The Zinc-Finger Protein Zat12 Plays a Central Role in Reactive Oxygen and Abiotic Stress Signaling in Arabidopsis[1][w]

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Plant acclimation to environmental stress is controlled by a complex network of regulatory genes that compose distinct stress-response regulons. In contrast to many signaling and regulatory genes that are stress specific, the zinc-finger protein Zat12 responds to a large number of biotic and abiotic stresses. Zat12 is thought to be involved in cold and oxidative stress signaling in Arabidopsis (*Arabidopsis thaliana*); however, its mode of action and regulation are largely unknown. Using a fusion between the Zat12 promoter and the reporter gene luciferase, we demonstrate that Zat12 expression is activated at the transcriptional level during different abiotic stresses and in response to a wound-induced systemic signal. Using Zat12 gain- and loss-of-function lines, we assign a function for Zat12 during oxidative, osmotic, salinity, high light, and heat stresses. Transcriptional profiling of Zat12-overexpressing plants and wild-type plants subjected to H_2O_2 stress revealed that constitutive expression of Zat12 in Arabidopsis results in the enhanced expression of oxidative- and light stress-response transcripts. Under specific growth conditions, Zat12 may therefore regulate a collection of transcripts involved in the response of Arabidopsis to high light and oxidative stress. Our results suggest that Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis.

Plants are sessile organisms that evolved complex regulatory networks to control their response to changes in environmental conditions (Arabidopsis Genome Initiative, 2000). Interestingly and in contrast to prior belief, little overlap in transcript expression was found between the response of plants to different environmental stress conditions (Kreps et al., 2002; Rizhsky et al., 2004b). Thus, transcriptome profiling of plants subjected to heat, drought, cold, salt, high light, or mechanical stress revealed that very few genes respond in a similar manner to all of these stresses (Cheong et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Rossel et al., 2002; Rizhsky et al., 2004b). Moreover, although reactive oxygen species (ROS) were implicated as signals and/or byproducts of many different biotic and abiotic stress conditions, different genes of the ROS gene network of Arabidopsis (*Arabidopsis thaliana*) were found to respond differently to different stress treatments (Mittler et al., 2004).

A complex network of transcription factors orchestrates the response of plants to changes in environmental conditions (Chen et al., 2002). These include WRKY and other zinc-finger proteins (72 WRKY genes and more than 600 zinc-finger proteins in Arabidopsis; Eulgem et al., 2000), MYB transcription factors (133 genes in Arabidopsis; Stracke et al., 2001), and heat shock transcription factors (21 genes in Arabidopsis; Nover et al., 2001). However, only a few of these transcription factors appear to respond in a similar manner to all or most of the different environmental stress conditions tested in Arabidopsis. One representative of the small group of genes that responds similarly to many different environmental stress conditions is the zinc-finger protein Zat12 (At5g59820). Zat12 was found to respond at the steady-state transcript level to ozone fumigation; wounding; bacterial, fungal, or nematode infection; H_2O_2; heat; cold; drought; elicitor; heavy metal; methanol; fumonisin; or UV application (Iida et al., 2000; Cheong et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Rizhsky et al., 2004b; Davletova et al., 2005; a search of 1,809 ATH1 Affymetrix chips in https://www.genevestigator.ethz.ch/). In addition, Zat12 is expressed in roots, flowers, and developing seeds, tissues associated with expression of stress-response genes (https://www.genevestigator.ethz.ch/).

Zat12 was originally isolated as a light stress-response cDNA (*rhl41*) by Iida et al. (2000), and was later identified by transcriptome analyses of plants subjected to different biotic and abiotic stress conditions. Recent analysis of Zat12 using transgenic plants suggested a role for Zat12 in cold acclimation and in the response of plants to oxidative stress (Rizhsky et al.,

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2004a; Vogel et al., 2005). However, the three different studies that used transgenic plants overexpressing Zat12 to study Zat12 function were very different in their description of plant phenotype (Iida et al., 2000; Rizhsky et al., 2004a; Vogel et al., 2005). In addition, very little overlap in transcript expression was found between the transcriptome of mature transgenic plants expressing Zat12 in experiments performed by Vogel et al. (2005) and Rizhsky et al. (2004a). Perhaps the involvement of Zat12 in many different stress-response pathways, the differences in experimental conditions used for the assays, and the differences in genetic background, age, and physical condition of plants might explain the variations observed in the effects of Zat12 overexpression in these experiments.

The enhanced expression of Zat12 in response to many different biotic and abiotic stress conditions makes Zat12 an interesting subject for analysis. What is the function of Zat12 in Arabidopsis? Is it involved in all of the stresses its expression is associated with? Is it related to ROS signaling as previously suggested (Rizhsky et al., 2004a)? To address these questions, we functionally characterized Zat12 in Arabidopsis. Using transgenic plants expressing the reporter gene luciferase under the control of the Zat12 promoter, we studied the transcriptional activation of Zat12 during different abiotic stresses. Using gain- and loss-of-function plants for Zat12, we studied the function of Zat12 during different stresses, and using microarray analysis we compared the transcriptome of Zat12-expressing plants to that of plants subjected to H_2O_2 stress. Our results identify a function for Zat12 during oxidative, osmotic, salinity, high light, and heat stresses. In addition, our analysis suggests that under certain growth conditions, Zat12 may control a collection of transcripts involved in plant acclimation to high light and oxidative stress.

RESULTS

Activation of Zat12 Transcription in Arabidopsis

The response of Zat12 to different stress treatments is typically measured as a transient increase in steady-state transcript level (mainly by RNA blots or Affymetrix chips; Iida et al., 2000; Cheong et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Rizhsky et al., 2004a; Davletova et al., 2005). To examine whether the increase in Zat12 steady-state transcript level results from an increase in Zat12 transcription or from a change in the stability of the Zat12 transcript, we generated a fusion between the Zat12 promoter and the reporter gene luciferase. For this analysis, a 1,000-bp fragment corresponding to position 1 to −1,000 of the *Zat12* gene (At5g59820) was amplified by PCR from genomic DNA, sequenced, and cloned into a binary vector that contained the luciferase reporter gene. Three independent transgenic lines were chosen for analysis.

As shown in Figure 1A, activation of Zat12 transcription occurred in response to cold (4°C, 2 h), heat (38°C, 1 h), salinity (150 mM NaCl, 4 h), drought (75% relative water content), wounding (arrows indicate leaves that were wounded 10 times each with a needle,

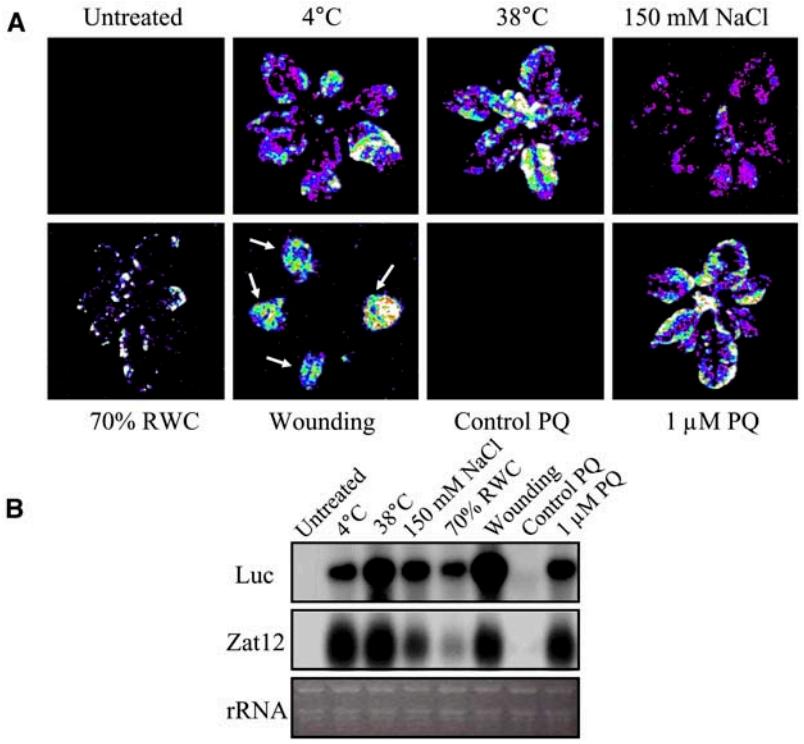


Figure 1. Promoter::reporter analysis of Zat12 expression in Arabidopsis. A, Luciferase imaging in transgenic Arabidopsis plants expressing luciferase under the control of the Zat12 promoter in response to different environmental stresses. B, RNA-blot analysis of luciferase and Zat12 steady-state transcript level in the plants shown in A in response to stress. Construction of transgenic plants, stress assays, and luciferase imaging were performed as described in "Materials and Methods." Abbreviations: Luc, luciferase; PQ, methyl viologen (paraquat); rRNA, ribosomal RNA; RWC, relative water content.

30 min), and application of the superoxide-generating agent methyl viologen (10^{-6} M, 1 h). These stresses were applied to soil-grown 3-week-old plants as described (Mittler and Zilinskas, 1992; Kreps et al., 2002; Rizhsky et al., 2004a, 2004b). As shown in Figure 1B, RNA-blot analysis of transgenic plants expressing the Zat12 promoter::luciferase construct confirmed that the expression of luciferase correlated with the steady-state level of Zat12 transcripts, the stress treatment, and the intensity of luciferase activity (compare the steady-state level of Zat12 and luciferase in Fig. 1B to luciferase activity in Fig. 1A). Luciferase transcription was also activated in response to light stress (1,000 μmol m^{-2} s^{-1}; 1 h), the application of H_2O_2 (1, 2, 5, or 20 mM, 1 h), ferric-citrate (1 mM, 5 h), dark treatment (4 h), or the application of the plant hormones auxin or abscisic acid (data not shown).

Several stresses, such as pathogen infection, high light, and wounding, induce a systemic response in plants (Alvarez et al., 1998; Karpinski et al., 1999; Orozco-Cardenas et al., 2001). Typically, this response requires a few hours to develop and is thought to involve salicylic acid, jasmonic acid, systemin, and/or H_2O_2. To test whether Zat12 expression is induced systemically in Arabidopsis, we wounded plants on three different leaves and followed the activation of luciferase transcription in the entire plant. As shown in Figure 2A, 30 min following wounding (middle section), luciferase activity was detected in the wounded leaves (wounded leaves are indicated by arrows in all sections). Two hours following wounding, luciferase activity in the wounded leaves subsided and strong luciferase activity was observed in upper young leaves that were not treated (right section). As shown in Figure 2B, RNA-blot analysis of treated and untreated local and systemic leaves confirmed that expression of the luciferase transcript is enhanced in wounded (local) and unwounded systemic (upper young leaves of wounded plants) tissues. The results presented in Figures 1 and 2 indicate that transcription of the Zat12 gene is activated in response to different stimuli. These may be directly related to stress or wounding, or may occur as a result of a systemic signal that is generated in the wounded leaves and transferred to untreated parts of the plant.

Tolerance of Gain- and Loss-of-Function Zat12 Lines to Abiotic Stress

The response of Zat12 to many different stresses is intriguing. Is Zat12 essential for plant tolerance to all of these stresses? To address this question, we subjected seedlings of knockout Zat12 plants (KO-Zat12; SALK_037357 and SAIL 792_F04) and seedlings of plants that overexpress Zat12 (OE-Zat12; three independent lines) to different abiotic stresses and scored them for root growth and percentage of germination, parameters that reflect overall health and stress tolerance of plants (Zhu, 2002). The following stresses were tested: heat (38°C; 1, 6, 24, and 48 h), cold (10°C; 1, 2, and 3 d), osmotic stress (50, 100, 200, and 300 mM sorbitol), salinity (50, 100, 150, and 200 mM NaCl), ferric-citrate (0.01, 0.1, 0.5, and 1 mM), high light (1,000 μmol m^{-2} s^{-1} for 24, 48, and 72 h), and methyl viologen (0.01, 0.1, 0.5, 1, and 10 μM).

As shown in Figure 3 and Supplemental Figures 1 and 2 (that represent different sets of experiments and include measurements of root growth, as well as data collected for percentage of germination), knockout Zat12 plants were more sensitive than wild-type plants to salinity stress (Fig. 3A) and osmotic stress (Fig. 3B). In contrast, knockout Zat12 plants were more tolerant than wild-type plants to heat stress (Fig. 3C). Compared to wild-type plants, transgenic plants overexpressing Zat12 were more tolerant to osmotic stress (Fig. 3D), light stress, and methyl viologen (data not shown; see

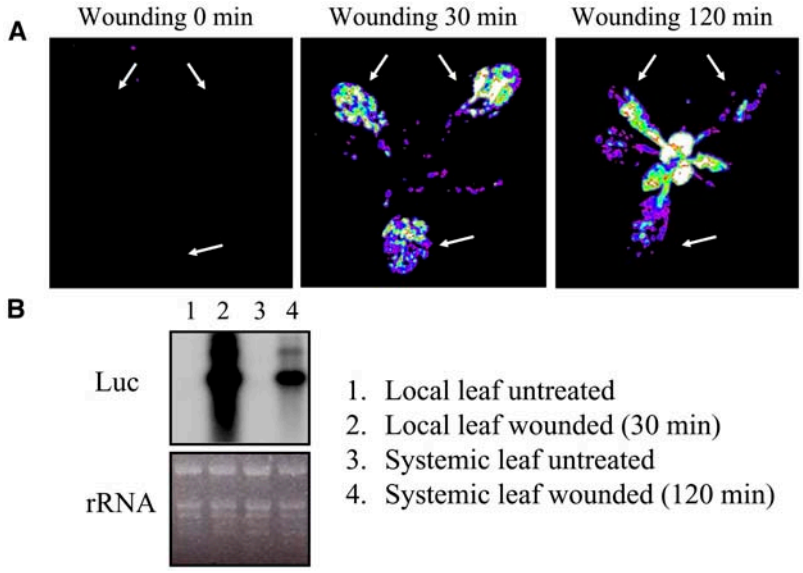


Figure 2. Systemic induction of the Zat12 promoter in response to wounding. A, Luciferase imaging in a transgenic Arabidopsis plant expressing luciferase under the control of the Zat12 promoter in response to wounding. Arrows indicate the wounded leaves. Luciferase imaging was performed at 0, 30, and 120 min following wounding. B, RNA-blot analysis of luciferase steady-state transcript level in transgenic Arabidopsis plant expressing luciferase under the control of the Zat12 promoter in response to wounding. Luciferase transcripts are shown to accumulate in local wounded leaves (lane 2) and systemic leaves of wounded plants (lane 4). Construction of transgenic plants, wounding, and luciferase imaging were performed as described in "Materials and Methods." Abbreviations: Luc, luciferase; rRNA, ribosomal RNA.

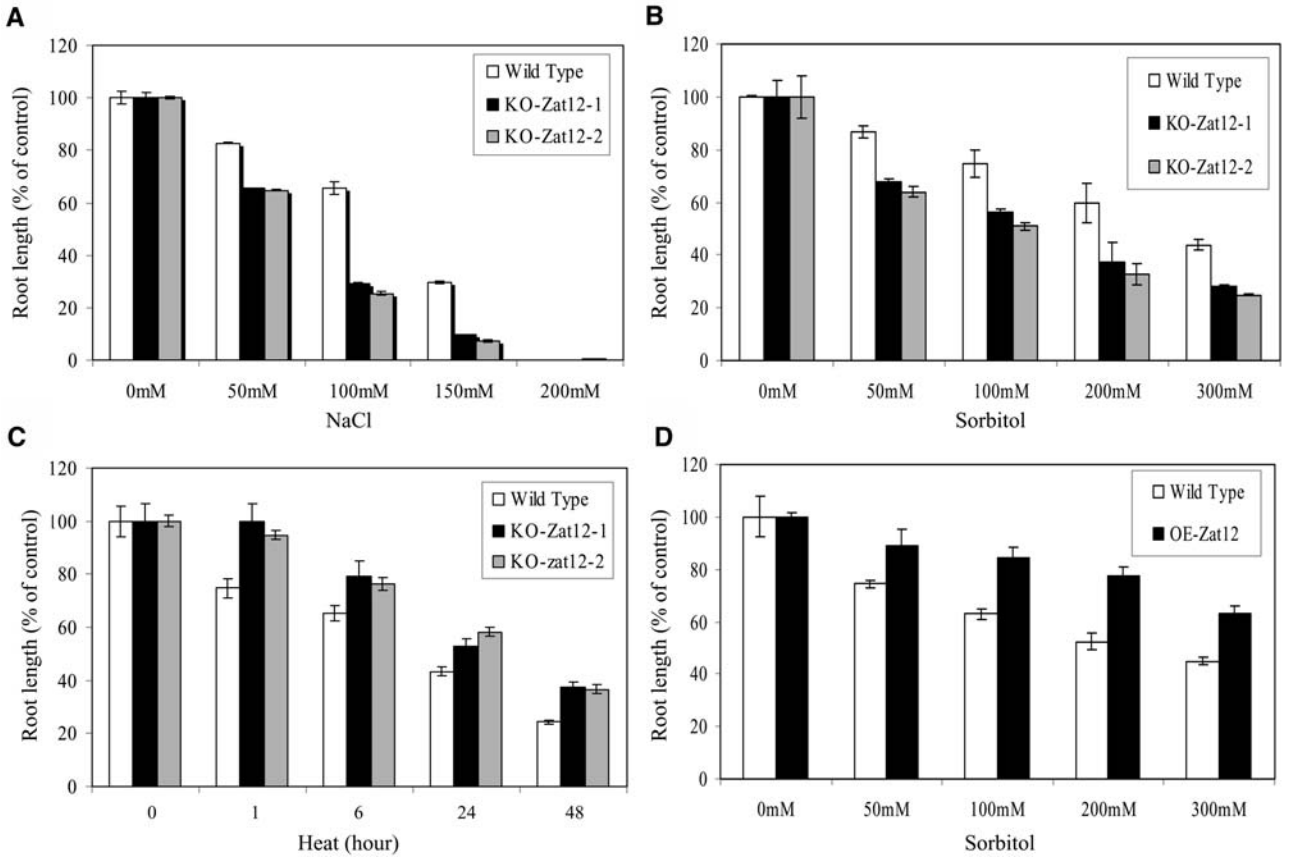


Figure 3. Tolerance of gain- and loss-of-function Zat12 Arabidopsis lines to abiotic stress. A, Root growth of wild-type and KO-Zat12 seedlings subjected to salinity stress. B, Root growth of wild-type and KO-Zat12 seedlings subjected to osmotic stress. C, Root growth of wild-type and KO-Zat12 seedlings subjected to heat stress. D, Root growth of wild-type and OE-Zat12 seedlings subjected to osmotic stress. Stress assays were performed as described in "Materials and Methods" using independent knockout and overexpression lines. Additional assays are shown in Supplemental Figures 1 and 2. Abbreviations: KO-Zat12-1, knockout Zat12, SALK_037357; KO-Zat12-2, knockout Zat12, SAIL 792_F04; OE-Zat12, transgenic seedlings overexpressing Zat12.

Iida et al. [2000] for enhanced tolerance of Zat12-overexpressing lines to light stress, and Rizhsky et al. [2004a] for enhanced tolerance of transgenic Zat12 plants to methyl viologen). These results suggest that, at least when tested with 5-d-old seedlings, Zat12 is required for plant tolerance to osmotic, oxidative, and salinity stresses. In contrast, Zat12 may have a negative effect on plant tolerance to heat stress.

Transcriptome Profiling of Transgenic Plants Overexpressing Zat12

To complement the characterization of abiotic stress tolerance performed with seedlings of gain- and loss-of-function lines (Fig. 3) and to address the question of Zat12 association with H_2O_2 (Rizhsky et al., 2004a), we performed transcriptome profiling of transgenic plants overexpressing Zat12 (obtained from a pool of three independent lines). For this analysis, we used 5-d-old seedlings, comparable in age and growth conditions to the seedlings used for all stress assays in this study (Fig. 3; Supplemental Figs. 1 and 2) and in Rizhsky et al. (2004a). Our profiling analysis was, however, not comparable to that reported by Rizhsky et al. (2004a) and Vogel et al. (2005) that used older or more mature plants grown in soil. In parallel to the profiling of Zat12-overexpressing seedlings, we performed profiling of wild-type seedlings subjected to H_2O_2 stress. This design enabled us to check for overlap between transcripts elevated or suppressed in transgenic seedlings overexpressing Zat12 and in wild-type seedlings treated with H_2O_2. Four different treatments were used: wild-type untreated (WT), wild-type treated with 20 mM H_2O_2 for 1 h (WT + H_2O_2), transgenic plants overexpressing Zat12 (Zat12), and transgenic plants overexpressing Zat12 treated with 20 mM H_2O_2 for 1 h (Zat12 + H_2O_2). All treatments were sampled at the same time and the experiment was repeated three times (total of 12 samples representing three biological repeats). We did not use loss-of-function (KO-Zat12) lines for transcriptome profiling because the quality of RNA extracted from these lines following the H_2O_2 treatment was insufficient for microarray analysis (data not shown).

As shown in Figure 4, expression of Zat12 and ascorbate peroxidase 1 (Apx1), a H_2O_2-response

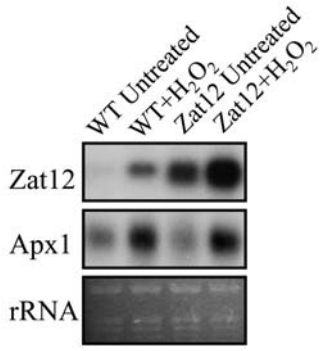


Figure 4. RNA blots showing the expression of Zat12 and Apx1 in H_2O_2-treated and untreated wild-type plants and transgenic plants overexpressing Zat12. RNA blots were performed as described in "Materials and Methods." Abbreviations: rRNA, ribosomal RNA; WT, wild type; Zat12, transgenic plants overexpressing Zat12.

transcript (Mittler and Zilinskas, 1992; Davletova et al., 2005), is elevated in wild-type plants following the application of H_2O_2 (WT + H_2O_2). The steady-state level of Zat12 transcripts found in transgenic plants overexpressing Zat12 (Zat12 untreated) was higher than that of treated wild-type plants (compare Zat12 untreated to WT + H_2O_2). The level of Zat12 found in untreated transgenic plants overexpressing Zat12 was elevated further following H_2O_2 application (Zat12 + H_2O_2).

Treatment of wild-type plants with H_2O_2 resulted in the significant elevation of 564 transcripts (more than 2-fold; Fig. 5; Supplemental Table II). To validate the results obtained with this treatment (i.e. H_2O_2 stress), we compared the transcripts enhanced in 5-d-old wild-type seedlings in response to H_2O_2 application with those enhanced in mature knockout plants deficient in *Apx1* (knockout-*Apx1*) plants in response to light stress, a stress that results in the accumulation of H_2O_2 in knockout-*Apx1* plants (Davletova et al., 2005). Overall, 32% of the transcripts elevated in wild-type plants in response to H_2O_2 application were also elevated in knockout-*Apx1* plants in response to a light stress treatment. Signal transduction, ROS-related, and defense-related transcripts significantly elevated in wild-type plants treated with H_2O_2 as well as in knockout-*Apx1* plants subjected to light stress (Davletova et al., 2005) are shown in Table I. They include heat shock transcription factor 21 (HSF21), NADPH oxidase D (RbohD), mitochondrial alternative oxidase, mitogen-activated protein kinase MAPK3, several WRKY transcription factors (40, 33, 18, and 70), several zinc-finger proteins including Zat10 and Zat12, and a number of transcripts associated with calcium signaling. We previously used dominant-negative lines for HSF21 and knockout lines for RbohD to show that these two proteins are directly involved in H_2O_2 sensing in Arabidopsis (Davletova et al., 2005). The transcripts shown in Table I might represent a collection of genes directly associated with H_2O_2 signaling and stress in Arabidopsis.

As shown in Figure 5, 42 transcripts were elevated and six transcripts suppressed (more than 2-fold) in transgenic plants overexpressing Zat12 in the absence of stress. Of the 42 transcripts elevated in Zat12-overexpressing lines, 23 were also elevated in wild-type plants following H_2O_2 application. As shown in Table II, the majority of these transcripts are encoded by chloroplastic genes (the 23 transcripts elevated in Zat12-overexpressing plants and in wild-type plants treated with H_2O_2 are indicated in bold in Table II). The transcriptome of transgenic plants overexpressing Zat12 resembled the transcriptome of plants subjected to light stress (Rossel et al., 2002; Rizhsky et al., 2003). Thus, chloroplastic transcripts elevated in Zat12-overexpressing lines included psbA, B and H, psaA, B and I, small ribosomal proteins, and clpP protease. The detection of chloroplastic transcripts by Affymetrix chips (Table II) indicates that these transcripts were not removed by the oligo(dT) purification step (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Because polyadenylation of chloroplastic transcripts is associated with increased turnover (Kudla et al., 1996), it is possible that the turnover of the chloroplastic transcripts shown in Table II is enhanced in Zat12-overexpressing plants and in wild-type plants subjected to a H_2O_2 treatment. This possibility is in accordance with the enhanced turnover of many chloroplastic

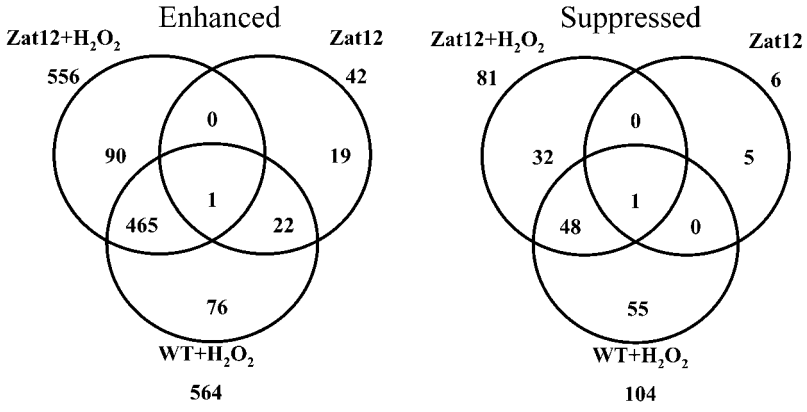


Figure 5. Venn diagrams showing the overlap between transcript expression in wild-type plants subjected to H_2O_2 stress (WT + H_2O_2), transgenic plants overexpressing Zat12 (Zat12), and transgenic plants overexpressing Zat12 subjected to H_2O_2 stress (Zat12 + H_2O_2). Transcriptome profiling and data analysis were performed as described in "Materials and Methods."

Table I. *Signal transduction, ROS-related, and defense-related transcripts significantly elevated in wild-type plants treated with H_2O_2 as well as in knockout-Apx1 plants subjected to light stress*

Signal transduction, ROS-related, and defense-related transcripts significantly elevated in wild-type plants treated with H_2O_2 and in knockout-*Apx1* plants subjected to light stress are shown. Affymetrix ATH1 accessions are given on left. Locus identification numbers are given in the second column from left. Gene annotation and fold change in $\log_2$ in response to H_2O_2 application to wild-type plants are given in the third and fourth columns from left, respectively. Transcriptome profiling and data analysis were performed as described in "Materials and Methods."

Chip Annotation	Locus Identifier	Gene Annotation	Fold (log2)
261892_at	AT1G80840	WRKY40 transcription factor	5.310
261648_at	AT1G27730	Zinc-finger (C_2H_2-type) family protein (ZAT10)	4.550
267028_at	AT2G38470	WRKY33 transcription factor	3.724
248657_at	AT5G48570	Peptidyl-prolyl cis-trans isomerase	3.415
263379_at	AT2G40140	Zinc finger (CCCH type), Ankyrin repeat	3.081
251745_at	AT3G55980	Zinc finger (CCCH type), Ankyrin repeat	2.955
254926_at	AT4G11280	Aminocyclopropane carboxylic acid synthase 6	2.923
262119_s_at	AT1G02920	Glutathione *S*-transferase	2.922
267083_at	AT2G41100	Touch-responsive/calmodulin-related protein 3	2.791
253414_at	AT4G33050	Calmodulin-binding family protein	2.537
248164_at	AT5G54490	Calcium-binding EF-hand protein	2.330
247655_at	AT5G59820	Zinc-finger (C_2H_2-type) family (ZAT12)	2.313
252592_at	AT3G45640	Mitogen-activated protein kinase (MPK3)	2.276
253915_at	AT4G27280	Calcium-binding EF-hand protein	2.245
259879_at	AT1G76650	Calcium-binding EF protein	2.169
246777_at	AT5G27420	Zinc finger (C_3HC_4-type RING finger)	2.114
254549_at	AT4G19880	Glutathione *S*-transferase	2.105
248719_at	AT5G47910	Respiratory burst oxidase protein D (RbohD)	2.000
252474_at	AT3G46620	Zinc finger (C_3HC_4-type RING finger)	1.996
253485_at	AT4G31800	WRKY18 transcription factor	1.906
254592_at	AT4G18880	HSF21	1.904
247137_at	AT5G66210	Calcium-dependent protein kinase/CDPK	1.858
259272_at	AT3G01290	Band 7 protein, hypersensitive induced	1.800
249417_at	AT5G39670	Calcium-binding EF-hand protein	1.663
248327_at	AT5G52750	Heavy-metal-associated protein	1.630
250676_at	AT5G06320	Harpin-induced family protein/HIN1	1.620
258921_at	AT3G10500	No apical meristem (NAM) protein	1.595
260399_at	AT1G72520	Lipoxygenase	1.509
253125_at	AT4G36040	DNAJ heat shock N-terminal domain containing	1.493
258452_at	AT3G22370	Alternative oxidase 1a	1.490
267293_at	AT2G23810	Senescence-associated protein 5	1.438
251705_at	AT3G56400	WRKY70	1.261
266746_s_at	AT4G02520	Glutathione *S*-transferase	1.238
267381_at	AT2G26190	Calmodulin-binding protein	1.217
260648_at	AT1G08050	Zinc finger (C_3HC_4-type RING finger)	1.166

transcripts during light stress (Kudla et al., 1996). Further studies are required to address this possibility and to elucidate the possible role of Zat12 in regulating light stress responses in Arabidopsis. Nuclear genes elevated in Zat12-overexpressing lines included chloroplastic and cytosolic copper/zinc superoxide dismutases and the superoxide dismutase copper chaperone (Table II). In addition to these transcripts, transcripts encoding six different Lea (late embryogenesis abundant) proteins were elevated in transgenic plants overexpressing Zat12. The elevated expression of these transcripts might explain the enhanced tolerance of Zat12-overexpressing lines to osmotic stress (Fig. 3D). The six transcripts suppressed in transgenic plants overexpressing Zat12 include the following: copper amine oxidase (At3g43670), copper homeostasis factor (At3g56240), Cys proteinase (At4g11310), harpin-induced protein (At2g35960), GDSL-lipase/hydrolase (At4g28780), and iron superoxide dismutase (At4g25100).

As shown in Figure 5, 465 transcripts were elevated in wild-type or Zat12-overexpressing plants treated with H_2O_2. Of these, only eight transcripts were elevated to a higher degree in Zat12-overexpressing lines compared to wild-type plants (HSP17.4-CIII, At1g54050; HSP17.6A-CI, At1g59860; HSP17.6B-CI, At2g29500; stress-inducible protein, At4g12400; and four expressed proteins of unknown function, At5g24660, At5g10695, At4g36500, and At3g10930; Supplemental Table III). In addition, as shown in Figure 5, 90 transcripts were specifically elevated in Zat12-overexpressing plants in response to H_2O_2 application. These transcripts are summarized in Supplemental Table IV. Interestingly, a number of ROS-response genes are found in this group of genes, suggesting that Zat12 expression during H_2O_2 stress might activate parts of the ROS-response pathway in Arabidopsis (Mittler et al., 2004).

Table II. *Transcripts significantly elevated (more than 2-fold) in transgenic Arabidopsis seedlings overexpressing Zat12*

Transcripts that are significantly elevated in transgenic plants overexpressing Zat12 compared to wild-type plants (more than 2-fold) are shown. Affymetrix ATH1 accessions are given on left. Locus identification numbers are given in the second column from left. Gene annotation and fold change in $\log_2$ are given in the third and fourth columns from left, respectively. Transcripts that are elevated in transgenic plants overexpressing Zat12, as well as in wild-type plants subjected to H_2O_2 stress, are indicated in bold. Locus identifiers that begin with an ATC denote chloroplastic genes. Transcriptome profiling and data analysis were performed as described in "Materials and Methods."

Chip Annotation	Locus Identifier	Gene Annotation	Fold (log2)
247655_at	**AT5G59820**	**Zinc-finger (C_2H_2-type) protein (ZAT12)**	**3.444**
245007_at	**ATCG00350**	**psaA PSI P700 apoprotein**	**2.895**
244965_at	**ATCG00590**	**orf31 hypothetical protein**	**2.632**
244940_at	**ATCG00900**	**rps7.1 ribosomal protein S12**	**2.563**
244992_s_at	**ATCG01240**	**rps7.2 ribosomal protein S7**	**2.372**
245003_at	**ATCG00280**	**psbC PSII 43-kD protein**	**2.226**
245025_at	**ATCG00130**	**atpF ATPase I subunit**	**2.151**
259511_at	AT1G12520	Superoxide dismutase copper chaperone	2.141
245006_at	**ATCG00340**	**psaB PSI P700 apoprotein A2**	**2.119**
266165_at	AT2G28190	Copper/zinc superoxide dismutase (CSD2)	1.983
245024_at	**ATCG00120**	**atpA ATPase α-subunit**	**1.919**
262128_at	AT1G52690	Lea group 3	1.903
251838_at	AT3G54940	Cys proteinase	1.893
244975_at	**ATCG00710**	**psbH PSII 10-kD phosphoprotein**	**1.887**
245026_at	**ATCG00140**	**atpH ATPase III subunit**	**1.865**
245047_at	**ATCG00020**	**psbA PSII 32-kD protein**	**1.793**
258224_at	AT3G15670	Lea group 3	1.736
254805_at	AT4G12480	Protease inhibitor/seed storage/lipid transfer protein	1.711
266544_at	AT2G35300	Lea group 3	1.689
246099_at	**AT5G20230**	**Plastocyanin like**	**1.686**
254550_at	AT4G19690	Iron-responsive transporter (IRT1)	1.651
244976_at	**ATCG00720**	**petB cytochrome B6**	**1.619**
244936_at	**ATCG01100**	**ndhA NADH dehydrogenase ND1**	**1.538**
244972_at	**ATCG00680**	**psbB PSII 47-kD protein**	**1.504**
244971_at	**ATCG00670**	**clpP ATP-dependent protease**	**1.503**
258498_at	AT3G02480	Abscisic acid-responsive, cold-induced protein kin1	1.497
264809_at	AT1G08830	Copper/zinc superoxide dismutase (CSD1)	1.495
250780_at	AT5G05290	Expansin At-EXP2	1.485
245009_at	**ATCG00380**	**rps4 ribosomal protein S4**	**1.447**
244961_at	**ATCG01040**	**ycf5 hypothetical protein**	**1.399**
244935_at	**ATCG01090**	**ndhI NADH dehydrogenase subunit**	**1.322**
254818_at	AT4G12470	Protease inhibitor/seed storage/lipid transfer protein	1.319
245002_at	**ATCG00270**	**psbD PSII D2 protein**	**1.263**
250648_at	AT5G06760	Lea group 5	1.263
260556_at	**AT2G43620**	**Basic endochitinase CHB4**	**1.216**
245017_at	**ATCG00510**	**psaI PSII protein**	**1.208**
245335_at	AT4G16160	Mitochondrial inner membrane translocase	1.179
265211_at	AT2G36640	Lea group 3	1.106
246242_at	AT4G36600	Lea group 3	1.058
265208_at	AT2G36690	Oxidoreductase, 2OG-Fe(II) oxygenase	1.050
249353_at	AT5G40420	Gly-rich protein/oleosin	1.030
250624_at	AT5G07330	Expressed protein	1.005

They include glutathione *S*-transferases, DNAJ heat shock proteins, heat shock transcription factors 4 and 6, thioredoxin, calmodulin-9, ubiquitin, and glutathione peroxidase. Supplemental Table V summarizes all transcripts significantly elevated in Zat12-overexpressing plants in response to H_2O_2 stress.

DISCUSSION

The enhanced expression of Zat12 in response to many different abiotic stress conditions (Figs. 1 and 2; Iida et al., 2000; Cheong et al., 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Rizhsky et al., 2004b; Davletova et al., 2005) suggests that Zat12 is involved in the response of plants to all of these stresses. However, functional characterization of Zat12 demonstrated that Zat12 is essential for plant tolerance to only a few of the stresses its expression is associated with (Fig. 3; Supplemental Figs. 1 and 2). How could this result be explained? We see at least two different possibilities. (1) Zat12 performs different functions as a transcriptional regulator during different stresses, and our functional characterization could not have detected all of the cellular and stress-response functions

associated with Zat12. (2) Zat12 expression is not directly linked to its function, and although in many stress situations Zat12 expression is enhanced, the Zat12 protein might not be required for stress tolerance to all stresses. The broad response of the Zat12 gene to different stresses might result from the activation of Zat12 expression by ROS such as H_2O_2 that accumulate in cells in response to almost all stress conditions (Dat et al., 2000; Apel and Hirt, 2004; Mittler et al., 2004).

The transcriptome profiling experiments performed in this study suggest that, under specific growth conditions, Zat12 regulates a collection of transcripts involved in light and oxidative stress responses (Table II; Fig. 5). Our profile analyses results are also in good agreement with the functional characterization of gain-of-function lines for Zat12 (Fig. 3). Thus, the six different Lea proteins expressed in Zat12-overexpressing lines (Table II) might explain the high tolerance of these lines to osmotic stress (Fig. 3D), and the copper/zinc superoxide dismutases (CSD1, CSD2, and the copper chaperone) expressed in Zat12-overexpressing lines (Table II) might explain the high tolerance of these lines to methyl viologen (data not shown; Rizhsky et al., 2004a) and high light stress (data not shown; Iida et al., 2000). If indeed the expression of the same Lea proteins enhanced in Zat12-overexpressing lines is linked to Zat12 function, then this result might also explain the sensitivity of KO-Zat12 lines to salinity (Fig. 3A) and osmotic stress (Fig. 3B). The similarity between the transcriptome of Zat12-overexpressing lines and plants subjected to light stress (Table II; Rizhsky et al., 2003) might explain the high tolerance of Zat12-overexpressing lines to light stress (Iida et al., 2000; D. Davletova, J. Coutu, and R. Mittler, unpublished data).

Zat12 contains an EAR-motif-like sequence that may function as a repression domain (Ohta et al., 2001; Hiratsu et al., 2002; Vogel et al., 2005). In response to cold stress, the Zat12 protein was suggested to act as a suppressor of CBF transcription factors (Vogel et al., 2005). Our transcriptional profiling analysis revealed several transcripts suppressed in Zat12-overexpressing plants in the absence of stress and in response to H_2O_2 application (Supplemental Table II). However, additional studies are required to confirm a possible role for Zat12 as a repressor of these transcripts. Our analysis of knockout Zat12 plants subjected to heat stress revealed that the absence of Zat12 might have resulted in an enhanced tolerance of knockout plants to this stress (Fig. 3C). Thus, at least in response to heat stress, Zat12 might function as a repressor. It should however be noted that transgenic plants overexpressing Zat12 were not found to be more sensitive to heat stress than wild-type plants (Supplemental Fig. 1). Further studies, including transcriptome profiling of wild-type and Zat12 knockout plants subjected to heat stress, are required to address the possibility that Zat12 functions as a repressor during stress.

We previously proposed that Zat12 expression is directly linked to reactive oxygen metabolism in Arabidopsis (Rizhsky et al., 2004a; see also Table I). This hypothesis was based on the enhanced expression of Zat12 in knockout-*Apx1* plants (Pnueli et al., 2003; Davletova et al., 2005), the lack of Apx1 expression in knockout Zat12 plants subjected to oxidative stress (Rizhsky et al., 2004a), the high sensitivity of knockout Zat12 plants to oxidative stress, and the enhanced tolerance of Zat12-overexpressing lines to methyl viologen application (Rizhsky et al., 2004a). Our current analysis reveals an overlap between transcript expression in Zat12-overexpressing seedlings grown under controlled conditions and transcript expression in wild-type seedlings subjected to H_2O_2 stress (Fig. 5; Table II). Surprisingly, the majority of transcripts that were expressed in Zat12-overexpressing seedlings, as well as in wild-type seedlings subjected to H_2O_2 stress, were encoded by the chloroplast genome and associated with the response of plants to high light stress (Rossel et al., 2002; Rizhsky et al., 2003). Reactive oxygen species such as H_2O_2, O_2^-, and $^1O_2$ are thought to function as early signals for high light stress in plants (Pnueli et al., 2003; Rizhsky et al., 2003; Apel and Hirt, 2004; Mittler et al., 2004). Because Zat12 expression is enhanced in response to light stress (Iida et al., 2000; Davletova et al., 2005) and its expression in transgenic plants enhances their tolerance to high light stress (Iida et al., 2000; D. Davletova, J. Coutu, and R. Mittler, unpublished data), it is possible that Zat12 functions as part of a signal transduction pathway that directly or indirectly alters chloroplast gene expression in response to an H_2O_2 signal generated during light stress.

We previously reported that Zat12 is required for Apx1 expression during oxidative stress (Rizhsky et al., 2004a). However, Zat12 overexpression did not result in the enhanced expression of Apx1 (more than wild type) in the presence or absence of oxidative stress (Fig. 4). This result suggests that the relationship between Zat12 and Apx1 expression is complex and that further studies are required to resolve it. It is possible, for example, that Zat12 functions as part of a transcriptional or signal transduction complex required for Apx1 expression. In its absence, Apx1 expression is prevented during oxidative stress (Rizhsky et al., 2004a). However, simply increasing the level of Zat12 in cells might not be sufficient to increase Apx1 expression because other components or subunits of the complex might be rate limiting.

Our analyses (Figs. 1–3; Table II; Rizhsky et al., 2004a) and that of others (Iida et al., 2000; Vogel et al., 2005) suggest that Zat12 plays an important role in abiotic stress signaling in Arabidopsis. The enhanced tolerance to different stress conditions and the presumed activation of a collection of transcripts involved in the defense response of plants against light and oxidative stress in Zat12-expressing plants suggest that Zat12, or its orthologs, could be used for the development of stress-tolerant crops. In addition, the Zat12

promoter::luciferase fusion plants (Figs. 1 and 2) generated during our study could be used as a valuable tool for the isolation of stress- and ROS-response signaling mutants (Zhu, 2002). These could be used in future studies to identify pathways and receptors involved in stress and reactive oxygen perception and defense in plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Five-day-old Arabidopsis (*Arabidopsis thaliana* cv Columbia) seedlings were used for all stress assays described in this study. Seedlings were grown on 1%, 0.5× Murashige and Skoog (MS) agar plates or in sterile 0.5× MS media on a shaker in growth chambers (Percival E-30) under controlled conditions (21°C–22°C, 18 h or constant light cycle, 100 μmol m^{-2} s^{-1}, and a relative humidity of 70%; Rizhsky et al., 2004a; Davletova et al., 2005). Two independent homozygous knockout Arabidopsis lines containing T-DNA inserts in the Zat12 gene (KO-Zat12; SALK_037357 and SAIL 792_F04) and three independent homozygous lines of transgenic plants overexpressing Zat12 (OE-Zat12; lines 4, 12, and 17, all with similar expression level) were obtained as described previously (Rizhsky et al., 2004a). Transformation of Arabidopsis plants with the Zat12 promoter (1,000-bp fragment)::luciferase fusion was performed as described (Bent and Clough, 2000; Rizhsky et al., 2003), and transgenic plants were screened by RNA blots (Rizhsky et al., 2004a). Three independent lines expressing the Zat12 promoter (1,000-bp fragment)::luciferase fusion construct were used for Zat12 expression analysis (lines 4-4, 5-4, and 12-1). These were similar to wild-type plants in all aspects of plant growth and development (data not shown). All experiments were performed with three to five technical replications and repeated at least three times.

Molecular and Biochemical Analysis

RNA was isolated and analyzed by RNA blots as described previously (Rizhsky et al., 2004a; Davletova et al., 2005). RNA staining or a ribosomal 18S rRNA probe was used to control for RNA loading. Luciferase imaging was performed with a Kodak 2000MM image station. Plants were sprayed with 1 mM luciferine (Promega) prepared in water, incubated for 30 min, placed on the imager, and exposed for 3 to 5 min using the luminescence setting with a fully opened aperture.

Stress Assays

For the analysis of stress tolerance, seeds of wild-type and Zat12-perturbed lines (two independent Zat12 knockout and three independent Zat12-overexpressing lines) were surface sterilized with bleach and placed in rows on 1% agar plates (0.5× MS medium), containing different concentrations of methyl viologen, NaCl, sorbitol, or ferric-citrate (Sigma; Rizhsky et al., 2003, 2004a). Each row of seeds placed on a plate was divided into two parts: wild-type seeds, and seeds of transgenic plants overexpressing Zat12 or seeds of knockout Zat12 plants. Thus, the different seeds were placed side by side on the same plate. Plates were maintained vertically in a growth chamber (21°C–22°C, constant light, 100 μmol m^{-2} s^{-1}), and percentage of germination and root length were scored 5 d after seed plating. Four- or five-day-old seedlings grown on 0.5× MS agar plates were also subjected to heat stress (38°C), cold stress (10°C), or light stress (1,000 μmol m^{-2} s^{-1}). For DNA chip analysis, 5-d-old seedlings grown in 0.5× MS media were subjected to H_2O_2 (1, 5, 10, or 20 mM) stress (Rizhsky et al., 2003, 2004a). Wounding was performed as described previously (Rizhsky et al., 2004a). All stress experiments were performed with three to five technical replications, each containing 15 to 30 seeds per line, and repeated at least three times.

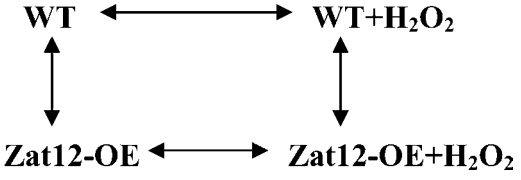
DNA Chip Analysis

In three independent experiments, RNA was isolated from control and H_2O_2 (20 mM, 1 h)-treated 5-d-old wild-type and Zat12-overexpressing seedlings (pooled from three independent lines) grown in 0.5× MS medium as described above. This treatment was not lethal to wild-type or Zat12-overexpressing lines as determined by continued growth of seedlings in culture (data not shown). A total of 12 RNA samples were used: three wild type untreated, three wild type treated with H_2O_2, three Zat12 overexpressing untreated, and three Zat12 overexpressing treated with H_2O_2. At least 150 seedlings were used per RNA sample, and RNA was isolated using Trizol (Davletova et al., 2005). RNA samples were used to perform chip hybridization analyses (Arabidopsis ATH1 chips; Affymetrix) at the Virginia Bioinformatics Institute Core Laboratory Gene Expression Facility (https://www.vbi.vt.edu/). Conditions for RNA isolation, labeling, hybridization, and data analysis are described (Davletova et al., 2005). Data visualization and analysis were performed with Silicon Genetics GeneSpring Version 5.1 and ArraAassit (IobionLab). Some of the results were confirmed by RNA blots. See details for data analysis below.

GeneChip Data Processing and Statistical Analysis

All GeneChip arrays were processed first by Robust Multi-Array Average (RMA; Irizarry et al., 2003) using the R package affy (Gautier et al., 2004). Expression values were computed from raw CEL files by first applying the RMA model of probe-specific correction of PM (perfect match) probes. These corrected probe values were then normalized via quantile normalization, and a median polish was applied to compute one expression measure from all probe values. Resulting RMA expression values were $\log_2$ transformed. These are standard methods for processing Affymetrix data (Davletova et al., 2005). Please see the affy manual at www.bioconductor.org/repository/devel/vignette/affy.pdf for details. A visual inspection of the distributions of raw PM probes values for all 12 arrays showed no outlying arrays; similarly, both density plots and boxplots of RMA expression value distributions of all arrays were very similar with no apparent outlying arrays. Curves describing trends in RNA degradation between the 5′ end and the 3′ end in each probe set were generated, and all 12 proved very similar, with an evident downward trend at the 5′ end. Pearson correlation coefficients and Spearman rank coefficients were computed on the RMA expression values (log base 2) for each set of biological triplicates. Spearman coefficients ranged from 0.985 to 0.997; Pearson coefficients ranged between 0.989 and 0.998 (for quality control measures, see Supplemental Figs. 3–8 and Supplemental Table I).

To determine whether genes were differentially expressed between genotypes across the temporal states, an ANOVA was performed on the RMA expression values. For an overview on the application of ANOVA to microarray data, see Kerr et al. (2000). The following model was used for this analysis: $y_{ijk} = V_i + (VT)_{ij} + \varepsilon_{ijk}$, where y_{ijk} denotes the $\log_2$ signal measured for variety i, treatment j, and biological replicate k, with $1 \le i \le 2$, $1 \le j \le 2$, and $1 \le k \le 3$. The terms V_i and T_j measure the effect of the variety and treatment, respectively, and the interaction term $(VT)_{ij}$ accounts for the interaction between variety and treatment. An ANOVA was performed on each gene using the linear model above, with four contrasts based on the comparisons between varieties and treatments as outlined in the diagram below.



These four comparisons were performed simultaneously, and a multiple testing correction (Benjamini and Hochberg, 1995) was applied to the P values of the test statistics to adjust the false discovery rate. Genes with adjusted P values < 0.05 were extracted for further analysis. This resulted in 5,417 genes with significant differential expression in at least one of the comparisons performed based on the ANOVA above. Expression values of this selection were then inverse-log transformed, and genes with differential expression in each of the four comparisons of more than 2-fold were selected. Specifically, 668 genes were found to be significantly differentially expressed by more than 2-fold in wild type compared to wild type treated with H_2O_2: 637 between Zat12 overexpressing and Zat12 overexpressing treated with H_2O_2; 48 between wild type and Zat12 overexpressing; and 54 between wild type treated with H_2O_2 and Zat12 overexpressing treated with H_2O_2. The R package limma was used for ANOVA methods (www.

bioconductor.org/repository/devel/vignette/affy.pdf). Microarray data from this experiment were submitted to NASCArrays at http://affymetrix.arabidopsis.info/.

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