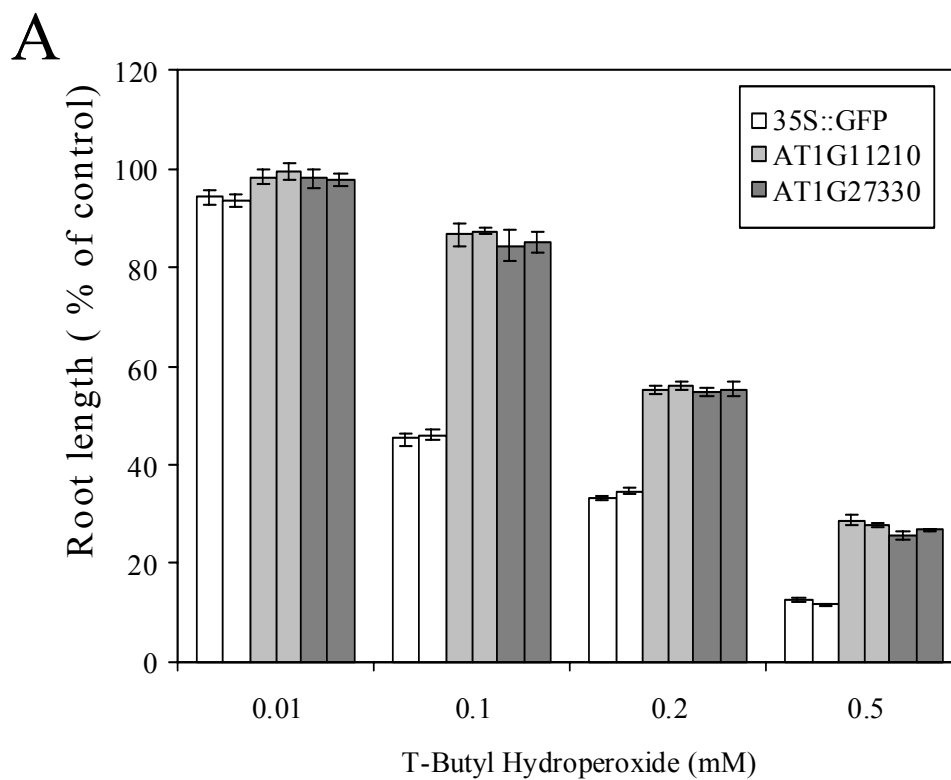


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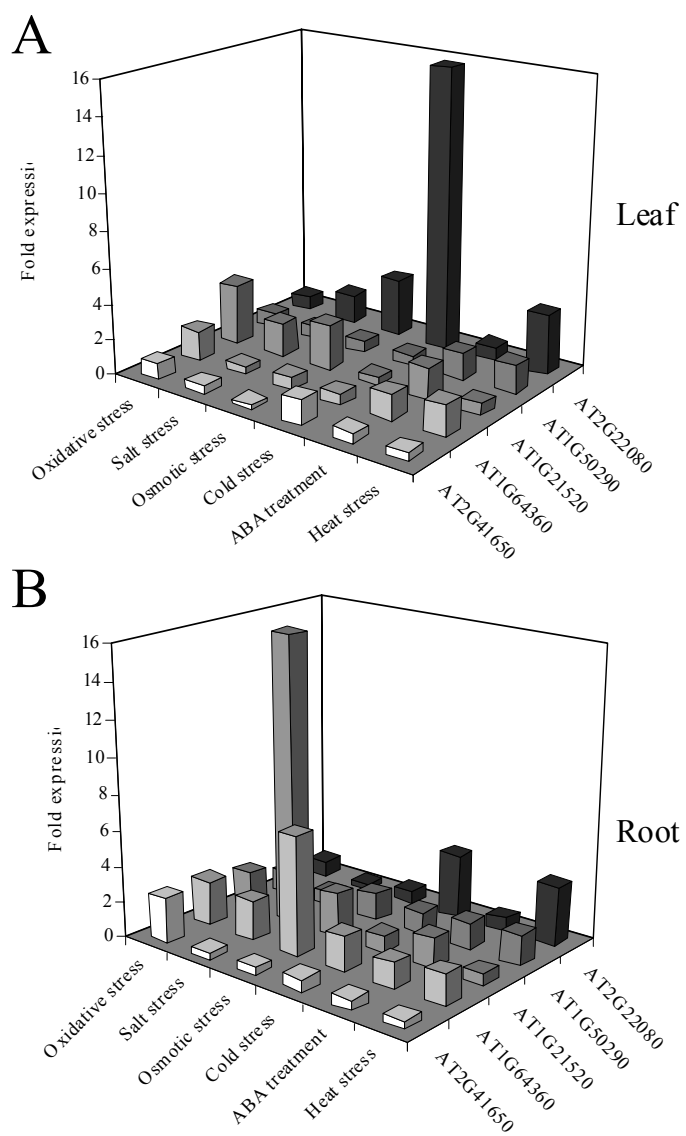


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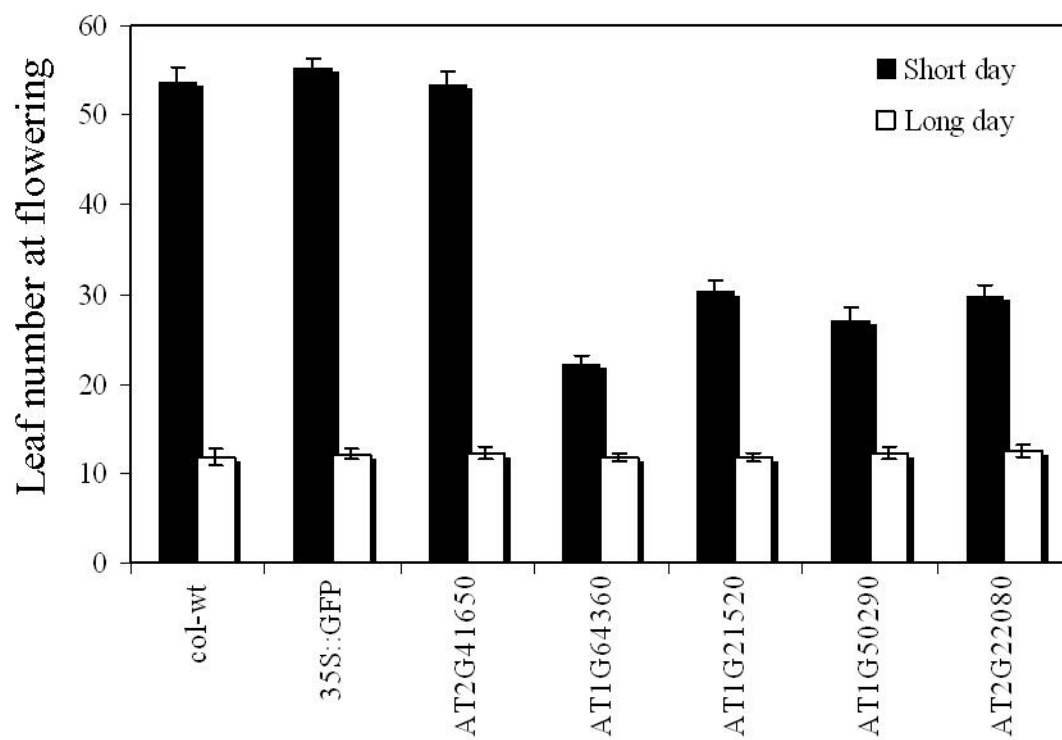


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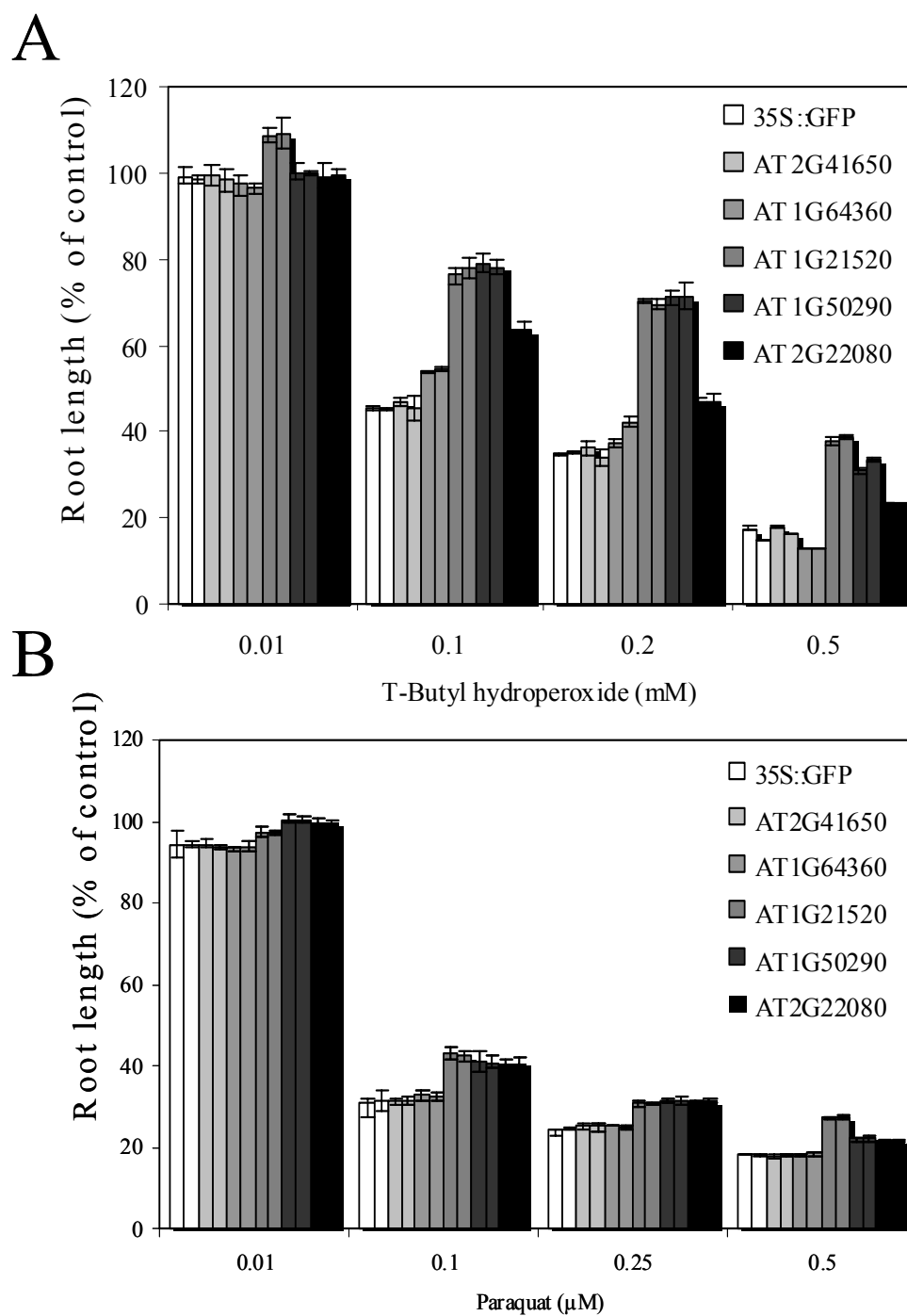


Figure 7

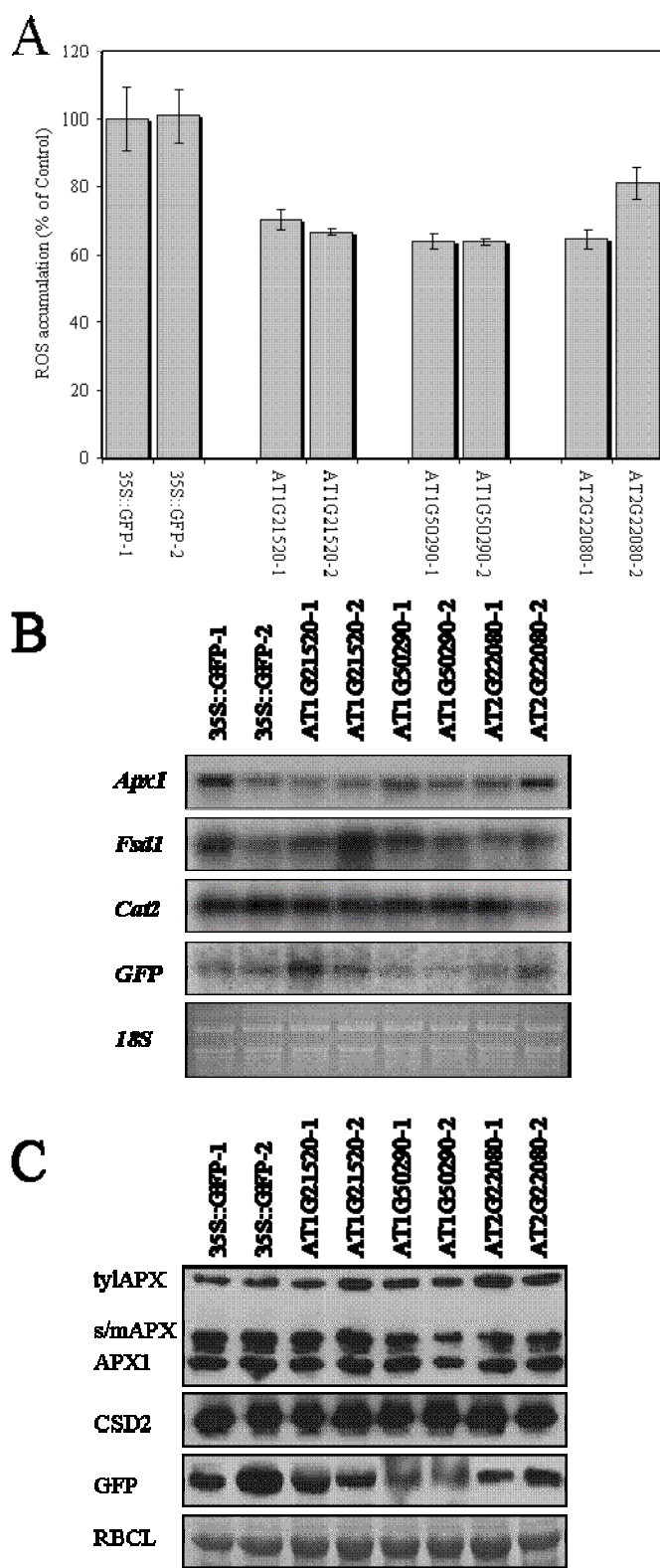


Figure 8

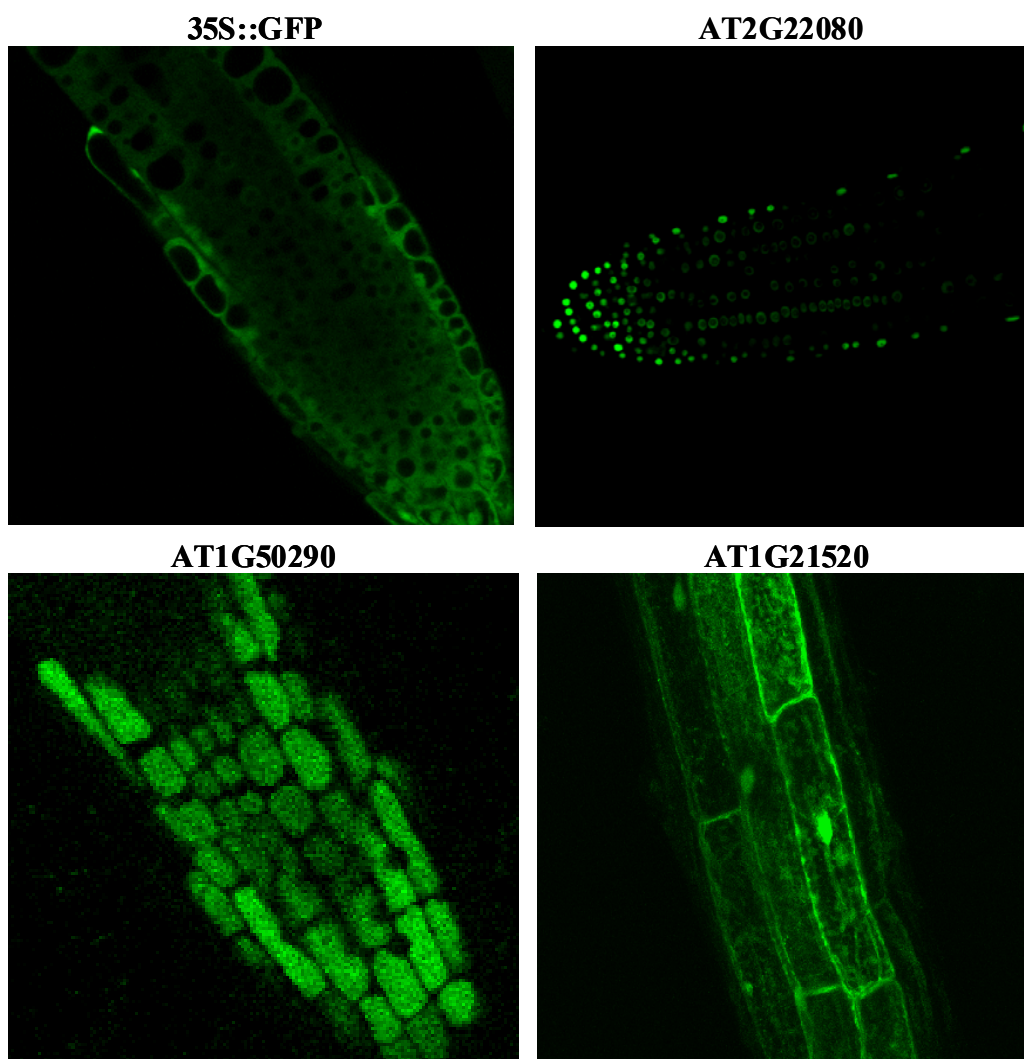
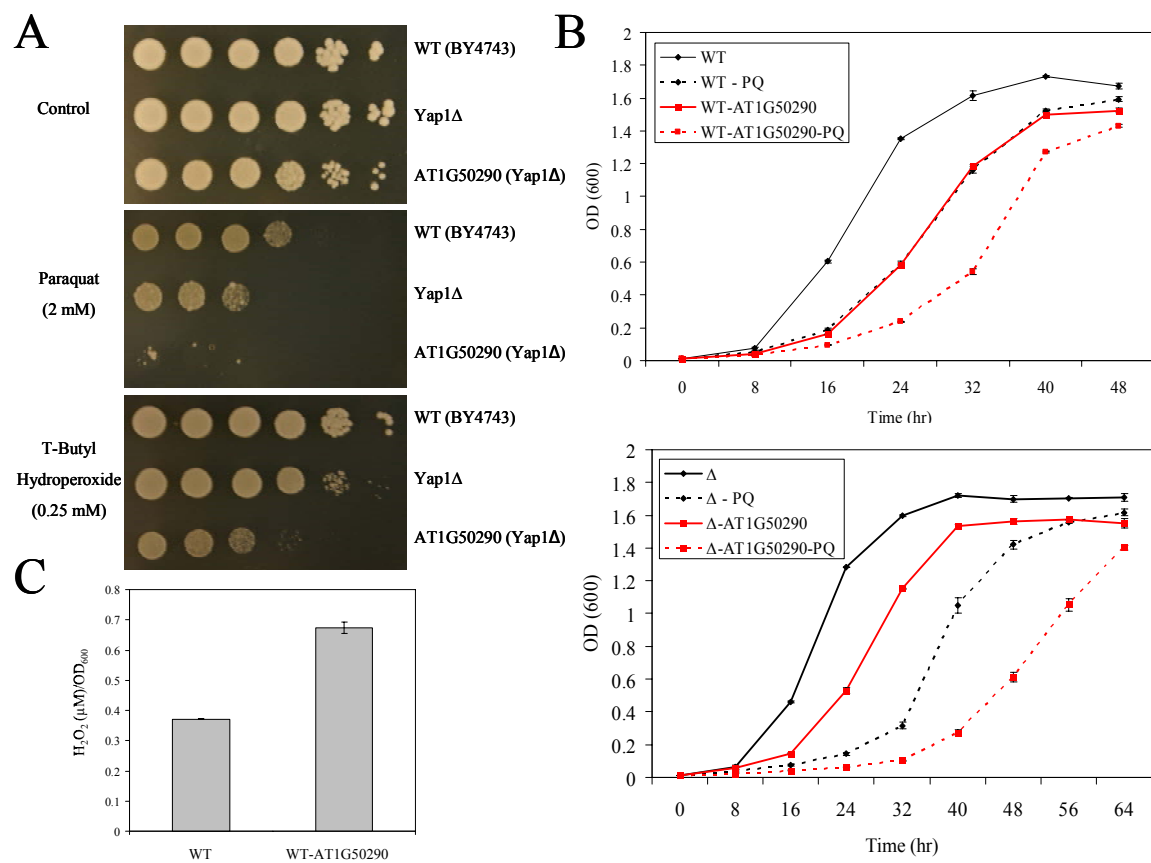


Figure 9



Supplementary Table 1

Suppl Table 1. Summary of stress tolerance of the different lines			Molecular weight of						
Locus	Annotation	fusion protein (kD)	t-butyl	Paraquat	Osmotic	Salinity	Heat	Cold	ABA
AT2G41650	Unknown (POF)	53.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT4G17070	Unknown (POF)	72.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G32220	Contain NAD-dependent epimerase/dehydratase	78	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT5G19875	Unknown (POF)	60.4	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT5G27830	Unknown (POF)	79	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT5G59080	Unknown (POF)	61	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT5G50350	Unknown (POF)	110	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT5G43750	Unknown (POF)	70	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT5G18040	Unknown (POF)	74.7	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G60980	Contain pentatricopeptide repeat domain	93.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G51610	Unknown (POF)	72.2	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT4G12000	Contain SNARE domain	80.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT4G08940	Contain Ubiquitin hydrolase domain	92.7	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G72060	Contain serine-type endopeptidase inhibitor domain	54.8	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G23910	Unknown (POF)	95	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G14430	Unknown (POF)	55.2	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G20340	Unknown (POF)	59.6	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G16670	Unknown (POF)	62	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G08670	Unknown (POF)	106	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT3G10020	Unknown (POF)	63	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G13340	Contain DUF292 domain	91.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G19020	Unknown (POF)	55	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G64360	Unknown (POF)	55.1	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G52200	Contain PLAC8 domain	67	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G78410	Contain VQ motif	58	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G21520	Unknown (POF)	53.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G80130	Unknown (POF)	80	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G73120	Unknown (POF)	58	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G50170	Contain Cbix domain	71	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G50290	Unknown (POF)	62	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G11210	Contain DUF761 domain	81	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G14870	Contain PLAC8 domain	63	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G22080	Contain zinc finger domain	65	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G04795	Unknown (POF)	56.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G21195	Unknown (POF)	56.5	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT1G27330	Contain ribosome associated RAMP4 domain	53.6	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G15560	Contain DUF537 domain	100.4	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G24150	Contain HlyIII domain	85	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G19310	Contain HSP20/alpha crystallin domain	64.7	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G44240	Contain DUF239 domain	90.8	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow
AT2G40000	Contain Hs1pro-1 domain	95.4	Yellow	Red	Blue	Blue	Blue	Yellow	Yellow

Yellow	No change
Red	More tolerant
Blue	Less tolerant

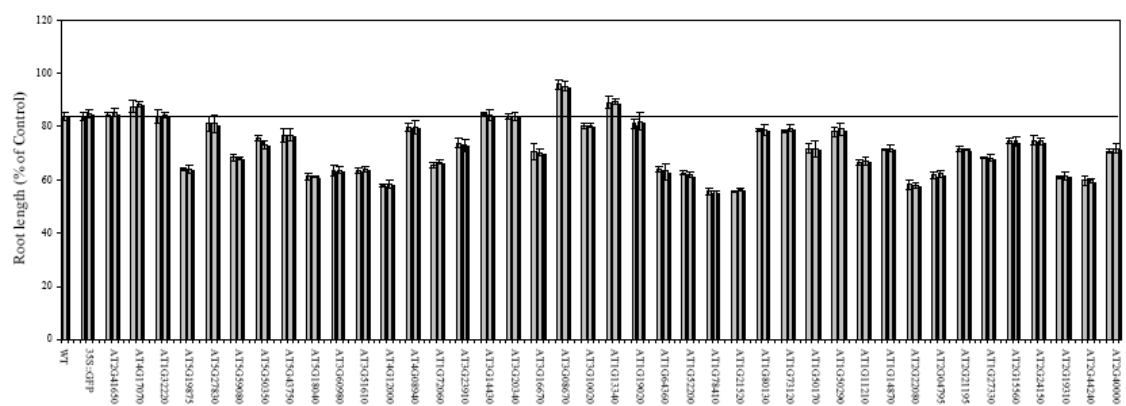
Supplementary Table 2

Suppl Table 2. Primers used for the cloning of the different proteins with unknown function

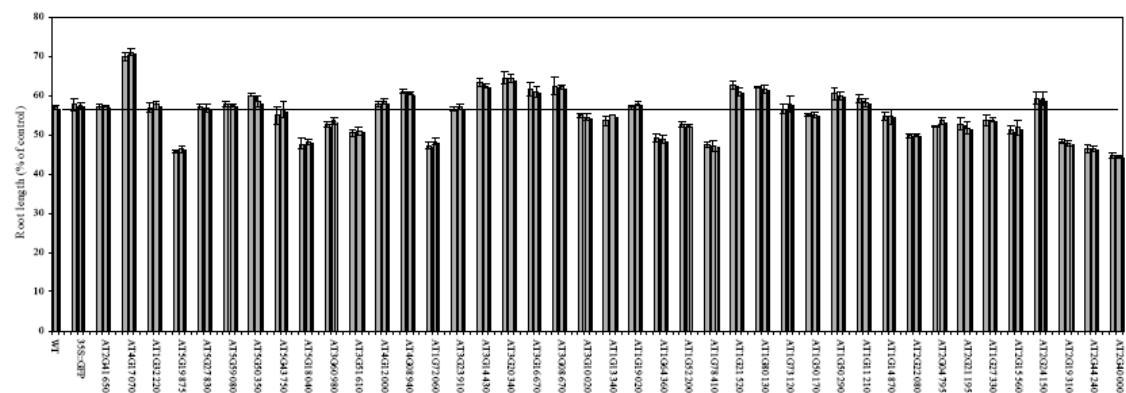
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AT1G32220	U17408		gagactcgagATGACTTCGTTTCTTAGT		gagaactagtGCTCTCATTTTTGC
AT5G18875	U61271		gagactcgagATGGCTAGATCACCATA		gagaactagtTTAGCCCCAACGGGAAC
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AT5G50350	U24198		gagagtcgacATGGCGACTTCTGCTTT		gagaactagtCCGATCAATGAGCGGCTAC
AT5G43750	U60098		gagactcgagATGGCAACGGTCACGAT		gagaactagtCGTGTGGCGATATCAT
AT5G18040	U11383		gagactcgagATGAATATGATTACAGCG		gagaactagtCCGTTGATAGATAAGCGT
AT3G80980	U24736		gagactcgagATGTCTCTGATCGGC		gagaactagtCCAAAGAGATGGGAGACCTC
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AT4G12000	U20042		gagactcgagATGACTTATCACGAAGA		gagaactagtTGTAACAACAAAGCTTC
AT4G08940	U61884		gagagtcgacATGGCTTGGCTTCGTTTCT		gagaactagtCCAACCTTGCTCCATCTG
AT1G72080	U62101		gagactcgagATGGTTACATACAAGAT		gagaactagtTTGCTACAATGGATAGT
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AT3G20340	U63078		gagactcgagATGGGAATTGTTTAAG		gagaactagtCCGTTAACTTCTGGTATGCT
AT3G16670	U22022		gagactcgagATGGCAGTTATCAAGAA		gagaactagtATTGTGGGGATAAAACC
AT3G08670	U61987		gagactcgagATGAATAGGAATCTCAG		gagacctaggAGTGGGGCAAATGGCTCT
AT3G10020	U13057		gagactcgagATGCAGGCGGTATCAGT		gagaactagtCGACCGACCAAGCGGAGG
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Supplementary Figure 1

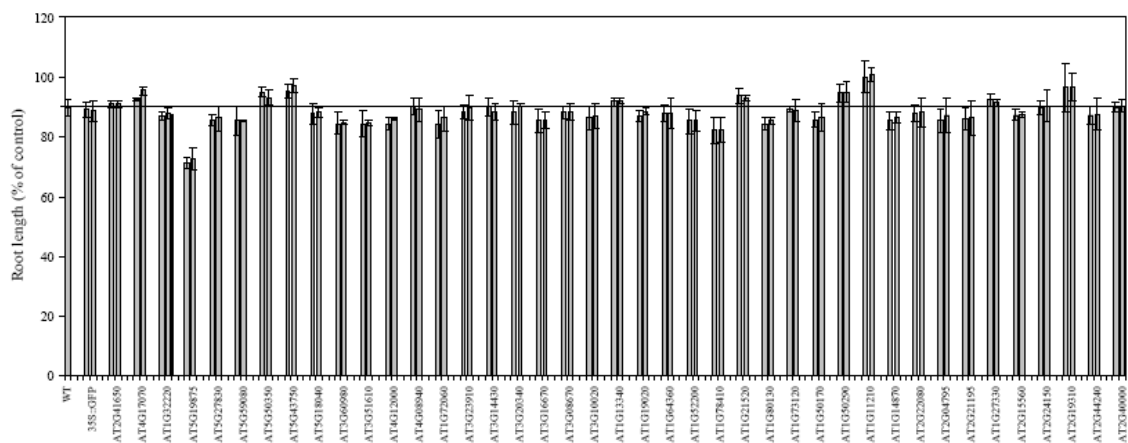
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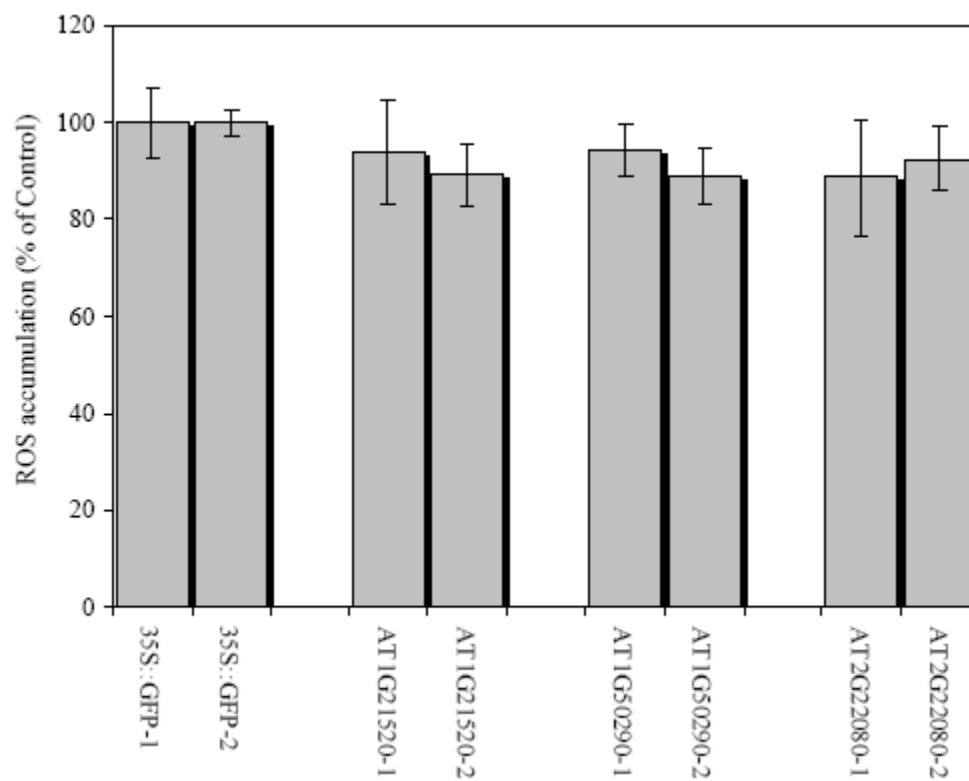
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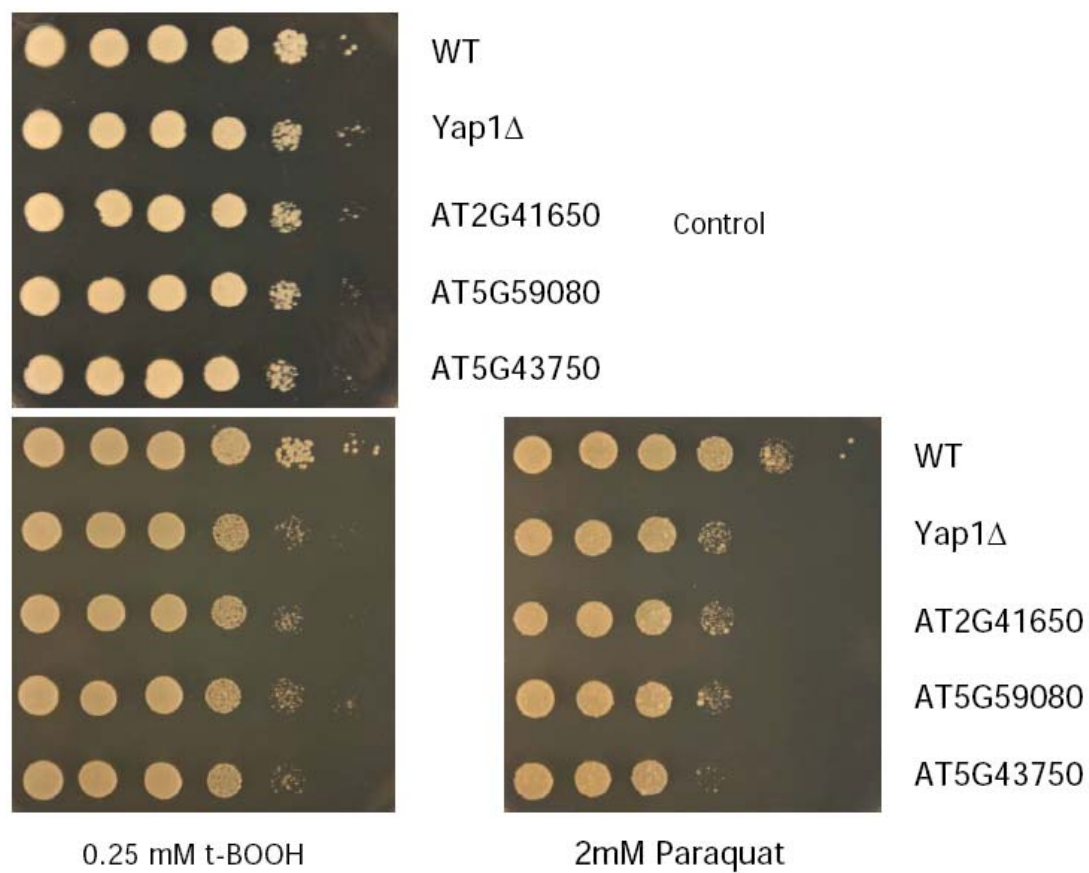
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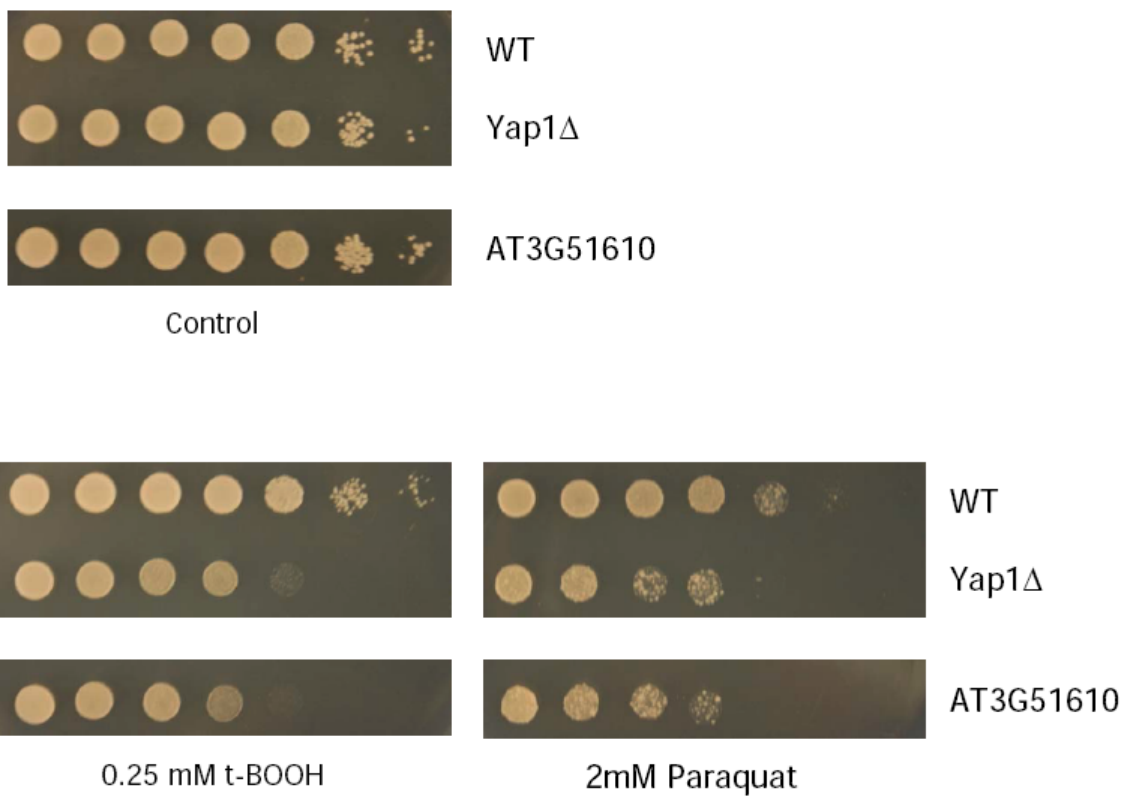


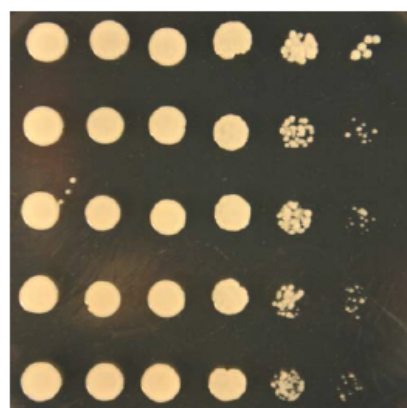
Supplementary Figure 3



Supplementary Figure 4







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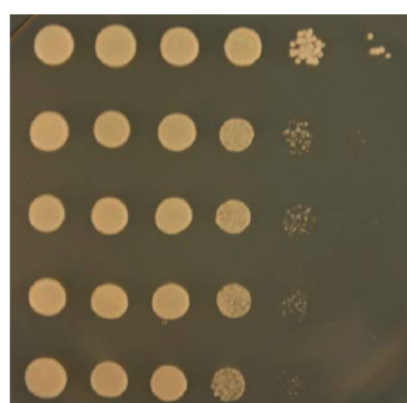
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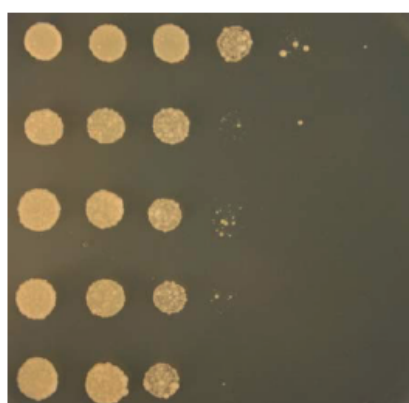
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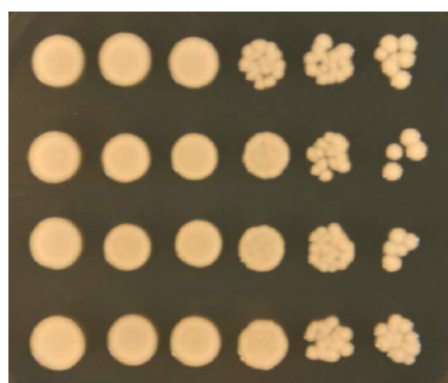
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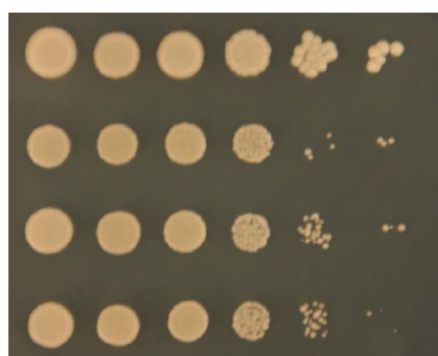
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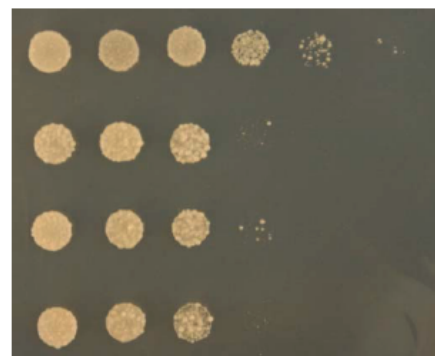
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Control



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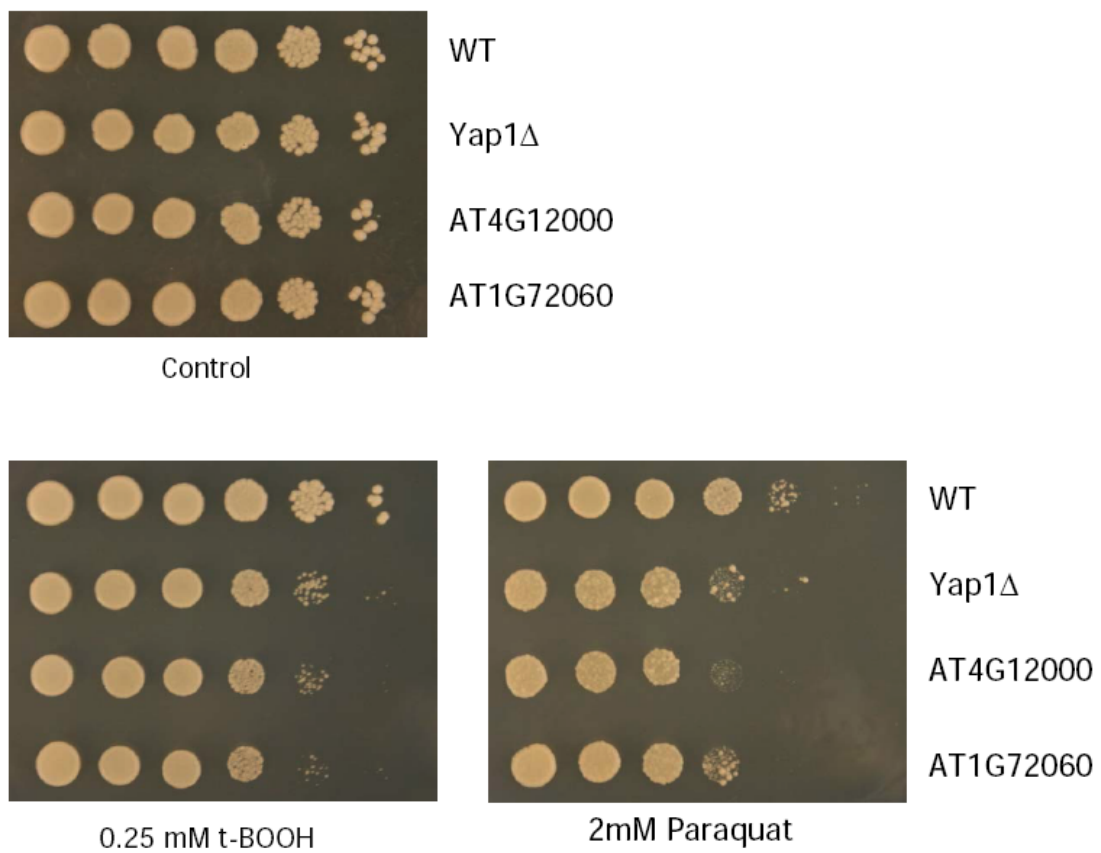
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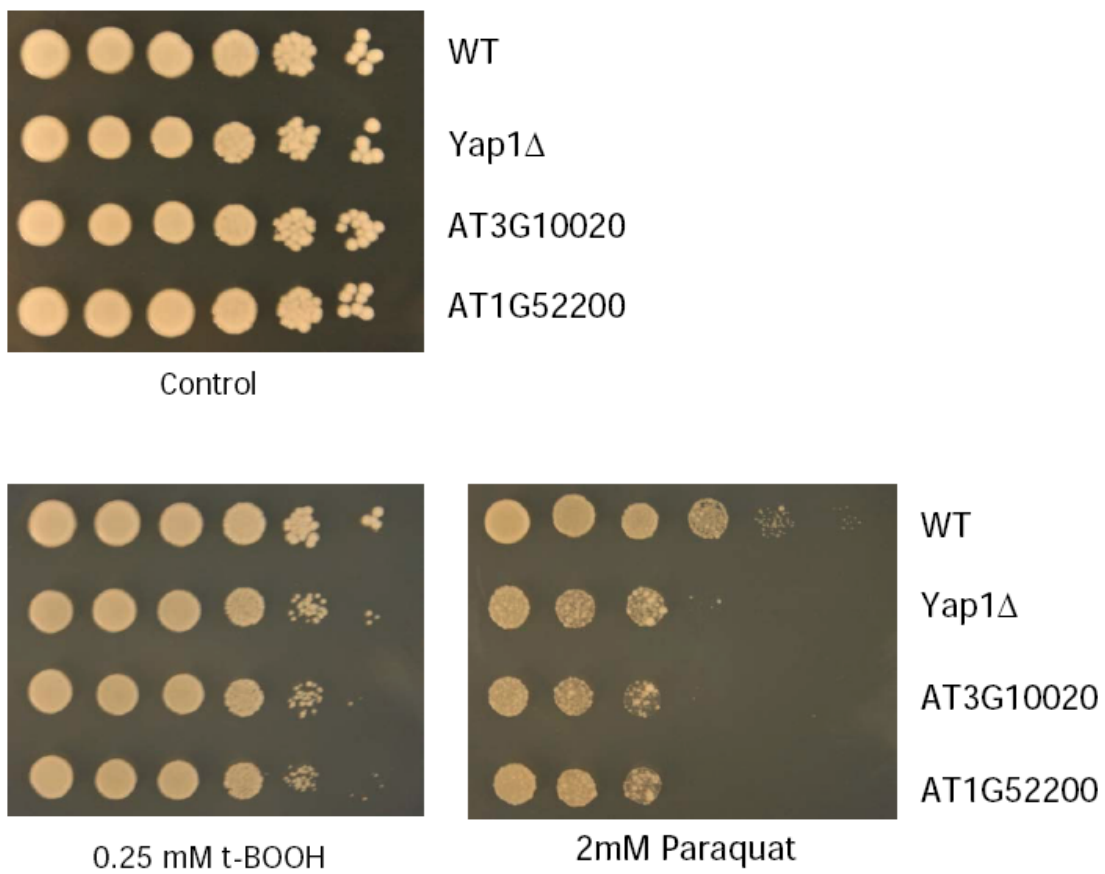
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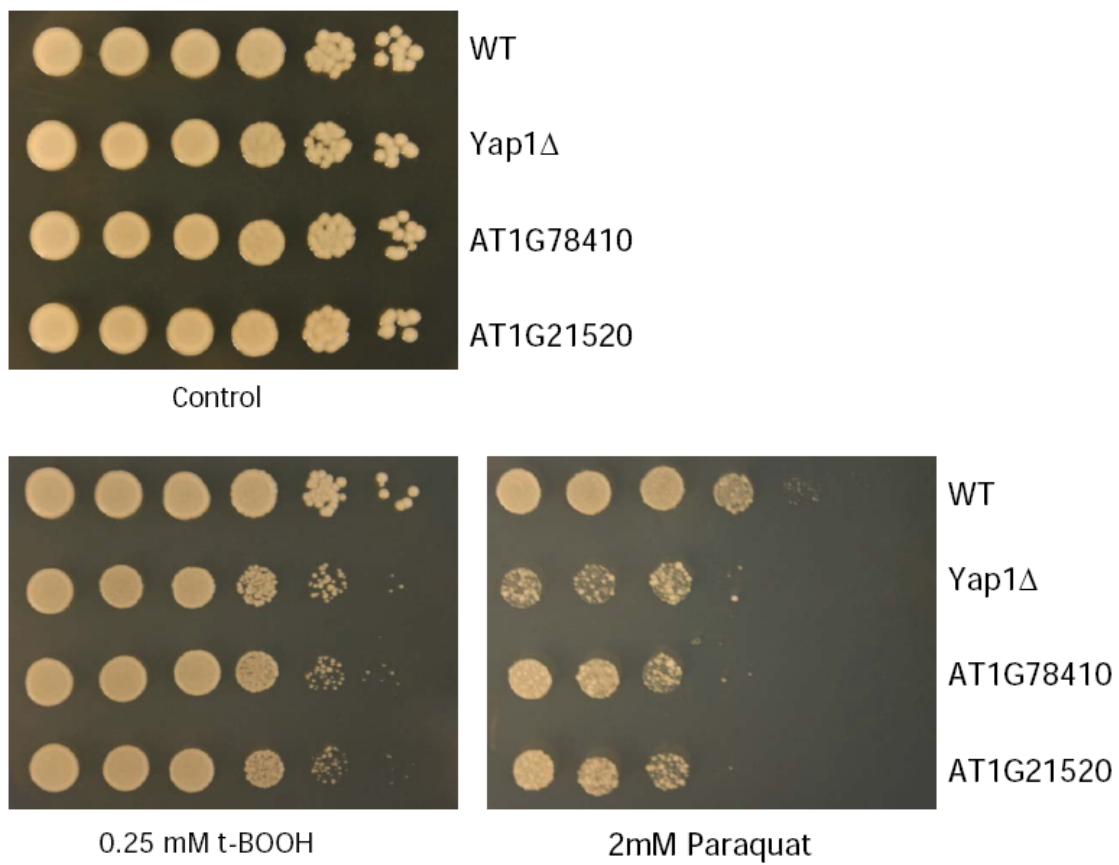
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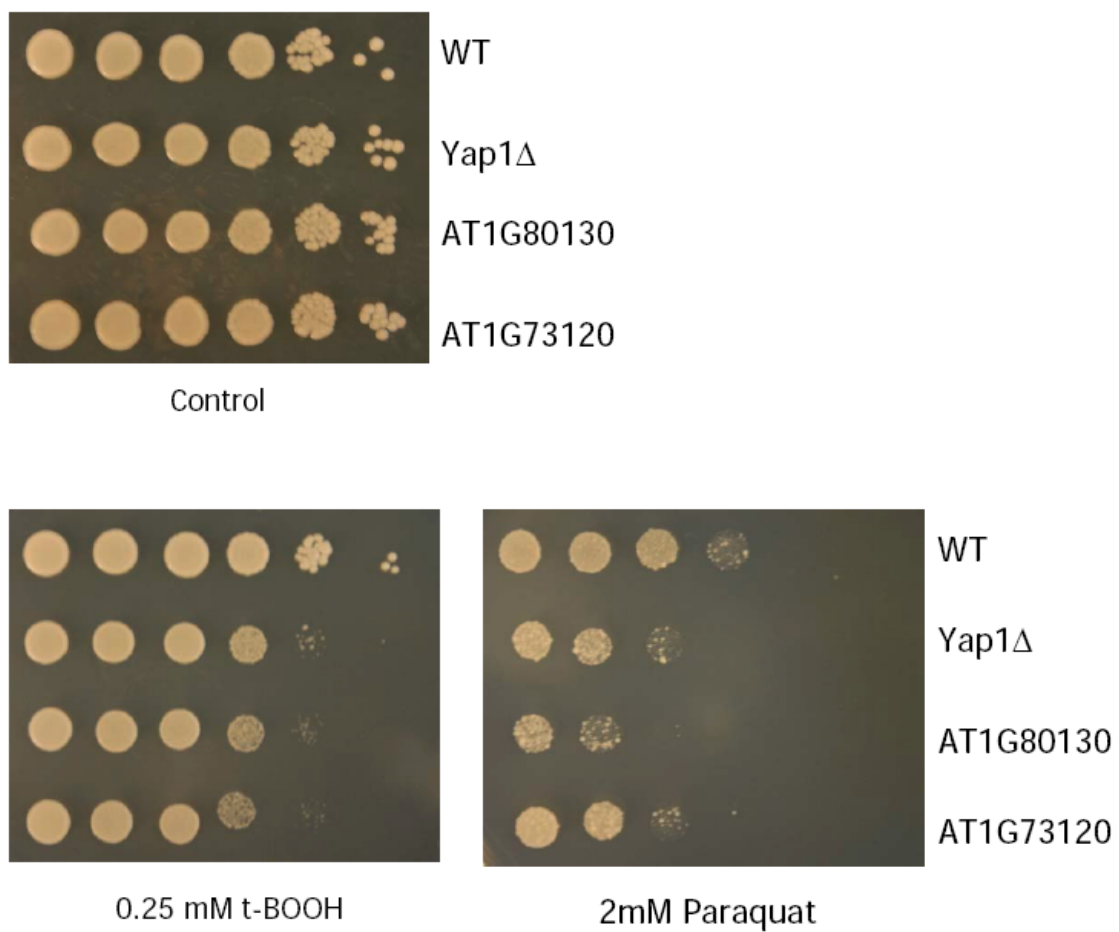
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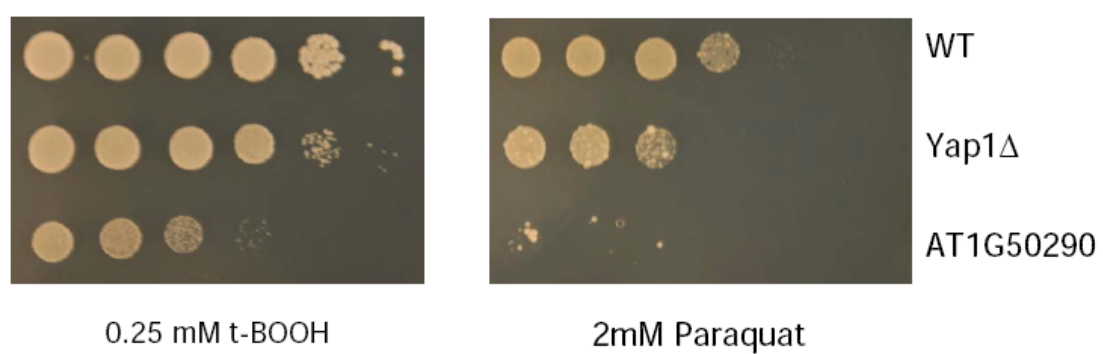
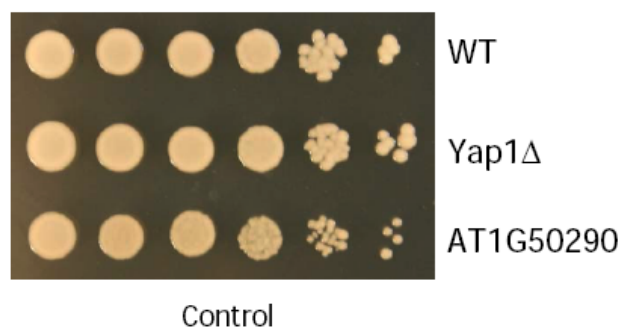
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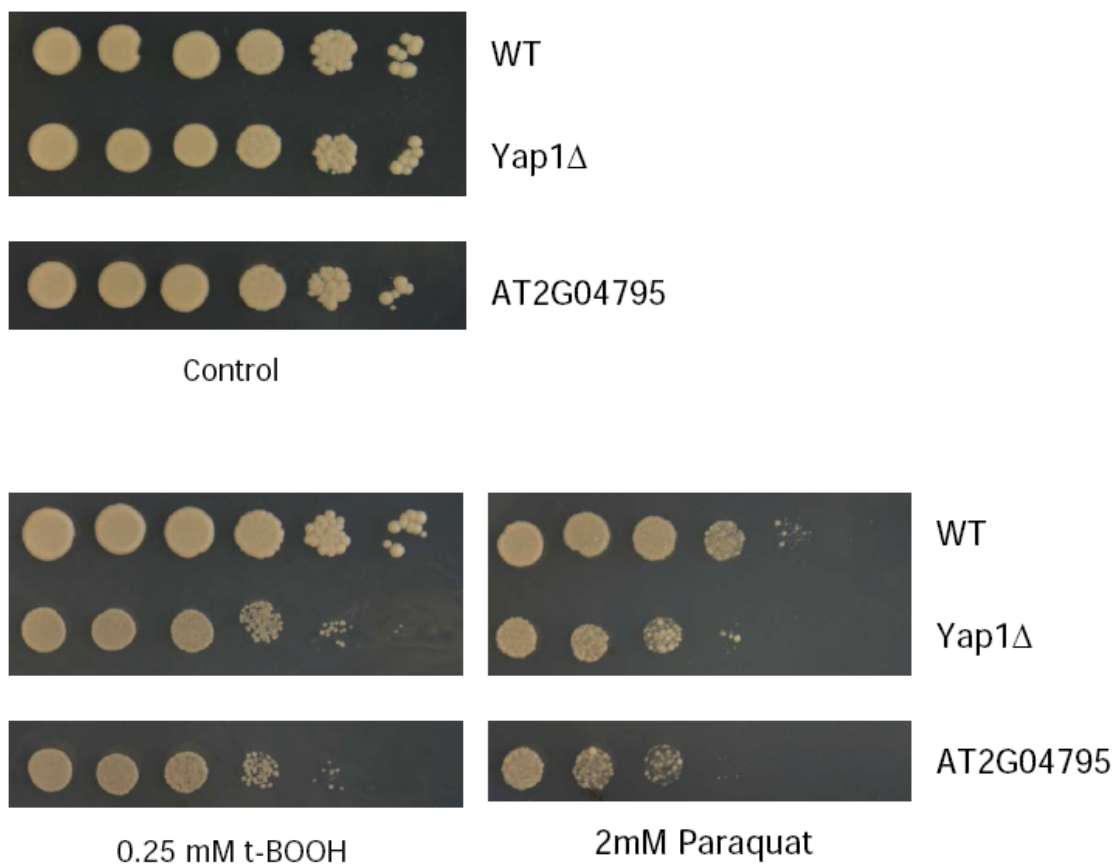


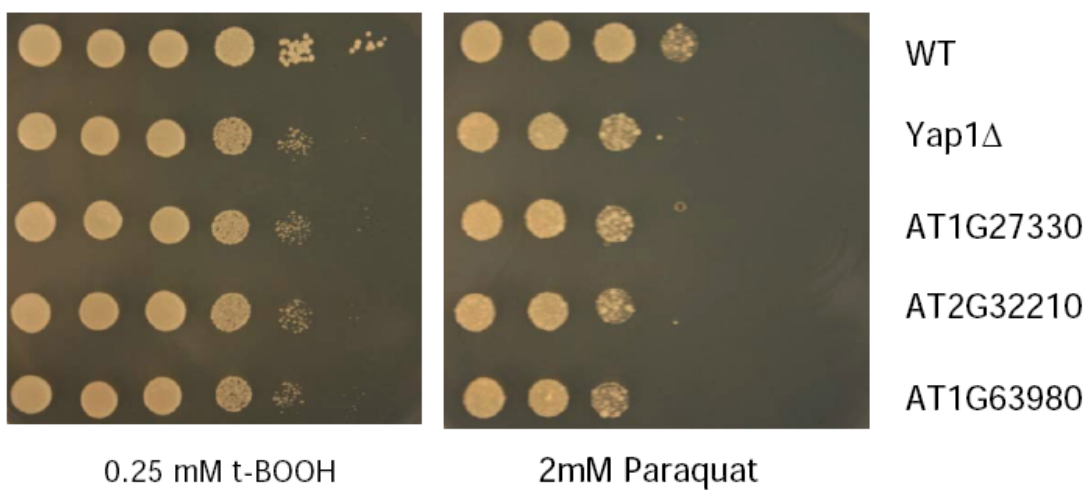
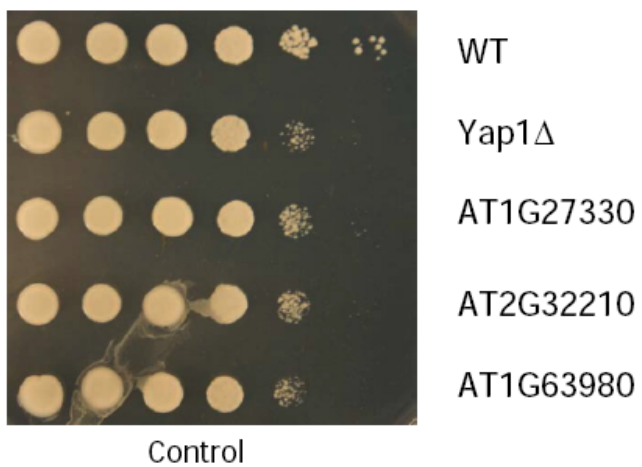


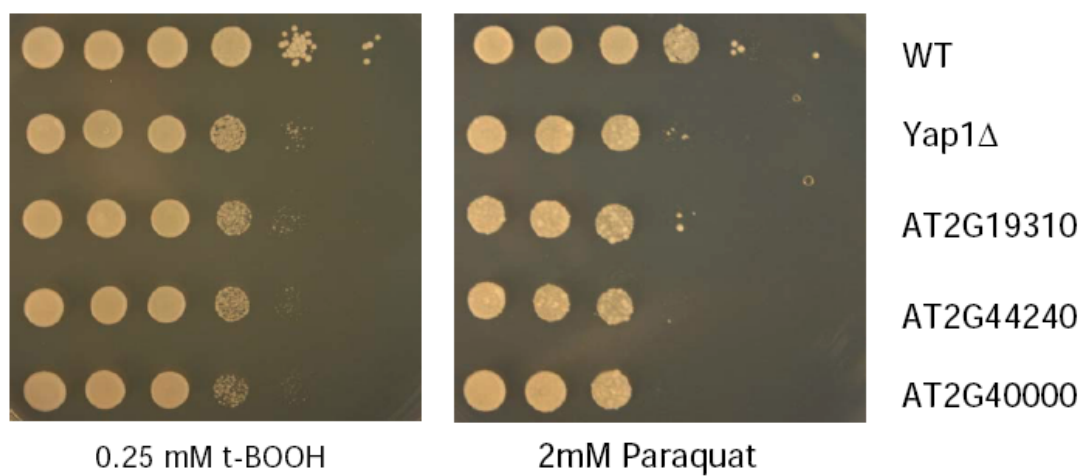
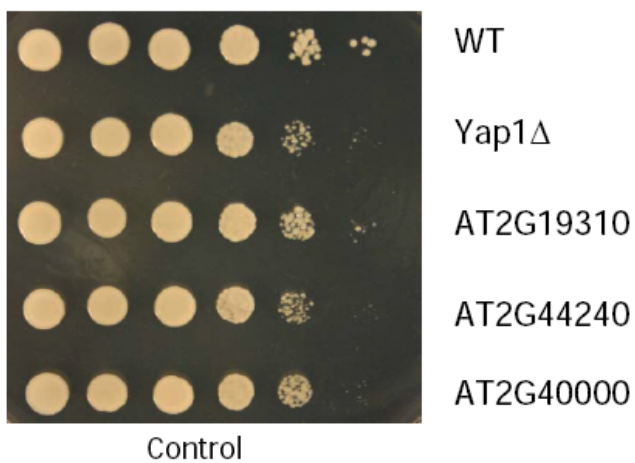












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Chapter 5

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 losses 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 the EAR domain of the zinc 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.

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