

# The EAR-motif of the Cys2/His2-type Zinc Finger Protein Zat7 Plays a Key Role in the Defense Response of *Arabidopsis* to Salinity Stress<sup>\*[5]</sup>

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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.

Transcriptional repressors are emerging as central regulators of development and stress responses in different organisms. By suppressing defense responses and keeping develop-

mental programs under control they are thought to prevent excessive waste of resources and the activation of programmed cell death caused by metabolic imbalances or runaway response pathways (1–4). 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 (1–4).

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 (3).

Cys2/His2-type (C2H2)<sup>3</sup> 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 (1, 5). Key members of this group include Zat12 (At5g59820) and Zat10/AZF (At1g27730). Zat12 was initially identified as a light stress response protein (6). 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 (7, 8). 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 (9). In accordance, transgenic plants constitutively expressing Zat12 are more tolerant to high light, osmotic and oxidative stresses, and knock-out plants lacking Zat12 are more sensitive to osmotic stress and salinity (7, 9). Zat10 was initially identified as a salt-, drought-, and cold-response protein (10). It was shown to contain a functional EAR-motif and to suppress the transcription of different reporter and defense genes (5, 11, 12). Constitutive expression of Zat10 was found to result in

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<sup>3</sup> The abbreviations used are: C2H2, Cys2/His2-type zinc finger; EAR, ERF-associated amphiphilic repression; *Ler*, *A. thaliana* cv Landsberg erecta; KO, knock-out; WT, wild type; APX1, ascorbate peroxidase 1; aa, amino acid; miRNA, micro-RNA.

growth suppression and enhanced tolerance of plants to drought stress, osmotic stress, and salinity (12, 13). 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 (13).

Although different *Zat* proteins were shown to suppress the transcription of reporter and defense genes (5, 11, 12), it is not clear whether the EAR-motif of these proteins is involved in this function (1). 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.

## EXPERIMENTAL PROCEDURES

### Generation of <sup>35</sup>S::*Zat7*-RNAi and <sup>35</sup>S::*Zat7*, *Zat7*Δ, and *Zat7m* Plants

For the *Zat7*-RNAi3' construct, a 115-bp fragment corresponding to 38 bp of the *Zat7* (At3g46090) coding sequence and 77 bp of the 3'-UTR was PCR-amplified from genomic DNA with *ZAT7*IR-1 (5'-CTCGAGGGATCCGGAAGTTG-GAGTTGGGAAGA-3') and *ZAT7*IR-2 (5'-GGTACCATCG-ATAATATTCACATCGATCGGTA-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 *ZAT7*IR-3 (5'-CTCGAGGGATCCATGGTTGCGAGAAG-TGAGGA-3') and *ZAT7*IR-4 (5'-GGTACCATCGATGAA-AACACGTTTCATCTCCCC-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 (14). The entire inverted repeat construct including the <sup>35</sup>S promoter was digested from pHannibal and subcloned into pART27 as a NotI fragment. The <sup>35</sup>S::*ZAT7*-3'IR/pART27 and <sup>35</sup>S::*ZAT7*-5'IR/pART27 plasmids were transformed into *Agrobacterium* ASE by electroporation.

For the <sup>35</sup>S::*Zat7* construct, the *Zat7* cDNA was amplified from genomic DNA using *ZAT7*-3 (5'-CCTAGAAGTCACT-AAGTCGATTCAAATGGTT-3') and *ZAT7*-2 (5'-ATTGT-ATCAAATAATATTCACAT-3') and cloned into pCRScript

(Stratagene). After verification of the sequence, the *Zat7* cDNA was subcloned as a XhoI/NotI fragment into <sup>35</sup>SpBARN (15). For the <sup>35</sup>S::*Zat7*Δ construct, *Zat7* was amplified from genomic DNA using *ZAT7*-3 and *ZAT7*-7 (5'-TTAACAAGCCACTC-TCTTCCCAC-3') and cloned into pCRScript. After verification of the sequence, *Zat7*Δ was subcloned as a XhoI/NotI fragment into <sup>35</sup>SpBARN. The resulting plasmid was transformed into *Agrobacterium* strain GV3101. <sup>35</sup>S::*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'-ATCTAAGGCCGCGACCAACAAGCCACTC-TCTTCC-3') for one piece and *ZAT7*-8 (5'-TGTTTGGCTG-CGGCCTTAGATTTCGATGGAGAGTTT-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 <sup>35</sup>SpBARN. The resulting plasmid was transformed into *Agrobacterium* strain EHA105.

*Arabidopsis Ler* plants were transformed using the floral dip method, and transformants were selected on Gamborg's B5 medium supplemented with the herbicide Basta. Plants were grown in soil at 21–22 °C, constant light, 100 μmol m<sup>-2</sup> s<sup>-1</sup> and watered with 1× Peters 20-20-20 solution. Transgenic plants were tested by RNA blot analysis as previously described (7, 9). Knock-out *Apx1* plants were obtained and analyzed as previously described (16).

### Nucleic Acid Sequence Analysis and Bioinformatics

RNA was isolated and analyzed by RNA blots as previously described (17). Analysis of microarray data (18), was performed as previously described (19). 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 (20). InterPro scan searches were performed using the InterPro Scan server (21). The WoLF protein subcellular localization prediction (PSORT) software was used to predict putative protein subcellular localization (22).

### Stress Assays

For the analysis of stress-tolerance, seeds of wild type and three independent <sup>35</sup>S::*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× MS medium), containing different concentrations of NaCl or sorbitol as previously described (7, 9). Plates were placed at 4 °C for 48 h and maintained vertically in a growth chamber for 5 days (21–22 °C, constant light, 100 μmol m<sup>-2</sup> s<sup>-1</sup>). Root length was scored at 3, 4, and 5 days after vernalization. Four- or five-day-old seedlings grown on 0.5× MS agar plates were also subjected to heat (38 °C) or cold stress (4 °C) for different times, allowed to recover for 24 h and analyzed (9).

For soil stress experiments, 7-day-old seedlings of wild type and three independent <sup>35</sup>S::*Zat7*, *Zat7m*, *Zat7*Δ lines were transferred to vermiculite, watered for 5 days with 0.1–0.5× Peter's fertilizer 20-20-20 solution and subjected to salinity

stress by watering with 0, 50, 75, 100, or 150 mM NaCl prepared in 0.1–0.5× Peter's fertilizer 20-20-20 solution for 20 days. Plants were then photographed and sampled for RNA blot analysis. All experiments were performed with 3–5 technical replications, each containing 15–30 seedlings or plants per line, and repeated at least 3 times. Statistical analysis was performed as described in Ref. 23.

### Yeast Two-hybrid Analysis

**Bait Construction**—The full-length ZAT7 and the last 25 amino acids were cloned into the pENTR vector with Gateway<sup>TM</sup> recombination sites. A Gateway<sup>TM</sup> cassette was introduced into the yeast two-hybrid vector pXDGATCY86 containing cycloheximide-sensitive gene (CYH<sup>S</sup>) (24). Recombination between pENTR vectors and destination vectors were performed according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After confirming that baits were in-frame with the DNA binding domain, constructs were transformed into MaV204K yeast strain (25) (*MAT*  $\alpha$ , *leu2-3,112*; *trp1-901*; *his3*  $\Delta$ 200; *ade2-101*; *cyh2<sup>R</sup>*; *can1<sup>R</sup>*; *gal4*  $\Delta$ ; *gal80*  $\Delta$ ; *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<sup>TM</sup> Library Construction and Screening kit (Clontech, Mountain View, CA) was used to construct a prey library in the pGADT7-Rec vector according to manufacturer's instructions. A mixture of 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  $\mu$ E/m<sup>2</sup>/sec), darkness, salicylic acid (100  $\mu$ M), ABA (100  $\mu$ M), benzylaminopurine (100  $\mu$ M), methyl jasmonate (100  $\mu$ M), indole acetic acid (100  $\mu$ M), gibberellic acid (100  $\mu$ M), paraquat (10  $\mu$ M), wounding (with a needle), 2,4-D (100  $\mu$ M), brassinolide (100  $\mu$ M), hydrogen peroxide (20 mM), desiccation (RT, 60% RH), and cell suspension (7-day-old cultures), control and water deficit-stressed roots, flowers, and siliques was used. Tissues for RNA extraction were pooled from samples collected 30, 60, 90, and 120 min after the start of each treatment. The cDNA prey library was introduced into AH109 yeast cells (*MAT*  $\alpha$ , *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4*  $\Delta$ , *gal80*  $\Delta$ , *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 medium lacking Ade, His, Trp, and Leu supplemented with 10 mM 3-AT. Positive and negative controls for interaction and mating were performed following the manufacturer's recommendations. Positive interactions grew on solid SD media were selected three times on SD -Ade/-His/-Trp/-Leu supplemented with 10 mM 3-AT. Bait plasmid was cured from positive clones that passed three rounds of selection by adding 1  $\mu$ g/ml of cycloheximide and grown overnight with shaking. Curing of bait plasmids was confirmed by PCR followed by plasmid prep-

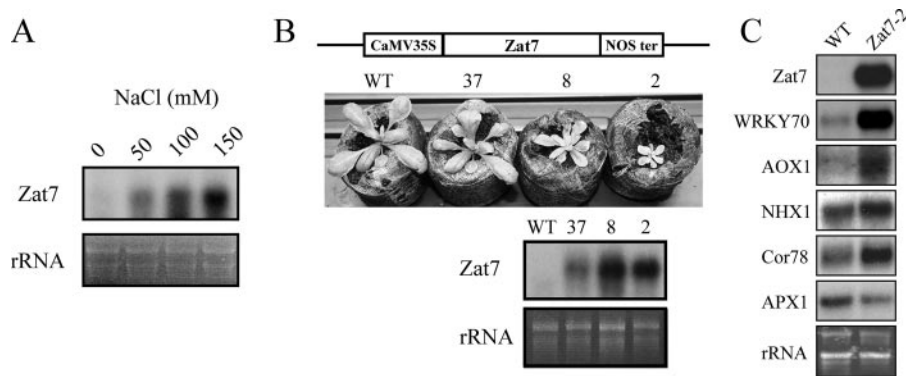
aration from cured clones containing only prey plasmids. Plasmids were then amplified from yeast using Rolling Circle Amplification (RCA) with the TempliPhi 100 Amplification kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) according to the manufacturer's recommendations. The RCA products were used to transform yeast strains containing specific baits to verify interaction (26). 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  $\beta$ -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 25 amino acids of *Zat7* as bait as well as mating between the empty prey vector as a negative control. AH109 strain harboring the pGADT7-RecT vector was mated with the MaV204K strain harboring the pGBKT7-53 vector as a positive interaction control. Mating was performed in 1:1 ratios between each bait and prey pairs on 2× YPAD medium for 24 h followed by plating on SD medium 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 h.  $A_{600}$  was measured for three independent 100- $\mu$ l cultures and then assayed for  $\beta$ -galactosidase activity using the yeast  $\beta$ -galactosidase assay kit (cat. 75768; Pierce Biotechnology, Inc.) following the manufacturer's instructions.  $A_{600}$  and  $A_{420}$  were measured using the PerkinElmer Victor 3v multiplate reader. The  $\beta$ -galactosidase activity was calculated using the equation:  $\beta$ -galactosidase activity =  $1000 \times A_{420}/T \times V \times A_{600}$  where T is time (in minutes) of incubation and V is volume of cells (ml) used in the assay.

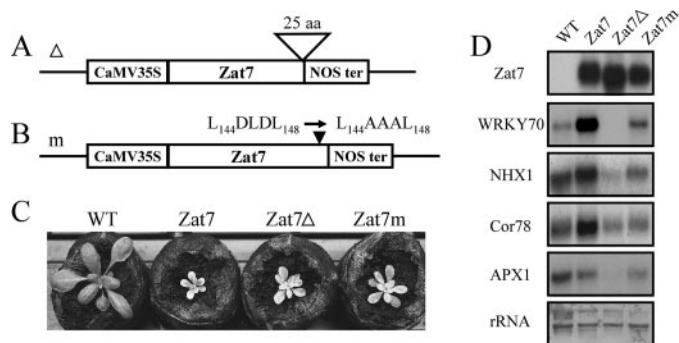
## RESULTS

**Expression of *Zat7* in *Arabidopsis* and Phenotype of Transgenic Plants with Constitutive Expression of *Zat7***—Transcripts encoding at least four different members of the *Zat* protein family show broad response to biotic and abiotic stresses (*Zat6*, 10, 11, and 12; Ref 13). In contrast, the expression of *Zat7* appears to be more specific, with enhanced expression mainly in roots during salinity stress (supplemental Fig. S1A, generated from transcriptome profiling data available at Ref. 18). In contrast to the early and transient expression of *Zat12* during stress (9, 17), the expression of *Zat7* in *Arabidopsis* roots subjected to salinity stress is enhanced at 6, 12, and 24 h, suggesting that *Zat7* functions relatively late during salinity stress (supplemental Fig. S1B). 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 (23), as well as in knock-out *Apx1* plants grown under controlled growth conditions (16, 17), 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 (7). Different studies on the expression of *Zat12* in transgenic plants produced conflicting reports on the suppression of growth induced by this



**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 under "Experimental Procedures."



**FIGURE 2. Growth suppression in transgenic plants with constitutive expression of Zat7, Zat7 $\Delta$ , or Zat7m.** *A*, DNA construct used to generate the Zat7 $\Delta$  lines. *B*, DNA construct used to generate the Zat7m lines. *C*, photograph of control and transgenic plants expressing Zat7, Zat7 $\Delta$ , or Zat7m. *D*, RNA gel blots of RNA obtained from the plants shown in *C*. Plant transformation, growth, and analysis by RNA blots were performed as described under "Experimental Procedures."

protein in transgenic plants (7–9). The discrepancies in phenotypes were linked to Zat12 expression level in the different plants, as well as to the use of different cultivars (7, 8). To test whether Zat7 expression would also induce growth suppression in *A. thaliana* cv *Landsberg erecta* (*Ler*), used in this study, we generated transgenic *Ler* lines with different levels of Zat7 expression. As shown in Fig. 1*B*, 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 (8, 9, 13). The enhanced expression of Zat7 in response to salinity stress (Fig. 1*A* and supplemental Fig. S1), 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. 1*C*, the expression of different transcripts associated with salinity tol-

erance in plants (27, 28) was elevated in transgenic plants expressing Zat7, grown under controlled conditions.

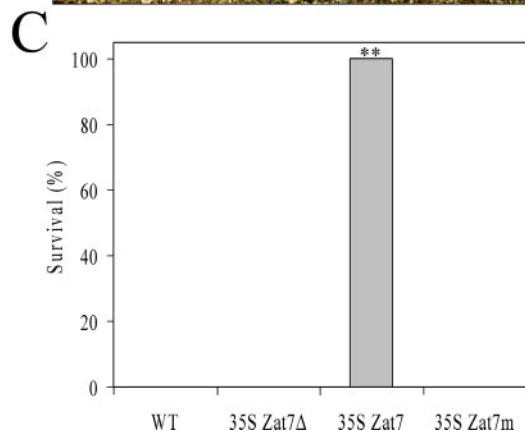
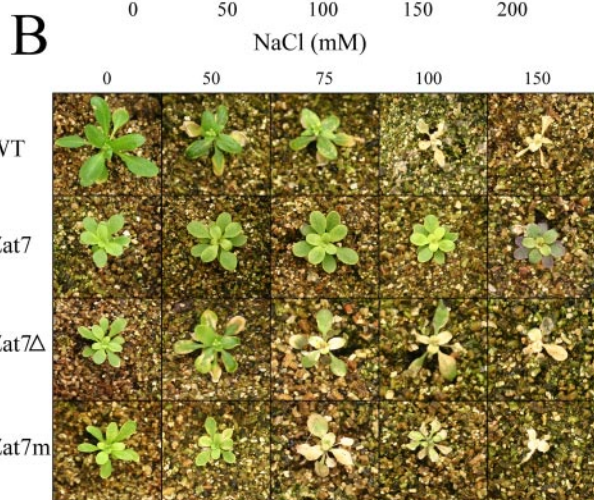
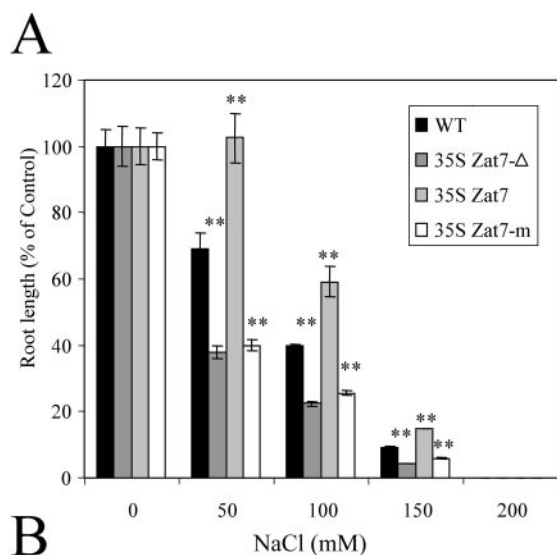
**Mutational Analysis of the EAR-motif of Zat7**—To perform functional characterization of the EAR-motif of Zat7 we generated transgenic lines expressing two different variants of Zat7: a deletion of the C-terminal 25 amino acids region that contains the EAR-motif (Zat7 $\Delta$ ), and a site-specific mutagenesis converting the LDLDL EAR-motif at position 144–148 to LAAAL at the same position (144–148; Zat7m). As shown in Fig. 2 and supplemental Fig. S2, deletion or mutation of the EAR-domain had no significant effect on the growth suppression

phenotype of Zat7. In contrast, expression of defense transcripts, associated with salinity tolerance, was not enhanced in transgenic plants with constitutive expression of Zat7 $\Delta$  or Zat7m (Fig. 2*D*). This result indicated that the EAR-motif of Zat7 is not involved in suppression of plant growth in transgenic plants. The EAR-motif of Zat7 could, however, be involved in the activation of defense responses. The results shown in Fig. 2 also suggest 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 $\Delta$ , or Zat7m showed a similar degree of growth suppression.

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

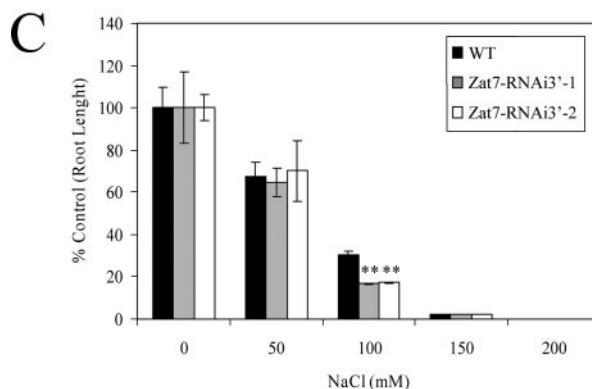
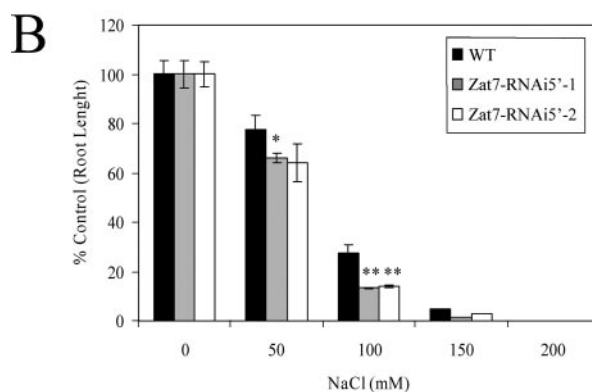
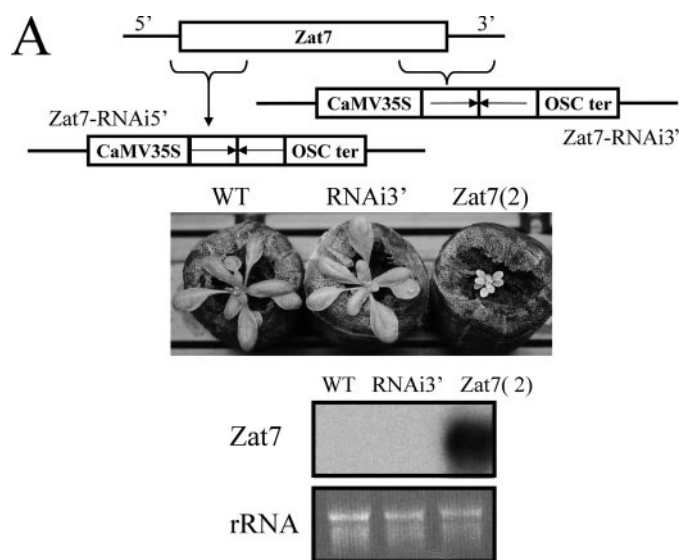
The enhanced sensitivity of transgenic plants expressing Zat7m or Zat7 $\Delta$  to salinity stress could indicate that Zat7 protein, which lacks a functional EAR-motif (*i.e.* Zat7m or Zat7 $\Delta$ ), functions in transgenic plants as dominant-negative suppressor of the endogenous Zat7 protein. Thus, 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 Zat7 $\Delta$  subjected to salinity stress (Fig. 3) suggested that suppression of Zat7 function in plants will result in enhanced



**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 under "Experimental Procedures." \*\*, Student's *t* test significant at  $p < 0.01$ .

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



**FIGURE 4. Characterization of RNAi lines for *Zat7*.** A, construction of RNAi lines. Top, DNA constructs used to generate the *Zat7*-RNAi 3' and 5' lines. Middle, photograph of wild type (WT), *Zat7*-RNAi3' (RNAi3'), and a <sup>35S</sup>::*Zat7* line [*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 under "Experimental Procedures." \*\*, Student's *t* test significant at  $p < 0.01$ ; \*, Student's *t* test significant at  $p < 0.05$ .

blot analysis of RNAi lines revealed that despite the use of a <sup>35S</sup> 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

TABLE 1

## Proteins that interact with Zat7 and/or the last 25 aa of Zat7 in a yeast two-hybrid screen

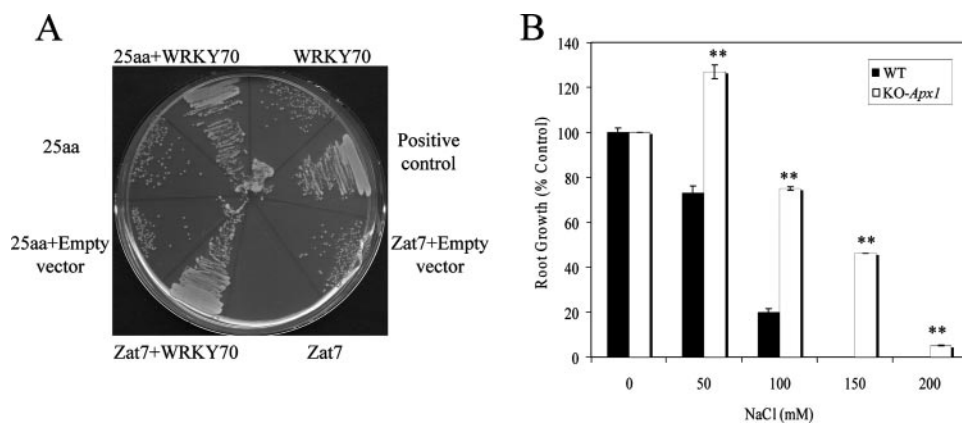
The proteins presented in the table were selected from the complete list of interactors (suppl. Table S1) based on their predicted subcellular localization and expression. Locus identifiers are given on the left followed by clone description, BLAST E-value for clone identification, predicted localization based on clone annotation in MIPS, TAIR, and PSORT, and expression in roots of *Arabidopsis* subjected to salinity stress as obtained from Ref. 18, or in knock-out plants deficient in cytosolic ascorbate peroxidase 1 grown under controlled conditions (KO-Apx1; 16, 17).

Locus	Description	BLAST E-value	Predicted localization	Expression (root, salinity)	Expression (KO-Apx1)
<b>Clones that interact with full-length and last 25aa</b>					
At3g56400	WRKY70	3.00E-24	Nucleus	Up <sup>a</sup>	Up
At3g05040	HASTY, importin/exportin, involved in miRNA transport	3.00E-66	Nuclear envelope	NC <sup>b</sup>	Up
At5g07140	Protein kinase family contains eukaryotic protein kinase domain	2.00E-20	Unknown	NF <sup>c</sup>	Down
At2g25670	Expressed protein	8.00E-09	Unknown	NC	NC
At5g06060	Short-chain dehydrogenase/reductase family protein	7.00E-42	Unknown	NC	NC
At1g24764	MAP70 protein family	4.00E-64	Microtubule	NC	Down
<b>Clones that interact with last 25aa only</b>					
At2g22780	Malate dehydrogenase	5.00E-54	Unknown	Up	Up
At3g16190	Isochorismatase hydrolase family	3.00E-13	Unknown	NC	Up
At5g49000	Kelch repeat containing F-box protein	8.00E-05	Unknown	NC	Up
<b>Clones that interact with full-length only</b>					
At1g24822	Hypothetical protein	8.00E-05	Unknown	NC	Down
At4g27130	Translation initiation factor SU11	1.00E-14	Unknown	NC	Down
At3g16640	Translationally controlled tumor protein-related	1.00E-05	Cytosol	NC	NC

<sup>a</sup> Up, expression enhanced by 2-fold or more.

<sup>b</sup> NC, no change in expression.

<sup>c</sup> NF, not found.



**FIGURE 5. Interaction of WRKY70 with Zat7 and tolerance of knock-out 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-amino acid fragment of Zat7 (25aa) that contains the EAR-domain. B, graph showing that knock-out plants deficient in Apx1 (KO-Apx1) are more tolerant to salinity stress than wild-type plants. KO-Apx1 plants were previously shown to constitutively co-express Zat7, WRKY70, and HASTY (16, 17). Yeast mating and stress assays were performed as described under "Experimental Procedures." \*\*, Student's *t* test significant at  $p < 0.01$ .

(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. 4, B and C, 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 amino acids 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 supplemental Table S1, several different proteins were found to interact in this system with both the full-length clone and the last 25 amino acids 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  $\beta$ -galactosidase reporter activity (see example in supplemental Fig. S3). Of the nine interactors, a protein involved in miRNA transport (HASTY; Ref. 29), WRKY70 (30), 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 $\Delta$  or Zat7m did not interact with WRKY70 or HASTY in a directed yeast two-hybrid interaction assay (supplemental Figs. S4 and S5). Interestingly, constitutive expression of Zat7, but not Zat7m or Zat7 $\Delta$ , resulted in enhanced expression of WRKY70 (Figs. 1C and 2D).

WRKY70, Zat7, and HASTY were reported to be co-expressed in knock-out Apx1 (KO-Apx1) plants grown under controlled conditions (Table 1; Refs. 16 and 17). 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-

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expression of these transcripts in KO-Apx1 plants is associated with enhanced tolerance to salinity stress, we subjected KO-Apx1 and wild-type plants to the same salinity stress assays shown in Fig. 3. Surprisingly, as shown in Fig. 5B, KO-Apx1 plants were significantly more tolerant to salinity stress than wild-type plants.

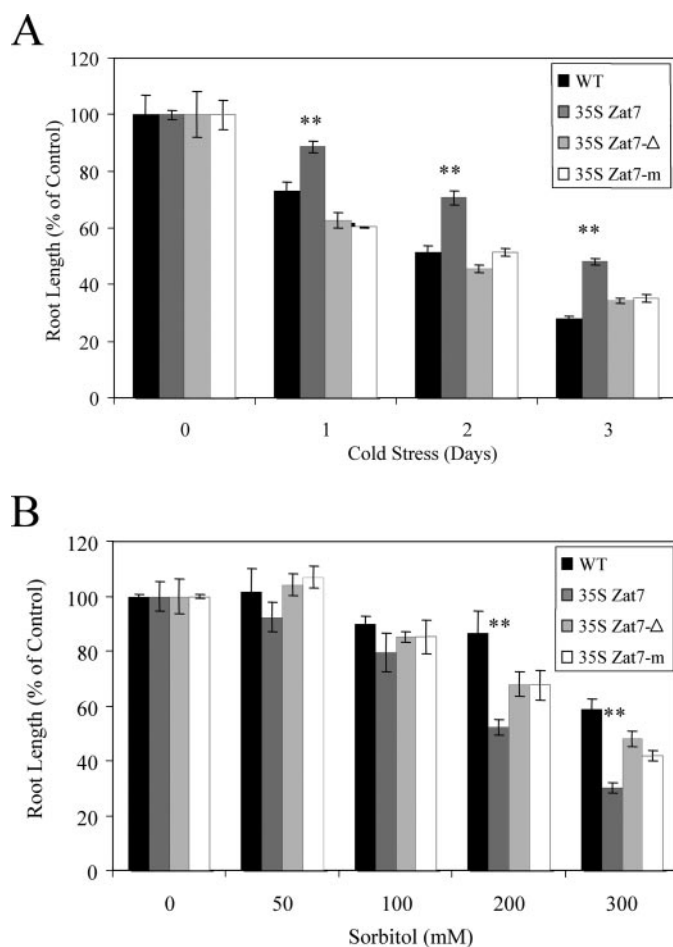
**Tolerance of Transgenic Plants That Constitutively Express *Zat7*, *Zat7Δ*, or *Zat7m* to Cold and Osmotic Stress**—The EAR-domain is found in several different C2H2 zinc finger proteins that are thought to be involved in regulating the response of plants to abiotic stress conditions (1). However, the specificity and the degree of cooperation between different *Zat* proteins such as *Zat10*, *Zat12*, and *Zat7* are unclear at present. The availability of transgenic plants that constitutively express *Zat7*, *Zat7Δ*, or *Zat7m* prompted us to test how these lines would behave in response to abiotic stresses other than salinity, and whether or not the *Zat7Δ* or *Zat7m* lines would suppress the tolerance of plants to these stresses (similar to their effect on the tolerance of plants to salinity stress; Fig. 3). To this end we tested the tolerance of transgenic plants that constitutively express *Zat7*, *Zat7Δ*, or *Zat7m* to osmotic, cold, and heat stresses.

As shown in Fig. 6A, transgenic plants that constitutively express *Zat7* were more tolerant to cold stress. Transgenic plants that constitutively express *Zat7Δ* or *Zat7m* were not, however, more susceptible to cold. In contrast, transgenic plants that constitutively express *Zat7* were more susceptible to osmotic stress (Fig. 6B). Although transgenic plants that constitutively express *Zat7Δ* or *Zat7m* were also more susceptible to osmotic stress, they appear to be less susceptible to this stress than transgenic plants that constitutively express *Zat7* (Fig. 6B). The response of transgenic plants that constitutively express *Zat7*, *Zat7Δ*, or *Zat7m* to heat stress was not significantly different from that of wild type (not shown). The results presented in Figs. 3 and 6, as well as those reported for *Zat12* by Davletova *et al.* (9), provide an initial glimpse into the complex nature of the C2H2-EAR-domain zinc finger network and its role in regulating abiotic stress responses.

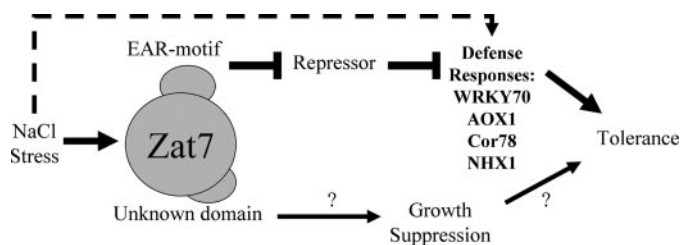
## DISCUSSION

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 (1, 7–12). Nonetheless, the role the EAR-domain of these proteins play in the defense response of plant to abiotic stress is unclear (1, 13). 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



**FIGURE 6. Differential tolerance of transgenic plants with constitutive expression of *Zat7*, *Zat7Δ*, or *Zat7m* to cold and osmotic stress.** A, differential tolerance of *Zat7*, *Zat7Δ*, or *Zat7m* to cold stress. B, differential tolerance of *Zat7*, *Zat7Δ*, or *Zat7m* to osmotic stress. Stress assays and statistical analysis were performed as described under "Experimental Procedures." \*\*, Student's *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 repressor 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 repressor 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).

(7–9, 12, 13). Based on previous findings that the EAR-domain functions as a transcriptional repressor (1, 5, 11), 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, 2D, and 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 (9) 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 (Ref. 9; see also Ref. 8). In the case of Zat7, several transcripts encoding defense and regulatory transcripts, involved in plant tolerance to salinity stress (27, 28), are elevated in Zat7-expressing plants, but not in plants expressing Zat7 with a deletion or a mutation in the EAR-motif (Figs. 1C, 2D, 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 (Figs. 2D and 3).

Constitutive expression of different Zat proteins was found to cause growth suppression in transgenic plants (7, 8, 12). 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 (27, 31, 32). 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-overexpressing 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 (33), 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 (13). 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 knock-out plants lacking APX1 (16, 17). Interestingly, compared with 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 (30, 34). Micro-RNAs were recently shown to be important for regulating defense responses to abiotic stress (35). 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*.

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