THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 12, Issue of March 19, pp. 11736–11743, 2004

Printed in U.S.A.

The Zinc Finger Protein Zat12 Is Required for Cytosolic Ascorbate Peroxidase 1 Expression during Oxidative Stress in *Arabidopsis**[S]

Received for publication, December 7, 2003, and in revised form, January 8, 2004
Published, JBC Papers in Press, January 13, 2004, DOI 10.1074/jbc.M313350200

Ludmila Rizhsky‡, Sholpan Davletova§, Hongjian Liang‡, and Ron Mittler§¶

From the ‡Department of Botany, Plant Sciences Institute, Iowa State University, Ames, Iowa 50011 and the §Department of Biochemistry, University of Nevada, Reno, Nevada 89557

Cytosolic ascorbate peroxidase 1 (Apx1) is a key H_2O_2 removal enzyme in plants. Microarray analysis of Apx1-deficient *Arabidopsis* plants revealed that the expression of two zinc finger proteins (Zat12 and Zat7) and a WRKY transcription factor (WRKY25) is elevated in knock-out Apx1 plants grown under controlled conditions. Because mutants lacking Apx1 accumulate H_2O_2, we examined the correlation between H_2O_2 and the expression of Zat12, Zat7, WRKY25, and Apx1. The expression of Zat12, Zat7, and WRKY25 was simultaneously elevated in cells in response to oxidative stress (*i.e.* H_2O_2 or paraquat application), heat shock, or wounding. In contrast, light or osmotic stress did not enhance the expression of these putative transcription factors. All stresses tested enhanced the expression of Apx1. Transgenic plants expressing Zat12 or Zat7 could tolerate oxidative stress. In contrast, transgenic plants expressing WRKY25 could not. Although the expression of Zat12, Zat7, or WRKY25 in transgenic plants did not enhance the expression of Apx1 under controlled conditions, Zat12-deficient plants were unable to enhance the expression of Apx1, Zat7, or WRKY25 in response to H_2O_2 or paraquat application. Zat12-deficient plants were also more sensitive than wild type plants to H_2O_2 application as revealed by a higher level of H_2O_2-induced protein oxidation detected in these plants by protein blots. Our results suggest that Zat12 is an important component of the oxidative stress response signal transduction network of *Arabidopsis* required for Zat7, WRKY25, and Apx1 expression during oxidative stress.

Plants are sessile organisms that evolved a complex and specialized network of regulatory genes to control their response to changes in environmental conditions. It is likely that many of these regulatory genes were initially created by gene duplication and that they later acquired roles specifically related to individual pathways or stresses as well as their combination (1, 2). Different members of gene families, such as WRKY and other zinc finger proteins (72 *WRKY* genes and over 600 zinc finger proteins in *Arabidopsis*; Ref. 3), MYB transcription factors (133 genes in *Arabidopsis*; Ref. 4), and heat shock transcription factors (21 genes in *Arabidopsis*; Ref. 5), were found to control and regulate diverse processes in plants ranging from development to response to biotic or abiotic stresses (1–5).

The different regulatory networks of plants are also involved in modulating the production and scavenging of reactive oxygen species (ROS)[1] in cells. These toxic intermediates of oxygen reduction not only control different plant responses to environmental and developmental cues but also potently inhibit essential metabolic pathways and may lead to cell death (6–9). Although a number of different enzymes and proteins produce or scavenge ROS in cells, little is known about how the different regulatory networks of plants control these enzymes and proteins and modulate the steady-state level of ROS (8–10). The steady-state level of a number of different transcripts encoding transcription factors such as MYB, WRKY, heat shock transcription factors, and different zinc finger proteins is elevated in plants in response to different forms of ROS-induced stress (11–14). However, genetic evidence supporting a direct regulatory role for these transcripts was only presented for two zinc finger proteins, Lsd1 and Lol1, which were recently found to mediate ROS signals and control programmed cell death in *Arabidopsis* (6), and for heat shock transcription factor 3, which was shown to enhance cytosolic ascorbate peroxidase (Apx) expression in the absence of stress (15)

We are studying the response of plants to overaccumulation of ROS in cells (*i.e.* oxidative stress; Ref. 9). Our goal is to identify and characterize the transcription factor network that controls the response of plants to oxidative stress. To dissect and study the ROS signal transduction network of plants, we are using knock-out plants deficient in key ROS-scavenging enzymes (13, 14). These plants provide an ideal experimental system to study plant responses to ROS accumulation, because they accumulate ROS and activate multiple defense mechanisms in the absence of externally applied stimuli such as stress, ROS, or ROS generators. Moreover, the ROS that accumulate in these mutants are ROS naturally produced in cells at the different cellular ROS-producing sites and not externally applied ROS that may activate additional signaling pathways, including pathogen or abiotic stress response pathways (13, 14). Knock-out plants deficient in cytosolic Apx1 are of particular interest. They maintain a high steady-state level of H_2O_2 in cells and activate ROS defense mechanisms when grown under controlled conditions (13). These plants are also altered

* This work was supported by National Science Foundation Grant NSF-0343866 and funding from the Plant Sciences Institute at Iowa State University, the Biotechnology Council of Iowa State University, the College of Liberal Arts and Sciences at Iowa State University, and the Nevada Agricultural Experimental Station (NAES publication number 03031429). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Supplementary Table I, which lists all transcripts elevated in transgenic plants expressing Zat12.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, University of Nevada, Mail Stop 330, Reno, NV 89557. Tel.: 775-784-1384; Fax: 775-784-1419; E-mail: ronm@unr.edu.

[1] The abbreviations used are: ROS, reactive oxygen species; Apx, ascorbate peroxidase; CaMV, cauliflower mosaic virus; KO, knock-out; MAPK, mitogen-activated kinase; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

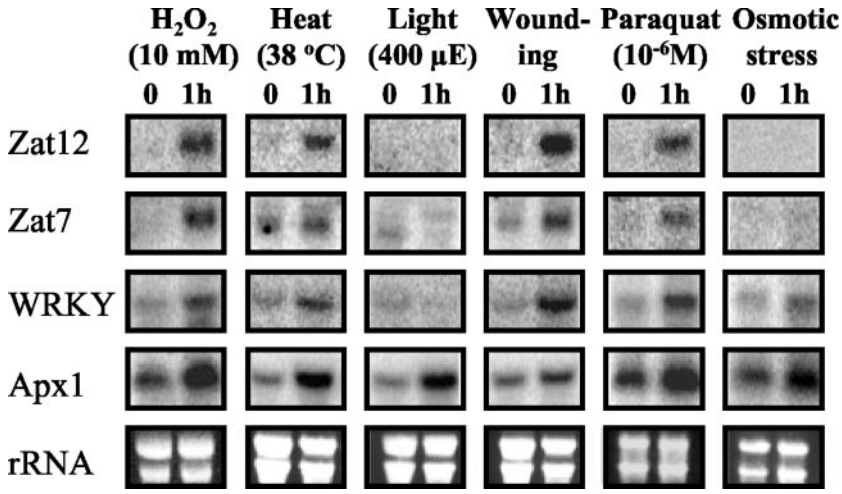

FIG. 1. **Changes in the steady-state level of transcripts encoding Zat12, Zat7, WRKY25, and Apx1 in response to abiotic stress or oxidative stress.** RNA blots showing the expression of Zat12, Zat7, WRKY25, and Apx1 in response to oxidative stress (applied by H_2O_2 or paraquat) or different abiotic stresses (heat shock, light stress, wounding, and osmotic stress) are presented. Stress treatments and molecular analysis were performed as described under "Experimental Procedures."

in their growth, flowering time, and stomatal responses and display an augmented induction of heat shock proteins and catalase in response to light stress.

Microarray analysis of knock-out Apx1 plants grown under controlled conditions revealed that the expression of at least two different zinc finger proteins (Zat7 and Zat12), a putative WRKY transcription factor (WRKY25), and a number of heat shock transcription factors is elevated in these plants (13). The expression of Zat12 is also elevated in cultured *Arabidopsis* cells in response to H_2O_2 application (12) and in mature *Arabidopsis* plants in response to cold stress, wounding, or high light stress (Refs. 16–18; stresses that result in ROS accumulation in cells). No direct link was reported between Zat12 expression and the expression of different ROS-scavenging transcripts such as those encoding Apx1. This finding stands in contrast to the established relationship between Apx1 expression and heat shock transcription factors (13, 15). The elevated expression of Zat12, Zat7, and WRKY25 in Apx1-deficient plants suggests a link between H_2O_2 accumulation, the expression of these putative regulatory genes, and Apx1 expression. In this study we examined the relationship between Zat12, Zat7, and WRKY25 expression, oxidative stress, and Apx1 expression. Our results suggest that Zat12 is essential for Zat7, WRKY25, and Apx1 expression during oxidative stress and that Zat12, Zat7, and WRKY25 are linked to H_2O_2 stress in plants.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—*Arabidopsis thaliana* (cv. Columbia and WS) plants were grown in growth chambers (Percival E-30) under controlled conditions (21–22 °C, 18 h or constant light cycle, 100 μmol m^{-2} sec^{-1}, and a relative humidity of 70%). Knock-out *Arabidopsis* lines containing a T-DNA insert in the *Zat12* gene (KO-Zat12; obtained through the SIGnAL project; signal.salk.edu/tabout.html) were outcrossed and selfed to check for segregation and to obtain pure homozygous lines as described (13, 14). Analysis of Zat12 knock-out and segregation was performed by PCR and genomic DNA blots (13). Screening for expression of Zat12 by RNA blots was performed with leaf tissues obtained from wounded and control wild type and knock-out plants 1 h following wounding. Wounding of plants was performed with needles as described (19). Transformation of *Arabidopsis* plants was performed as described (14, 20), and transgenic plants were screened by RNA blots (14). All experiments were performed in triplicate and repeated at least three times (with the exception of DNA arrays, which were performed as described below).

Molecular and Biochemical Analysis—RNA and protein were isolated and analyzed by RNA and protein blots as described previously (13, 14). Detection of protein oxidation was performed with the OxyBlot protein oxidation kit (Chemicon International, Temecula, CA) as recommended by the manufacturer. The identity of the major protein bend oxidized in KO-Zat12 plants was determined by immunoprecipitation with an antibody against Rubisco as described (21). RNA staining or a ribosomal 18 S rRNA probe were used to control for RNA loading. Coomassie Blue staining of protein gels was used to control for protein loading. Zat12 (At5g59820), Zat7 (At3g46080), and WRKY25 (At2g30250) detection by RNA blots was performed with gene-specific probes. Full-length clones for WRKY25 were obtained from RIKIN (www.brc.riken.go.jp/lab/epd/Eng/index.html), and full-length cDNA clones for Zat7 and Zat12 were cloned by reverse transcription PCR using mRNA prepared from cells 1 h following wounding (13, 19). Clones were sequenced and compared with genomic sequences of Zat12 and Zat7. Gene-specific probes (~200bp) were prepared by PCR according to (22) using the following primers: Zat12 left, 5′-CACAAACCACAAGAGGATCATTTC-3′ and Zat12 right, 5′-GACGTTTTCACCTTCTTCATCAAT-3′; Zat7 left, 5′-TCAAAACCCTAG AAGTCACTA-3′ and Zat7 right, 5′-CAAGAAGTGATGGATTGTC AC-3′; and WRKY25 left, 5′-AGAAATCTTAAAGTTGTCTCCTTT-3′ and WRKY25 right, 5′-TGGAAACGTTCCTGTTGTTGGAG-3′.

DNA Chip Analysis—In two independent experiments, RNA was isolated from 40–50 wild type, KO-Zat12, or KO-Apx1 plants (a total of 80–100 plants per line, in triplicate) grown under controlled conditions as described above. This RNA was used to perform chip analyses (*Arabidopsis* ATH1 chips; Affymetrix, Santa Clara, CA) at the University of Iowa DNA facility (dna-9.int-med.uiowa.edu/microarrays.htm). Conditions for RNA isolation, labeling, hybridization, and data analysis are described (13, 14). Comparative analysis of samples was performed with the GeneChip mining tool version 5.0 and the Silicon Genetics GeneSpring V 5.1. Some of the comparison results were confirmed by RNA blots.

Stress Assays—For the analysis of oxidative stress tolerance of transgenic plants constitutively expressing Zat12, Zat7, and WRKY25, seeds of wild type and transgenic lines were surface-sterilized with bleach and placed in rows on 1% agar plates (0.5× Murashige and Skoog medium) containing different concentrations of paraquat (Sigma). Each row of seeds placed on a plate was divided into two parts, wild type seeds and seeds of transgenic plants. Thus, the different seeds were placed side by side on the same plate. Plates were maintained vertically in a growth room (21–22 °C, constant light, and 80–100 μmol m^{-2} sec^{-1}), and the percentage of germination and root length were scored 5 days after seed sterilization and plating. Heat shock and light stress were performed as described (13). Osmotic stress and H_2O_2 and paraquat stresses were performed by subjecting 5-day-old seedlings grown in liquid culture (0.5× Murashige and Skoog medium, 21–22 °C, and 100 μmol m^{-2} sec^{-1}) to polyethylene glycol (PEG 6000, 0–2%), paraquat (0–1 μM), or H_2O_2 (0–20 mM) for 0, 0.5, 1, and 2 h.

RESULTS

Expression Analysis of Zat12, Zat7, and WRKY25—To examine the correlation between Zat12, Zat7, WRKY25, and Apx1 expression in response to oxidative stress or different abiotic stresses, we subjected wild type plants to H_2O_2 stress, heat shock, a moderate level of light stress (400 μmol m^{-2} sec^{-1}), wounding, paraquat (a superoxide-generating agent), and osmotic stress. As shown in Fig. 1, the steady-state level of transcripts encoding Zat12, Zat7, and WRKY25 was elevated in cells in response to H_2O_2, heat shock, wounding, or paraquat

TABLE I

Putative regulatory sequences found in the promoters of Zat12, Zat7, WRKY25, and Apx1

Only putative regulatory elements found in two or more of the promoters analyzed are included. Putative elements involved in light responses (found in all promoters) are not included in the table. Promoter sequences were analyzed with the PlantCARE software (sphinx.rug.ac.be:8080/PlantCARE/cgi/index.html). The length of promoter sequences included in the analysis was as follows: *Zat12*, 2512 bp; *Zat7*, 2486 bp; *WRKY25*, 1771 bp; and *Apx1*, 1841 bp.

Gene and corresponding regulatory elements				Function of regulatory element
Zat12	*Zat7*	*WRKY25*	*Apx1*	
A-box	A-box	A-box	A-box	Common to α-amylase promoters
ABRE	ABRE		ABRE	ABA response
AuxRR		AuxRR	AuxRR	Auxin respone
As1	As1			Root expression
CGTCA-m	CGTCA-m		CGTCA-m	MeJA response
ELI-box3	ELI-box3	ELI-box3	ELI-box3	Elicitor response
ERE	ERE	ERE	ERE	Ethylene response
	EIRE	EIRE	EIRE	Elicitor response
GCN4-m	GCN4-m		GCN4-m	Endosperm expression
HSE	HSE	HSE	HSE	Heat shock response
LTR	LTR			Low temp response
MRE	MRE	MRE	MRE	MYB binding
MSA		MSA	MSA	Cell cycle
P box	P box	P box	P box	Gibberellin response
Prolamin box		Prolamin box		Endosperm expression
Skn-1	Skn-1	Skn-1	Skn-1	Endosperm expression
TATC box	TATC box	TATC box	TATC box	Gibberellin response
TCA element	TCA element	TCA element	TCA element	Salicylic acid response
TGA box	TGA box	TGA box		Auxin response
TGACG-m	TGACG-m	TGACG-m	TGACG-m	MeJA response
WUN-m	WUN-m	WUN-m	WUN-m	Wound-response

application. In contrast, a moderate level of light stress or osmotic stress did not enhance the expression of these transcripts. The level of transcripts encoding Apx1 was elevated by all treatments, suggesting specifically that oxidative stress (H_2O_2 or paraquat), wounding, and heat shock (Fig. 1) present a clear correlation between Zat12, Zat7, WRKY25, and Apx1 expression. Analysis of the promoter regions of *Zat12*, *Zat7*, and *WRKY25*, performed with the plantCARE software (sphinx.rug.ac.be:8080/PlantCARE/cgi/index.html), revealed that a number of putative DNA binding sites are common among all three genes and Apx1 (Table I). These include a number of light response elements (not shown in Table I), methyl jasmonate (MeJA), ethylene, gibberellin, and salicylic acid response elements, a wound response element (WUN), a MYB-binding site, and the heat shock transcription factor binding site (heat shock element or HSE; Table I). In addition, two conserved motifs with an unknown function (5′-GAG**ACGCGGT**GACAC-3′ and 5′-T**C**G**T**CC**CAGCC**-3′) were identified in the promoters of *Zat12*, *Zat7*, *WRKY25*, and *Apx1* using the MEME software (meme.sdsc.edu/meme/website/intro.html). Because these motifs were found in the promoters of all four genes, it is possible that they are involved in regulating the expression of these genes during abiotic stress or oxidative stress (Fig. 1). In addition, the expression of *Zat12*, *Zat7*, *WRKY25*, and *Apx1* during wounding or heat shock could be explained by the presence of the wound response element (WUN) or heat shock element (HSE) binding sites in the promoters of these genes. Interestingly, the expression of Zat12 was not elevated in response to a moderate level of light stress (400 μmol m^{-2} sec^{-1}). This result conflicts with a previous report on the expression of Zat12 during high light stress (400–1800 μmol m^{-2} sec^{-1}; Ref. 18) and with the presence of many different light response elements in the promoter of Zat12 (light response elements in the promoters of Zat7 and WRKY25 were also found to be non-responsive to the same moderate light stress treatment; Fig. 1).

Time course analysis of Zat12, Zat7, WRKY25, and Apx1 expression during H_2O_2 stress (Fig. 2) revealed that the steady-state level of transcripts encoding Zat12, Zat7, and WRKY25 is elevated in plants prior to the elevation in Apx1 expression. In addition, the expression of Zat12, Zat7, and WRKY25 was transient and declined within 2 h of H_2O_2 application. The expression of Zat12 was found to further decline to an undetectable level 4 h following the application of H_2O_2 (not shown). These findings are in accordance with previous reports on the transient expression of Zat12 during cold stress, wounding, and anoxic stress (16, 17, 23) and the transient expression of Zat7 during wounding (16).

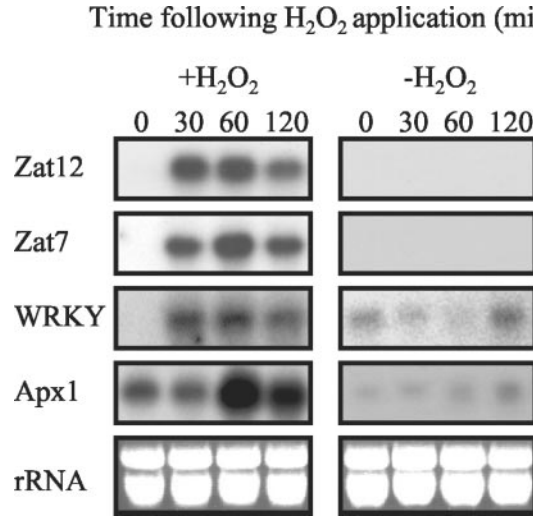

FIG. 2. **Time course analysis of Zat12, Zat7, WRKY25, and Apx1 expression during oxidative stress.** RNA blots showing the expression of Zat12, Zat7, WRKY25, and Apx1 during oxidative stress applied by H_2O_2. H_2O_2 stress and molecular analysis were performed as described under "Experimental Procedures."

Analysis of Zat12, Zat7, and WRKY25 in Transgenic Plants—To test the function of Zat12, Zat7, and WRKY25 in plants, we expressed full-length cDNA clones for these putative transcription factors in transgenic plants. For this purpose, we used the 35S-CaMV promoter. We then tested the tolerance of seedlings obtained from transgenic plants that constitutively express Zat12, Zat7, or WRKY25 to oxidative stress using a plate assay that measures root length and percentage of ger-

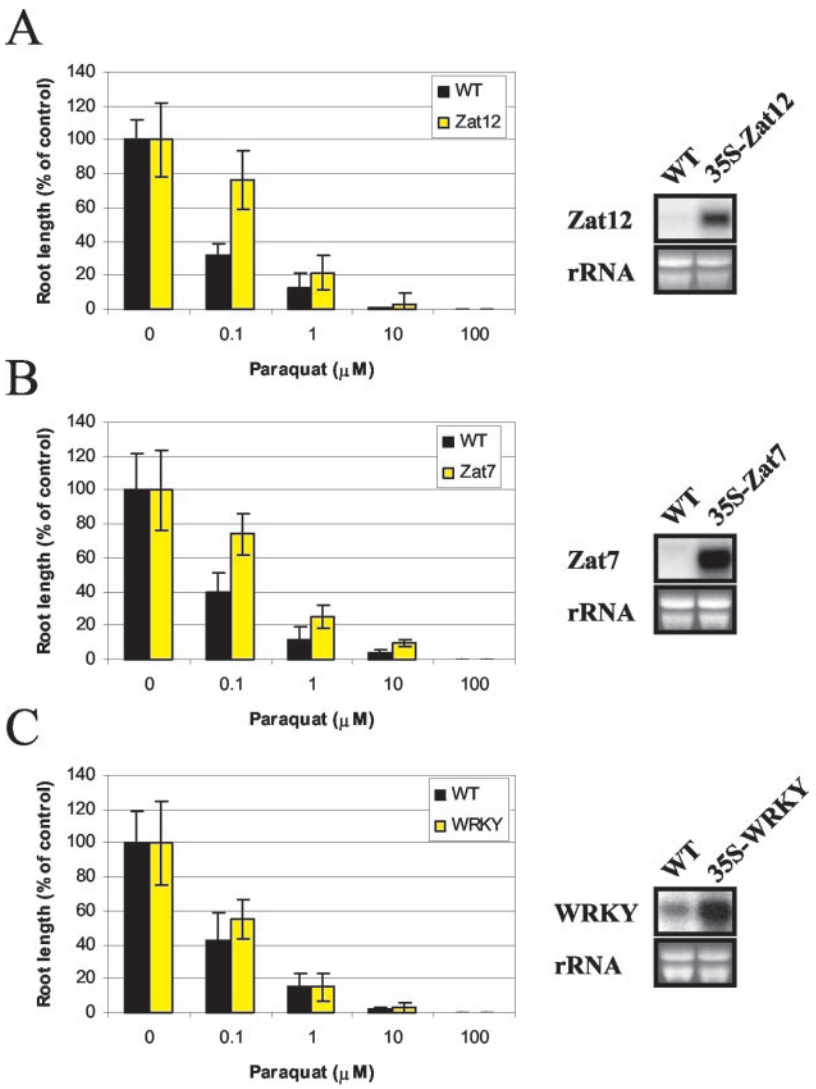

FIG. 3. **Tolerance of seedlings of transgenic plants that constitutively express Zat12 or Zat7 to oxidative stress.** *A*, *left*, root length of 5-day-old wild type (*WT*) and transgenic seedlings expressing Zat12 germinated on agar plates in the presence or absence of the superoxide-generating agent paraquat. *Right*, RNA blots showing the expression of Zat12 in transgenic plants grown under controlled conditions. *B* and *C*, similar to *panel A* but with transgenic seedlings and plants expressing Zat7 (*B*) or WRKY25 (*C*). Production of transgenic plants, oxidative stress assays, and molecular analysis were performed as described under "Experimental Procedures."

mination of seedlings in the presence or absence of the superoxide-generating agent paraquat (14). As shown in Fig. 3, seedlings of transgenic plants expressing Zat12 or Zat7 were more tolerant than seedlings of wild type plants to the oxidative stress applied in this assay. In contrast, seedlings of transgenic plants expressing WRKY25 were not more tolerant than seedlings of wild type plants to this treatment. These results were obtained with at least two independent transgenic lines for each of the different putative transcription factors.

Using RNA blots, we tested the expression of Zat12, Zat7, WRKY25, and Apx1 in all transgenic lines grown under controlled growth conditions. As shown in Fig. 4*A*, constitutive expression of Zat12, Zat7, or WRKY25 in the different transgenic plants did not result in the elevated expression of Apx1 or any of the three putative transcription factors not controlled by the 35S-CaMV promoter. This result suggests that, under the controlled conditions we used to grow plants, the different putative transcription factors expressed in each of the different lines were unable to elevate the expression of each other or the expression of Apx1. A very high level of expression of Zat7, obtained in certain lines at the homozygous state, resulted in a delayed growth and development phenotype (Fig. 4*B*). However, this level of expression did not elevate the expression of Zat12 or WRKY25 and suppressed the expression level of Apx1 (Fig. 4*B*). A similar phenotype was not observed in transgenic plants expressing Zat12 or WRKY25 (not shown).

Microarray Analysis of Transgenic Plants Constitutively Expressing Zat12—Because Zat12 expression enhanced the tolerance of plants to oxidative stress (Fig. 3) but did not result in the enhanced expression of Zat7, WRKY25, or Apx1 (Fig. 4*A*), we examined transgenic plants constitutively expressing Zat12 by microarrays to identify transcripts that may be involved in the response of plants to ROS (elevated in Zat12-expressing plants in the absence of an external stimuli). For these studies, we used leaf tissues of 2-week-old plants grown under controlled conditions and compared the pattern of transcript expression between transgenic plants expressing Zat12, wild type plants, and knock-out plants deficient in Apx1 (KO-Apx1). As shown in Table II, ten different transcripts that were elevated in transgenic plants expressing Zat12 were also elevated in KO-Apx1 plants (cutoff 0.8 $\log_2$-fold; transcripts elevated in Zat12-expressing plants as well as in KO-Apx1 plants are indicated in boldfaced type). It is possible that the expression of these transcripts in Apx1-deficient plants is controlled by Zat12. Our microarray experiments further confirmed that the expression of Zat7, WRKY25, or Apx1 is not elevated in transgenic plants expressing Zat12 (Table II; Fig. 4*A*). The steady-state level of a number of transcripts with a putative signaling function was elevated in transgenic plants expressing Zat12. These included a monomeric G-protein, MAPK kinase 4, a number of putative transcription factors (*i.e.* TINY, MYB, and zinc finger proteins), two different kinases, and a calcium-

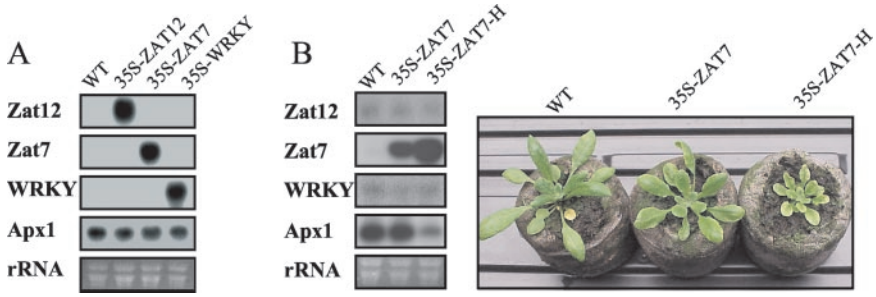

FIG. 4. **Expression of Zat12, Zat7, WRKY25, and Apx1 in transgenic plants expressing Zat12, Zat7, or WRKY25.** *A*, RNA blots showing the expression of Zat12, Zat7, WRKY25, and Apx1 in transgenic plants expressing Zat12, Zat7, or WRKY25. *B*, *left*, RNA blots showing the expression of Zat12, Zat7, WRKY25, and Apx1 in different transgenic lines expressing Zat7 (*35S-Zat7* and *35S-Zat7H* indicate moderate and high expression of Zat7, respectively). *Right*, a photograph of wild type (*WT*) and transgenic plants expressing Zat7 at different levels. Production of transgenic plants and molecular analysis were performed as described under "Experimental Procedures."

binding protein. Transcripts related to ROS metabolism enhanced in plants constitutively expressing Zat12 included superoxide-generating NADPH oxidase, peroxidase 2a, and glutathione *S*-transferase. Transcripts related to pathogen response and auxin, ethylene, and methyl jasmonate signaling were also elevated in Zat12-expressing plants (Table II). Supplementary Table I, available in the on-line version of this article, lists all transcripts elevated in transgenic plants expressing Zat12 (cutoff 0.5 $\log_2$).

Analysis of Knock-out Plants for Zat12—To test the function of Zat12 in plants during oxidative stress, we obtained and purified Zat12-deficient knock-out lines. When grown under controlled conditions, Zat12-deficient plants were similar in their growth and appearance to wild type plants (not shown). However, when the oxidative stress response of KO-Zat12 plants was compared with that of wild type plants (tested with H_2O_2 or paraquat), it was found that the steady-state level of transcripts encoding Zat7, WRKY25, and Apx1 was not elevated in KO-Zat12 plants during oxidative stress (Fig. 5*A*). The expression of Apx1 was, however, elevated in KO-Zat12 plants in response to a moderate level of light stress (Fig. 5*A*).

To test whether the suppression of Zat7, WRKY25, and Apx1 during oxidative stress (Fig. 5*A*) resulted in greater damage to cells during oxidative stress, we used a protein blot approach to detect protein oxidation in plants. We first tested protein oxidation in wild type plants during H_2O_2 stress (10 mM, 1 h), and found that the major protein bend oxidized in *Arabidopsis* seedlings subjected to this treatment corresponds in its molecular weight to that of the large subunit of Rubisco. Immunoprecipitation assays of Rubisco in protein extracts obtained from treated and untreated plants confirmed that the oxidized protein is indeed Rubisco[2] (not shown; see Ref. 24 for a detailed study of Rubisco protein oxidation during oxidative stress). As shown in Fig. 5*B*, the H_2O_2-induced oxidation of a Rubisco large subunit in KO-Zat12 plants was higher than that in wild type plants subjected to the same H_2O_2 stress. This finding suggests that the lack of Apx1 expression in knock-out Zat12 plants during oxidative stress results in greater oxidative damage to Rubisco protein, providing further evidence that cytosolic Apx1 is involved in protecting the chloroplast from oxidative stress (9, 13).

DISCUSSION

Enhanced expression of transcripts encoding different regulatory proteins, *e.g.* 2-component histidine kinase, different receptor-like protein kinases, WRKY transcription factors, calcium-binding proteins, calmodulin-like proteins, and MAPKs, was associated with oxidative stress in plants (11–14). However, genetic evidence supporting a regulatory role for many of these proteins during oxidative stress was not presented. In *Arabidopsis*, two different MAP kinases (MAPK3 and MAPK6) were shown to be involved in H_2O_2 responses, and two different zinc finger proteins (Lsd1 and Lol1) were shown to have an antagonistic effect on cytosolic copper/zinc-superoxide dismutase expression during pathogen response (6, 25). In addition, constitutive expression of a heat shock transcription factor (HSF3) in transgenic *Arabidopsis* plants was shown to enhance the expression of Apx1 and Apx2 in the absence of stress (15). Here, we report that the zinc finger protein Zat12 is required for cytosolic Apx1 expression during oxidative stress (Fig. 5). Furthermore, we show that Zat12 is also essential for the expression of Zat7 and WRKY25 and that these putative transcription factors are involved in the response of plants to oxidative stress (Figs. 3 and 5). The elevation in Zat12, Zat7, and WRKY25 expression in cells prior to the elevation in Apx1 expression during oxidative stress (Fig. 2) and the lack of Zat7, WRKY25, and Apx1 expression during oxidative stress in knock-out Zat12 plants (Fig. 5) provide strong evidence that Zat12, Zat7, and WRKY25 are integral components of the oxidative stress response signal transduction pathway of *Arabidopsis*. Based on our findings (Fig. 5), we propose that Zat12 acts upstream of Zat7, WRKY25, and Apx1 on the ROS signal transduction pathway of *Arabidopsis* (Fig. 6).

Interestingly, constitutive expression of Zat12, Zat7, or WRKY25 did not enhance the expression of Apx1 in the absence of stress (Fig. 4*A*). This result suggests that an additional factor(s), unknown at present, may be required to enable the expression of Apx1 in these plants. This factor may only be present in cells during oxidative stress (Fig. 6). Because Zat12, Zat7, and WRKY25 are transiently induced in cells during stress (Refs.16–18; Fig. 2), it is possible that their expression is coordinated with that of other factors transiently induced during stress and that the absence of these factors in transgenic plants grown under controlled conditions prevented the induction of Apx1 (see also Ref. 26 for a discussion on expressing inducible transcription factors in plants). To test this possibility, we applied H_2O_2 stress to wild type plants and transgenic plants that constitutively express Zat12 (similar to the treatment shown in Fig. 2), and compared the expression of Apx1 between wild type plants and transgenic plants that constitutively express Zat12. However, the expression of Apx1 in transgenic plants constitutively expressing Zat12 in response to oxidative stress was only slightly higher than that of wild type plants (1.5–2-fold higher than wild type; not shown). Further studies are therefore required to identify the factors involved in Apx1 expression during stress and determine whether constitutive expression of Zat12, Zat7, and/or WRKY25 in transgenic

[2] R. Mittler, L. Rizhsky, S. Davletova, and H. Liang, manuscript in preparation.

TABLE II

Transcripts elevated in transgenic plants constitutively expressing Zat12

Results are presented as fold difference in steady-state transcript level ($\log_2$) over control wild type plants (cutoff 0.8 $\log_2$). Transcripts indicated in boldfaced type are also elevated in knock-out plants lacking Apx1. Wild type plants, transgenic plants expressing Zat12, and Apx1-deficient plants were grown under controlled conditions. Changes in transcript abundance were measured in leaves of 2-week-old plants with Affymetrix chips. All measurements were performed as described under "Experimental Procedures." NLS, nuclear localization signal.

Gene number	Fold increase ($\log_2$)		Transcript name
	Exp1	Exp2	
At5g59820	5.1	5.2	Zinc finger protein Zat12
At4g32800	4.5	4.4	Transcription factor TINY
At5g38550	4.4	4.1	Myrosinase binding protein, jasmonate-induced
At2g44070	3.8	3.9	Translation initiation factor eIF-2Bδ
At1g15580	3.1	3	Auxin-induced protein IAA5
At4g23680	2.4	2.7	Major latex protein type 1
At1g09080	2.3	2.2	Luminal-binding protein
At1g22650	2.2	2.1	Putative invertase
At4g30270	1.7	1.6	Xyloglucan endo-1,4-β-D-glucanase
At3g01850	1.6	1.6	D-Ribulose-5-phosphate 3-epimerase
At4g20860	1.6	1.5	Berberine bridge-like
At5g54840	1.6	1.4	SGP1 monomeric G-protein
At1g35140	1.6	1.4	Phosphate-induced (phi-1) protein
At2g27190	1.5	1.3	Purple acid phosphatase
At4g16770	1.5	1.2	Gibberellin oxidase-like
At1g51660	1.4	1.4	MAPK kinase 4
At4g38600	1.4	1.3	Putative NLS receptor
At2g26560	1.4	1.3	Latex allergen
At2g37760	1.3	1.3	Alcohol dehydrogenase
At3g27170	1.3	1.3	**CLC-b chloride channel**
At5g58770	1.3	1.2	Dehydrodolichyl diphosphate
At5g09220	1.3	1.1	Amino acid transport protein AAP2
At1g23020	1.2	1.1	Superoxide-generating NADPH oxidase
At2g35060	1.1	1.1	Potassium transporter
At2g37130	1.1	1.1	Peroxidase ATP2a
At1g04250	1.1	1.1	Auxin-induced protein IAA17
At1g49160	1.1	1	Serine/threonine protein kinase
At1g52880	1.1	1	NAM-like protein
At1g29460	1.1	1	Auxin-induced protein
At3g04720	1.1	1	Hevein-like protein (PR-4)
At3g09600	1.1	1	MYB-related protein
At2g47070	1	1	Squamosa promoter-binding protein
At2g21060	1	1	Glycine-rich protein (AtGRP2)
At2g24850	1	1	Tyrosine aminotransferase
At1g56120	1	1	Wall-associated kinase 2
At3g26590	1	0.9	Integral membrane protein
At4g11280	1	0.9	**ACC synthase (AtACS-6)**
At4g19420	1	0.9	Pectinacetylesterase
At3g47420	1	0.9	*sn*-Glycerol-3-phosphate permease
At5g37540	1	0.9	Nucleoid DNA-binding protein cnd41, chloroplast
At5g39670	1	0.9	Calcium-binding protein
At5g62000	1	0.9	**Auxin response factor**
At2g46830	0.9	0.9	**MYB transcription factor (CCA1)**
At2g29650	0.9	0	Na^+-Dependent phosphate cotransporter
At1g78670	0.9	0.9	γ-Glutamyl hydrolase
At1g06570	0.9	0.9	4-Hydroxyphenylpyruvate dioxygenase
At3g26280	0.9	0.9	Cytochrome P450 monooxygenase
At3g18290	0.9	0.9	**Zinc finger protein**
At3g26450	0.9	0.9	Major latex protein
At1g17990	0.9	0.9	12-Oxophytodienoate reductase
At4g08290	0.9	0.9	Nodulin-like protein
At4g19170	0.9	0.9	Neoxanthin cleavage enzyme
At4g37560	0.9	0.9	**Formamidase**
At3g55610	0.9	0.9	**δ-1-Pyrroline-5-carboxylate synthetase**
At5g57190	0.9	0.8	Phosphatidylserine decarboxylase
At5g64800	0.9	0.8	CLAVATA3/ESR-related 21
At5g28020	0.9	0.8	Cysteine synthase
At4g15530	0.9	0.8	Pyruvate,orthophosphate dikinase
At4g16990	0.9	0.8	**RPP5-like protein**
At2g46340	0.8	0.8	Photomorphogenesis repressor protein
At2g21320	0.8	0.8	CONSTANS-like B-box zinc finger
At1g02930	0.8	0.8	Glutathione *S*-transferase
At1g64500	0.8	0.8	Peptide transporter
At1g21910	0.8	0.8	TINY-like protein
At1g73220	0.8	0.8	Similar to organic cation transporter 3
At1g75040	0.8	0.8	Thaumatin-like protein
At1g71030	0.8	0.8	**Similar to MYB-related transcription factor 24**
At1g13260	0.8	0.8	**DNA-binding protein RAV1**

plants would result in enhanced expression of Apx1 in cells in the absence of stress.

Constitutive expression of Zat12 resulted in the elevated expression of different transcripts involved in ROS metabolism and hormonal signaling (Table II). The enhanced expression of transcripts encoding an NADPH oxidase gene in Zat12-ex-

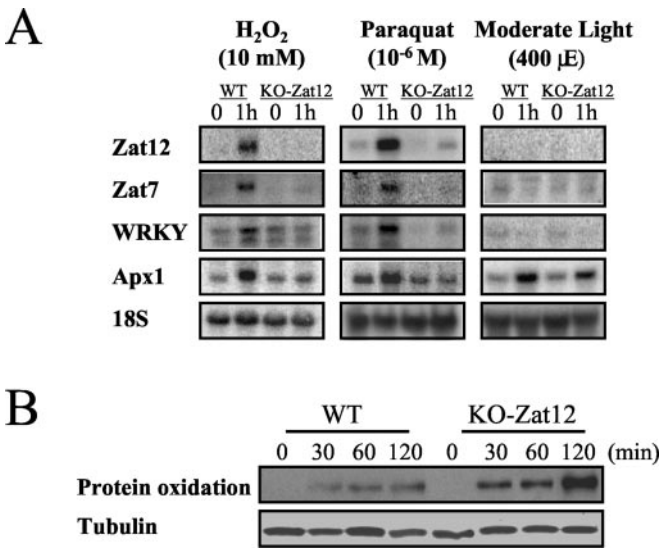

FIG. 5. **Expression of Zat12, Zat7, WRKY25, and Apx1 in knockout plants lacking Zat12 in response to oxidative stress.** *A*, RNA blots comparing the expression of Zat12, Zat7, WRKY25, and Apx1 between wild type plants (*WT*) and Zat12-deficient plants (*KO-Zat12*) in response to oxidative stress (H_2O_2 or paraquat application) or light stress. *B*, a protein oxidation blot showing the level of Rubisco large subunit oxidation during H_2O_2 stress in wild type and KO-Zat12 plants (protein oxidation; *top*) and a regular protein blot showing the level of tubulin in the different samples (used to control for protein loading; *bottom*). Stress treatments and biochemical and molecular analysis were performed as described under "Experimental Procedures."

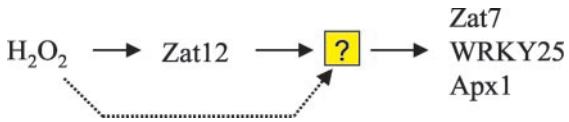

FIG. 6. **A hypothetical model for Zat12 function in plants.** Zat12 expression is shown to be enhanced by H_2O_2 (*solid arrow*). However the enhancement of Zat7, WRKY25, and Apx1 expression by Zat12 (*solid arrows*) requires additional factors (indicated by *question mark*), most likely enhanced in cells in response to oxidative stress (*dotted arrow*). Therefore, in the absence of stress, constitutive expression of Zat12 in plants does not enhance the expression of Zat7, WRKY25, or Apx1 (Fig. 4).

pressing plants may suggest that Zat12 can facilitate the production of ROS in cells. Because NADPH oxidases were shown to regulate the response of plants to different biotic, abiotic, and developmental signals via enhanced production of ROS in cells (27–29), the finding that Zat12 enhances the expression of an NADPH oxidase may suggest that NADPH oxidases are also involved in regulating the response of plants to oxidative stress. The possibility that NADPH oxidases and ROS are used to regulate the response of plants to ROS stress should be tested in future studies, because it suggests that limited and localized production of ROS, and not a global enhancement in the steady-state level of ROS in cells, is required to trigger the defense response of plants against oxidative stress. The expression of the same NADPH oxidase gene elevated in Zat12-expressing plants (At1g23020; Table II), was also found to be elevated in plants subjected to cold or salt stress, stresses that enhance the expression of Zat12, as well as the expression of different ROS-scavenging enzymes (30). These findings further support the hypothesis that Zat12 expression can enhance the expression of this NADPH gene during abiotic stress.

Compared with seedlings of wild type plants or plants expressing WRKY25, seedlings of plants expressing Zat12 or Zat7 were tolerant to oxidative stress applied on agar plates by paraquat (Fig. 3). In a previous study, seedlings of tobacco plants expressing a constitutively activated form of the oxidative stress signal transduction protein ANP1, a MAPK similar to MAPK3/6 in *Arabidopsis*, were found to be more tolerant than wild type seedlings to different abiotic stresses such as freezing, heat shock, and salt stress (25). Our findings suggest that additional components of the oxidative stress signal transduction pathway of *Arabidopsis* could be used in a similar manner to enhance the tolerance of plants to oxidative stress. Because Zat12 expression in transgenic plants did not activate multiple defense mechanisms in plants in the absence of stress (Table II) and did not result in a deleterious side effect on plant growth and yield (not shown), Zat12 may be an ideal signal transduction protein to express in plants and enhance their tolerance to oxidative stress or, potentially, other abiotic stresses. Further studies examining the tolerance of Zat12- and Zat7-expressing plants to different abiotic stresses may reveal whether these proteins could be used for different biotechnological applications such as the enhancement of plant tolerance to biotic or abiotic stress.

In contrast to many of the different transcription factors characterized in plants, the steady-state level of transcripts encoding Zat12 is elevated in *Arabidopsis* in response to a very large number of different biotic and abiotic stresses. These include stresses such as heat shock, salt, cold, wounding, pathogen, and high light (Refs. 12, 13, and 16–18, as well as a search of stress response *Arabidopsis* microarray results available at www.arabidopsis.org/servlets/Search). Common to these stresses, as well as to other stresses that do not enhance Zat12 expression, is the accumulation of ROS in cells during different stages of stress and stress recovery (31). Although it is not known which signals are involved in enhancing Zat12 expression in cells, it is tempting to speculate that a combination of different signals such as ROS and/or different stress response hormones control the expression of Zat12 in cells during stress. Analysis of the *Zat12*, *Zat7*, and *WRKY25* promoters (Table I) supports a link between different stress hormones and Zat12 expression. However, with the exception of the heat shock factor-binding site that may regulate Zat12 expression during heat shock or oxidative stress, no known DNA binding site for ROS responses was identified in the promoter of Zat12. We are currently using Zat12 promoter-luciferase fusions to study the Zat12 promoter and isolate different mutants deficient in Zat12 expression during stress.

Acknowledgments—We thank Drs. Eve Syrkin-Wurtele, Carol Foster, and Hailong Zhang for their help with Affymetrix data analysis.

REFERENCES

1. Chen, W., Provart, N. J., Glazebrook, J., Katagiri, F., Chang, H. S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S. A., Budworth, P. R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J. A., Harper, J. F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J. L., Wang, X. & Zhu, T. (2002) *Plant Cell* **14,** 559–574
2. The *Arabidopsis* Genome Initiative (2000) *Nature* **408,** 796–815
3. Eulgem, T., Rushton, P. J., Robatzek, S. & Somssich, I. E. (2000) *Trends Plant Sci.* **5,** 199–206
4. Stracke, R., Werber, M. & Weisshaar, B. (2001) *Curr. Opin. Plant Biol.* **4,** 447–456
5. Nover, L., Bharti, K., Doring, P., Mishra, S. K., Ganguli, A. & Scharf, K. D. (2001) *Cell Stress Chaperones* **6,** 177–189
6. Epple, P., Mack, A. A., Morris, V. R. & Dangl, J. L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 6831–6836
7. Foreman, J., Demidchik, V., Bothwell, J. H., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D., Davies, J. M. & Dolan, L. (2003) *Nature* **422,** 442–446
8. Neill, S., Desikan, R. & Hancock, J. (2002) *Curr. Opin. Plant Biol.* **5,** 388–395
9. Mittler, R. (2002) *Trends Plant Sci.* **9,** 405–410
10. Vranova, E., Inze, D. & Van Breusegem, F. (2002) *J. Exp. Bot.* **53,** 1227–1236
11. Vranova, E., Atichartpongkul, S., Villarroel, R., Van Montagu, M., Inze, D. & Van Camp, W. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 10870–10875
12. Desikan, R., Mackerness, A. H., Hancock, J. T. & Neill, S. J. (2001) *Plant Physiol.* **127,** 159–172
13. Pnueli, L., Liang, H., Rozenberg, M. & Mittler, R. (2003) *Plant J.* **34,** 187–203

14. Rizhsky, L., Liang, H. & Mittler, R. (2003) *J. Biol. Chem.* **278,** 38921–38925
15. Panchuk, I. I., Volkov, R. A. & Schoffl, F. (2002) *Plant Physiol.* **129,** 838–853
16. Cheong, Y. H., Chang, H. S., Gupta, R., Wang, X., Zhu, T. & Luan, S. (2002) *Plant Physiol.* **129,** 661–677
17. Fowler, S. & Thomashow, M. F. (2002) *Plant Cell* **14,** 1675–1690
18. Iida, A., Kazuoka, T., Torikai, S., Kikuchi, H. & Oeda, K. (2000) *Plant J.* **24,** 191–203
19. Rizhsky, L. & Mittler, R. (2001) *Plant Mol. Biol.* **46,** 313–323
20. Bent, A. F. & Clough, S. J. (2000) *Plant Molecular Biology Manual* (Gelvin, S. B. & Schilperoort, R. A., eds) 2nd Ed., pp. B7:1–B7:14, Kluwer Academic Publishers, Dordrecht, The Netherlands
21. Mittler, R., Feng, X. & Cohen, M. (1998) *Plant Cell* **10,** 461–474
22. Mittler, R. & Zilinskas, B. (1994) *Plant J.* **5,** 397–406
23. Klok, E. J., Wilson, I. W., Wilson, D., Chapman, S. C., Ewing, R. M., Somerville, S. C., Peacock, W. J., Dolferus, R. & Dennis, E. S. (2002) *Plant Cell* **14,** 2481–2494
24. Mehta, R. A., Fawcett, T. W., Porath, D. & Mattoo, A. K. (1992) *J. Biol. Chem.* **267,** 2810–2816
25. Kovtun, Y., Chiu, W. L., Tena, G. & Sheen, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2940–2945
26. Zhang, J. Z. (2003) *Curr. Opin. Plant Biol.* **6,** 430–440
27. Torres, M. A., Dangl, J. L. & Jones, J. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 517–522
28. Knight, H. & Knight, M. R. (2001) *Trends Plant Sci.* **6,** 262–267
29. Pastori, G. M. & Foyer, C. H. (2002) *Plant Physiol.* **129,** 460–468
30. Kreps, J. A., Wu, Y., Chang, H. S., Zhu, T., Wang, X. & Harper, J. F. (2002) *Plant Physiol.* **130,** 2129–2141
31. Zhu, J.-K. (2002) *Annu. Rev. Plant Biol.* **53,** 247–273