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Gain- and loss-of-function mutations in *Zat10* enhance the tolerance of plants to abiotic stress

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Abstract C_2H_2-zinc finger proteins that contain the EAR repressor domain are thought to play a key role in modulating the defense response of plants to abiotic stress. Constitutive expression of the C_2H_2-EAR zinc finger protein *Zat10* in *Arabidopsis* was found to elevate the expression of reactive oxygen-defense transcripts and to enhance the tolerance of plants to salinity, heat and osmotic stress. Surprisingly, knockout and RNAi mutants of *Zat10* were also more tolerant to osmotic and salinity stress. Our results suggest that *Zat10* plays a key role as both a positive and a negative regulator of plant defenses.

Keywords: Abiotic stress; C_2H_2-zinc finger; EAR motif; Stress tolerance; *Zat10*; *Arabidopsis thaliana*

1. Introduction

The acclimation of plants to changes in environmental conditions is orchestrated by a complex network of regulatory genes and signaling molecules [1–3]. These include signal transduction proteins such as mitogen-activated protein kinases and phosphatases, signaling molecules such as calcium, different stress-response hormones and reactive oxygen species (ROS), and an array of transcriptional regulators that include activators, co-activators and suppressors. Transcriptional suppressors were recently proposed to play a key role in modulating the defense response of plants to biotic and abiotic stresses [4]. A subset of these transcriptional suppressors belongs to the C_2H_2 zinc finger gene family and includes an ERF-associated amphiphilic repression (EAR) domain [4–6]. Key members of this group include the stress-response proteins *Zat12* (At5g59820) [7–10] and *Zat10*/*STZ* (At1g27730) [5,6,11,12].

Zat10 was initially identified as a salt- and cold-response protein [11]. It was shown to contain a functional EAR motif and to suppress the transcription of different reporter and defense genes [5,6,12]. Constitutive expression of *Zat10* was found to result in growth suppression and enhanced tolerance of plants to drought stress [5]. However, whether *Zat10* functions to enhance stress tolerance in transgenic plants as a suppressor or as an activator of gene expression is unknown at present [4]. Transcriptome profiling studies performed with plants subjected to external or internal oxidative stress suggest that, similar to the function of *Zat12* as a key mediator of responses to hydrogen peroxide stress [8–10], *Zat10* may also be involved in the response of plants to oxidative stress [10,13,14].

Here we report that constitutive expression of *Zat10* results in the enhanced expression of different ROS-response transcripts. Constitutive expression of *Zat10* enhances the tolerance of plants to osmotic stress, salinity and heat stress. Surprisingly, knockout and RNAi plants for *Zat10* were also found to have enhanced tolerance to osmotic and salinity stresses. Our results suggest that *Zat10* plays a key role as both a positive and a negative regulator of plant defenses and may act to modulate the activation of defense responses during different stresses, as well as stress combination [15].

2. Materials and methods

2.1. Plant material

Transgenic *Arabidopsis thaliana* plants (ecotype Columbia) expressing the full-length *Zat10* cDNA were constructed as described previously [8,10]. Transgenic lines (30) were screened by RNA blots and three independent homozygous lines expressing *Zat10* under the control of the 35S CaMV promoter were used for further analysis.

To generate the *Zat10*-RNAi plants, the coding region of the *Arabidopsis STZ*/*Zat10* gene (from +1 to +515 bp) was amplified with primers containing the following restriction enzyme sites: 5′-most primer with *SpeI* and *AscI* sites, and the 3′-most primer with *BamHI* and *SwaI* sites. The resulting PCR product was digested first with *AscI* and *SwaI* and ligated into an *AscI*–*SwaI*-cleaved pFGC1008 vector (template plasmid). For the second PCR fragment for the inverted repeat construct, the same PCR product was digested with *BamHI* and *SpeI* and inserted into the *BamHI*–*SpeI* sites of the template plasmid. The *Zat10*-RNAi plasmid was introduced into the C24 ecotype of *Arabidopsis* expressing *RD29A-LUC* and twenty-one hygromycin resistant seedlings were obtained. Homozygous T3 or T4 generations were screened for hygromycin resistance and confirmed by RNA blots as described by [8]. Three independent lines were used for all stress studies.

T-DNA insertion mutant of *STZ*/*Zat10*, SALK_054092, was obtained from the Arabidopsis Biological Resource Center (ABRC). To verify the presence of T-DNA in the *STZ*/*Zat10* locus, a pair of Lba1 and Zat10-R (5′-CGAGCTCGCAAACGAAATCTTATCGTC-TAAGT-3′) primers was used for genotyping. For the gene-specific

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Abbreviations: APX, ascorbate peroxidase; EAR, ERF-associated amphiphilic repression; FSD, Fe-superoxide dismutase; ROS, reactive oxygen species

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PCR genotyping, a pair of *Zat10*-R and *Zat10*-F (5′-GCTCTAGAGCCTCGAGAGACAAGAAATCCTC-3′) primers was used.

2.2. Molecular analysis and bioinformatics

Methods for isolation of total RNA, preparation of RNA blots and hybridization with radiolabeled probes for *COR47*, *COR15A*, *RD29A*, *RD29B*, *AZF2*, *RD22*, *ADH*, *FSD1*, *APX2*, *ZAT10* and *Tubulin* were as described [10,12,13]. Analysis of microarray data available from <https://www.genevestigator.ethz.ch> [16] was performed as previously described [17].

2.3. Stress assays

For the analysis of stress-tolerance, seeds of wild type and *Zat10*-perturbed lines (two independent *Zat10* over-expressing lines, a knockout and three independent RNAi lines) were surface-sterilized with bleach and placed in rows on 1% agar plates (0.5 × MS medium), containing different concentrations of NaCl, Sorbitol or paraquat as previously described [8,10,13]. Plates were maintained vertically in a growth chamber (21–22 °C, constant light, 100 μmol m^{-2} s^{-1}) and root length, root growth and % germination were scored at different times after seed plating [10]. Four- or five-day-old seedlings grown on 0.5 × MS agar plates were also subjected to heat (38 °C) or cold stress (10 °C) for different times, allowed to recover for 24 h, and analyzed [10]. C24 wild type lines containing empty vectors and expressing *RD29A-LUC* were used as controls for the *Zat10*-RNAi lines, and Columbia wild type lines expressing empty vectors were used as controls for the overexpressor lines. All stress experiments were performed with 3–5 technical replications, each containing 15–30 seeds per line, and repeated at least three times. Statistical analysis was performed as described in [10].

For RNA blot analysis: Light stress experiments were performed with three-week-old plants grown at 21–22 °C, constant light, 100 μmol m^{-2} s^{-1}. Light stress was performed by changing the light intensity to 1000 μmol m^{-2} s^{-1} (21–22 °C) for 0, 1 and 3 h. For cold treatment, seven-day-old seedlings grown in MS agar plates containing 3% sucrose were incubated at 0°C for 24 h. For ABA treatment, 100 μM ABA (mixed isomers in water) was sprayed on leaves of seedlings and incubated for 3 h. For NaCl treatment, seedlings were transferred onto filter paper saturated with 300 mM NaCl in 1 × MS liquid media for 5, 6 and 24 h. Plants were sampled at different times, frozen in liquid nitrogen and used for RNA blot analysis [13].

3. Results

3.1. Expression of different Zat transcripts in response to stress

To obtain a comprehensive overview of the relative expression of different Zat transcripts during stress in *Arabidopsis* we examined the expression pattern of *Zat2* (At2g17180), *Zat3* (At4g35280), *Zat4* (At2g45120), *Zat5* (At2g28200), *Zat6* (At5g04340), *Zat7* (At3g46070), *Zat9* (At3g60580), *Zat10*/*STZ* (At1g27730), *Zat11* (At2g37430), *Zat12* (At5g59820), *Zat13* (At3g49930), *Zat14* (At5g03510), *Zat15* (At3g10470), and *Zat17* (At2g28710), during different abiotic stresses and pathogen infection utilizing transcriptome profiling data available at <https://www.genevestigator.ethz.ch> [16,17]. As shown in Fig. 1, the Zat transcripts that demonstrated the highest level of expression in leaves (Fig. 1A) or roots (Fig. 1B) during different stresses were *Zat6*, *Zat10*, *Zat11* and *Zat12* (see also Supplementary Fig. 1 for expression pattern of all Zat transcripts tested).

Expression of *Zat10* was elevated in leaves in response to cold, UV-B, oxidative stress, osmotic stress and genotoxic stress [16] (Fig. 1A). *Zat10* expression was also elevated in roots in response to salinity and cold stress [16] (Fig. 1B). Interestingly, whenever the expression of *Zat10* was elevated in leaves or roots so was the expression of *Zat12* (Fig. 1).

3.2. Characterization of transgenic Arabidopsis plants with constitutive expression of Zat10

Constitutive expression of *Zat10* in transgenic plants was previously reported to result in suppressed growth and enhanced tolerance to drought stress [5]. The expression of *Zat10* in transgenic plants was not however linked to the expression of particular defense genes [5]. As shown in Fig. 2A, growth suppression was observed only in lines that exhibited a high level of constitutive expression of *Zat10* (i.e., *Zat10*-OE2). Constitutive expression of *Zat10* in *Arabidopsis* enhanced the expression of three different ROS-defense transcripts [18] in plants grown under controlled conditions (Fig. 2B). These include ascorbate peroxidase 2 (*APX2*), Fe-superoxide dismutase 1 (*FSD1*) and to a lesser degree *APX1*. Interestingly, the enhanced expression of *APX2* and *FSD1* occurred in all lines that had elevated expression of *Zat10*, regardless of their degree of growth suppression (Figs. 2A and B). A survey of microarray data [16] revealed that *Zat10* expression correlated with the expression of *FSD1* during heat stress and with the expression of *APX2* during wounding and salt, osmotic or cold stresses (not shown).

In contrast to the elevated expression of oxidative stress-response transcripts in plants with constitutive expression of *Zat10* (Fig. 2B), the expression of different defense transcripts involved in salinity, drought and cold tolerance was not altered

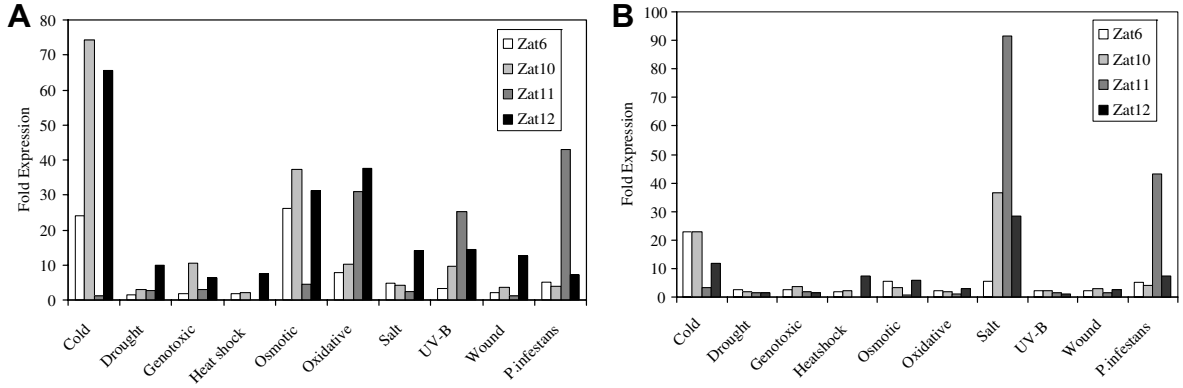

Fig. 1. Expression pattern of different Zat transcripts during stress. (A) Relative expression of *Zat6*, *Zat10*, *Zat11* and *Zat12* transcripts in leaves of *Arabidopsis* subjected to different stresses. (B) Relative expression of *Zat6*, *Zat10*, *Zat11* and *Zat12* transcripts in roots of *Arabidopsis* subjected to different stresses. Data acquisition and calculation of relative expression for each stress treatment were performed as described in Section 2.

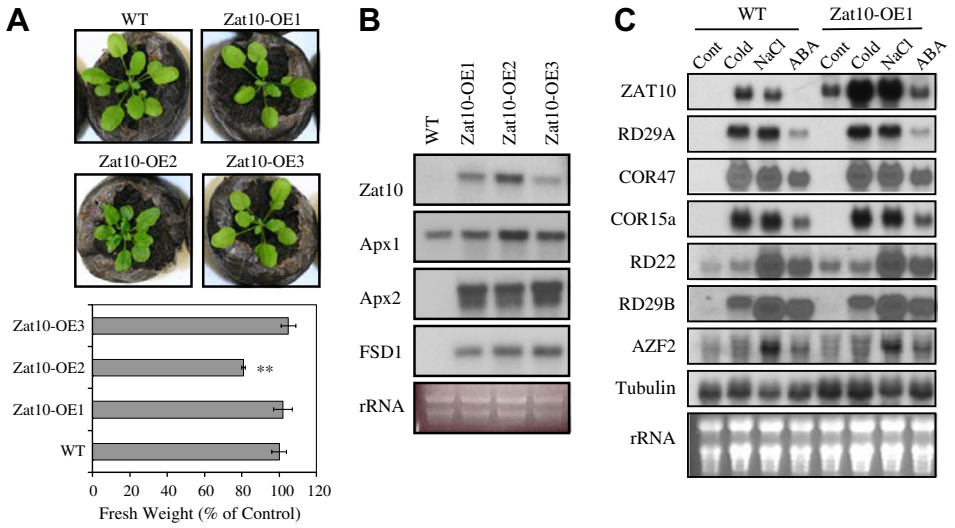

Fig. 2. Characterization of transgenic *Arabidopsis* plants with constitutive expression of *Zat10*. (A) Top: Photographs of three-week-old control (WT) and three independent lines expressing *Zat10* (*Zat10*-OE1-3) grown under controlled growth conditions. Bottom: Biomass (fresh weight) of four-week-old WT and *Zat10*-OE lines. (B) RNA blot analysis showing the expression of *Zat10*, *APX1*, *APX2* and *FSD1* in control (WT) and three independent lines expressing *Zat10* (*Zat10*-OE1-3) grown under controlled growth conditions. (C) RNA blot analysis showing the expression of different abiotic stress-response transcripts in WT and *Zat10*-OE plants grown under controlled conditions or subjected to cold stress, salinity stress or ABA application. Plant growth and RNA blot analysis were performed as described in Section 2. **, *t*-test significant at $P < 0.01$ ($n = 40$).

in *Zat10*-expressing plants grown under controlled conditions, or subjected to different stresses (Fig. 2C).

3.3. Expression of defense transcripts during stress in *Zat10*-perturbed lines

To further test the correlation between *Zat10* expression and the expression of different defense transcript we examined the changes in steady-state mRNA level of different defense transcripts in loss-of-function *Zat10* lines during stress. As shown in Fig. 3A, the expression *FSD1* and *APX2* in response to light stress was not abolished in knockout *Zat10* plants (KO-*Zat10*). The absence of *Zat10* did not appear to have a significant effect on the accumulation of different cold-, salt-, or drought-response transcripts in knockout plants subjected to stress (Fig. 3B). Similar results were found in *Zat10* RNAi lines subjected to salinity stress (*Zat10*-RNAi; Fig. 3B).

3.4. Stress tolerance of *Zat10* gain- and loss-of-function lines

A previous study showed that transgenic plants expressing *Zat10* are more tolerant to drought stress [5]. However, the plants used in that study were also suppressed in their growth, a phenotype that complicates the interpretation of stress tolerance results obtained with transgenic plants [19]. To test the relative contribution of *Zat10* to abiotic stress tolerance in *Arabidopsis* we subjected seedlings of transgenic plants with constitutive expression of *Zat10* (*Zat10*-OE), seedlings of knockout *Zat10* (KO-*Zat10*), and seedlings of RNAi lines for *Zat10* (*Zat10*-RNAi) to different abiotic stresses, and scored

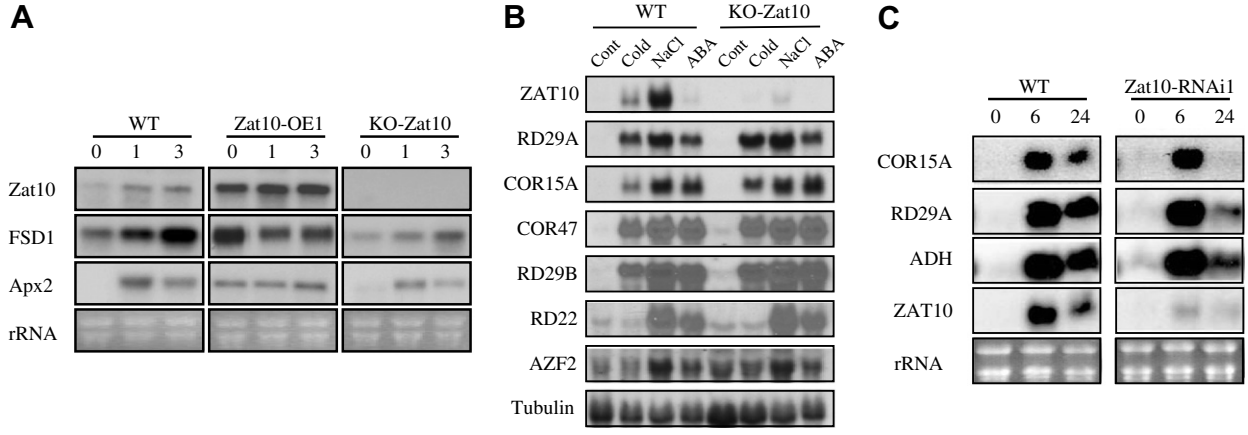

Fig. 3. Expression of defense transcripts in loss-of-function *Zat10* lines during stress. (A) RNA blots showing the expression of *APX2* and *FSD1* in control plants (WT), knockout plants for *Zat10* (KO-*Zat10*), and transgenic plants with constitutive expression of *Zat10* (*Zat10*-OE) subjected to a light stress treatment for 0, 1 and 3 h. (B) RNA blot analysis showing the expression of different abiotic stress-response transcripts in WT and KO-*Zat10* plants grown under controlled conditions or subjected to cold stress, salinity stress or ABA application. (C) RNA blot analysis showing the expression of different abiotic stress-response transcripts in WT and *Zat10* RNAi (*Zat10*-RNAi1) plants subjected to salinity stress for 0, 6 and 24 h. Plant growth, light stress treatments and RNA blot analysis were performed as described in Section 2. Abbreviations: HL, high light; LL, low light.

seedlings for root growth and % germination, parameters that reflects overall health and stress tolerance of plants [20].

As shown in Fig. 4, significant differences were found in the tolerance of *Zat10*- perturbed lines to osmotic stress, salinity and heat. In contrast, no differences were found in the tolerance of these lines to cold stress, and tolerance to oxidative stress imposed by paraquat was only observed at very low concentrations (0.01 μM; not shown). Significant differences in % germination were not observed between control and overexpressor or knockout lines, or between control and RNAi lines subjected to the different stresses (Supplementary Fig. 2).

Interestingly, both transgenic plants expressing *Zat10*, as well as knockout and RNAi lines were more tolerant to osmotic and salinity stress. These results are in contrast to our previous findings with *Zat12* plants in which overexpressor lines were more tolerant to osmotic stress, whereas knockout lines were more sensitive to osmotic and salinity stress [10]. Drought experiments performed with control and overexpressor or knockout lines, or control and RNAi lines, of similar size and age, grown in the same pots, failed to show a significant difference in drought tolerance (not shown).

4. Discussion

The expression patterns of *Zat10* and *Zat12* during stress (Fig. 1), suggest that *Zat10* function could be linked during stress to the function of *Zat12*. *Zat12* was previously shown to play a central role in ROS signaling during abiotic stress, and to be essential for the expression of *APX1* [8,10]. Here, we report that constitutive expression of *Zat10* results in the enhanced expression of *APX2*, *FSD1* and *APX1* in plants

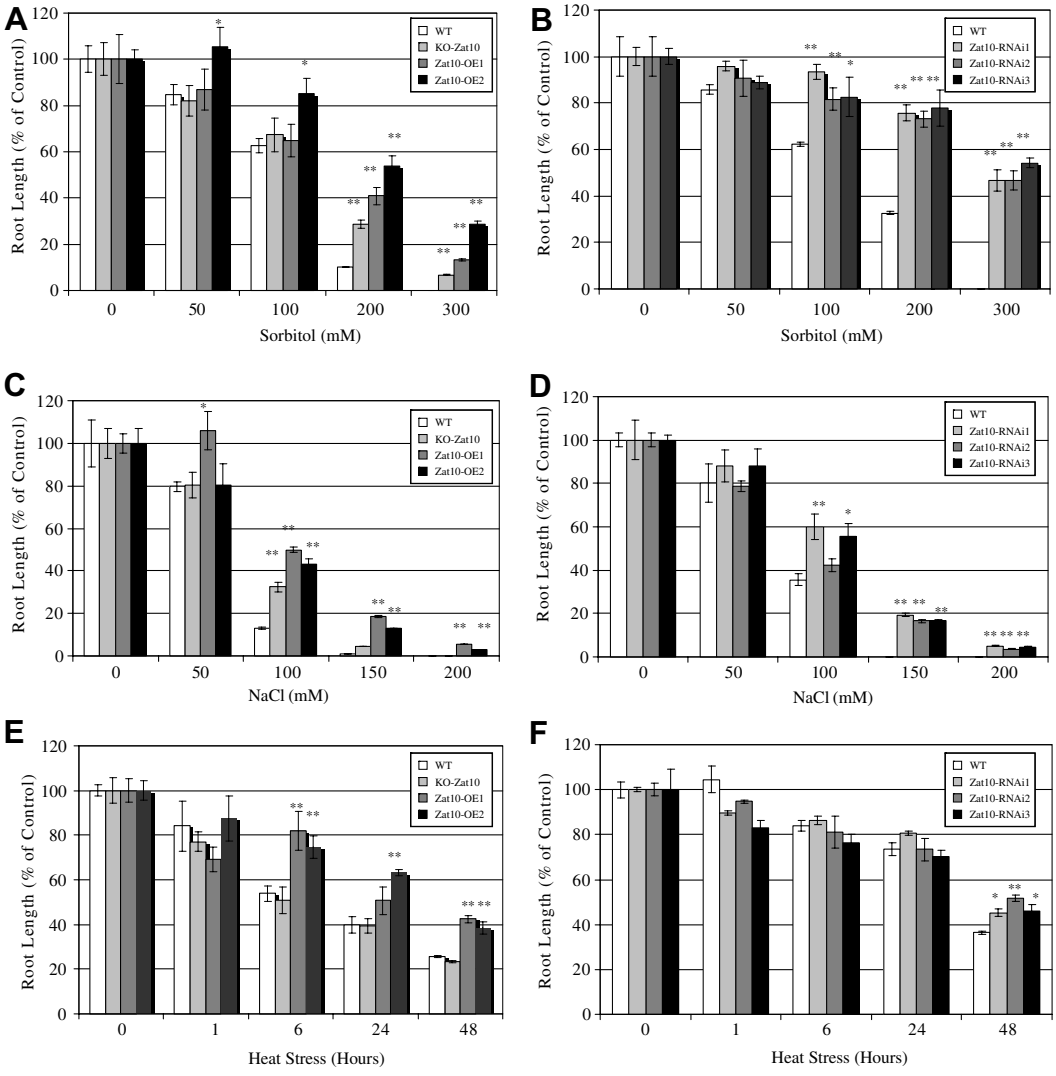

Fig. 4. Tolerance of gain- and loss-of-function *Zat10* Arabidopsis seedlings to abiotic stress. (A) Root growth of control (WT), knockout *Zat10* (KO-*Zat10*), and two independent lines with constitutive expression of *Zat10* (*Zat10*-OE1 and 2) subjected to osmotic stress. (B) Root growth of control (WT) and three independent RNAi lines for *Zat10* (*Zat10*-RNAi) subjected to osmotic stress. (C) Root growth of control (WT), knockout *Zat10* (KO-*Zat10*), and two independent lines with constitutive expression of *Zat10* (*Zat10*-OE1 and 2) subjected to salinity stress. (D) Root growth of control (WT) and three independent RNAi lines for *Zat10* (*Zat10*-RNAi) subjected to salinity stress. (E) Root growth of control (WT), knockout *Zat10* (KO-*Zat10*), and two independent lines with constitutive expression of *Zat10* (*Zat10*-OE1 and 2) subjected to heat stress. (F) Root growth of control (WT) and three independent RNAi lines for *Zat10* (*Zat10*-RNAi) subjected to heat stress. Stress assays were performed as described in Section 2. **, *t*-test significant at $P < 0.01$; *, *t*-test significant at $P < 0.05$ ($n = 45$).

grown under controlled growth conditions (Figs. 2 and 3). These findings suggest that *Zat10* is involved in the response of plants to oxidative stress, and that it could be directly involved in the control of *FSD1* and *APX2* expression during light stress. Interestingly, transgenic plants with constitutive expression of *Zat12* did not have elevated expression of *APX1*, *APX2* or *FSD1* [8], suggesting that *Zat10* and *Zat12* could play different roles during stress. In contrast to the observation that *Zat10* expression enhances the expression of several ROS-response transcripts (Fig. 2), *Zat10* does not appear to be involved in the control of different salt, cold or drought response transcripts (Figs. 2 and 3).

High level constitutive expression of *Zat10* resulted in growth suppression (Fig. 2). However, growth suppression was not required for enhanced expression of *APX2* or *FSD1*, or for enhanced tolerance to abiotic stress (Figs. 2 and 4). Growth suppression was previously reported in transgenic plants with constitutive expression of *Zat7* [8]. It is possible that aberrant expression of certain Zat proteins could cause growth alterations, but this phenotype appears not to be linked to the activation of defense mechanisms or the enhanced tolerance of transgenic plants to stress.

Functional characterization of *Zat10* using gain- and loss-of-function lines subjected to abiotic stress revealed that transgenic plants with constitutive expression of *Zat10* are more tolerant to osmotic, salinity and heat stresses (Fig. 4). An unexpected result was however the finding that knockout and RNAi lines for *Zat10* were also more tolerant to osmotic and salinity stress (Fig. 4). It was previously shown that the EAR motif of *Zat10* can function as a transcriptional suppressor [5,6,12]. If this domain is directly involved in the suppression of defense responses, then suppressing *Zat10* by RNAi or knockout mutagenesis would result in enhanced tolerance. However, constitutive expression of *Zat10* would likewise be expected to result in suppressed tolerance to stress, a result that is not reflected by our findings (Fig. 4).

Assuming the results obtained with the *Zat10* over-expressing lines reflect a true gain-of-function situation (i.e., they do not reflect a non-specific effect of *Zat10* expression on plant metabolism), it is possible that *Zat10* plays a dual role in the control of plant defenses. On the one hand it causes the activation of defense responses such as *FSD1* and *APX2* during stress, either directly as an activator, or indirectly by repressing a repressor of these defenses. On the other hand, however, it functions as a repressor of a different set of defense mechanisms that enhances the tolerance of plants to osmotic and salinity stresses. Thus, constitutive expression of *Zat10* results in the activation of ROS responses and the enhanced tolerance to stress (Figs. 2–4), whereas repression of *Zat10* releases the suppression of a different defense response pathway(s) that enhances plant tolerance to abiotic stress (Fig. 4). The differences observed between the enhanced tolerance of gain- and loss-of-function *Zat10* lines to heat stress (Fig. 4) might support the model described above and suggest that the defense mechanisms activated in transgenic plants that constitutively express *Zat10* are different than the defense mechanisms activated in loss-of-function lines for *Zat10*. Further studies are required to identify the defense mechanisms activated in *Zat10* loss-of-function lines during stress (Fig. 3).

The response of plants to abiotic stress involves a subset of responses directed at controlling the steady-state level of ROS in cells [15,18]. This is important to prevent damages caused by ROS, but also to control ROS signaling that is an integral part of the plant defense response to stress [18]. Our characterization of *Zat10* indicates that this protein could function to modulate or balance the response of plants to ROS and abiotic stress. Thus, on the one hand it could activate ROS responses, such as *FSD1* and *APX2*, whereas on the other hand it could suppress defense responses that enhance the tolerance of plants to osmotic and salinity stress (Fig. 4).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.002.

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