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Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (*Apx1*)-deficient *Arabidopsis* plants

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Summary

The accumulation of hydrogen peroxide (H_2O_2) in plants is typically associated with biotic or abiotic stresses. However, H_2O_2 is continuously produced in cells during normal metabolism. Yet, little is known about how H_2O_2 accumulation will affect plant metabolism in the absence of pathogens or abiotic stress. Here, we report that a deficiency in the H_2O_2-scavenging enzyme, cytosolic ascorbate peroxidase (APX1), results in the accumulation of H_2O_2 in *Arabidopsis* plants grown under optimal conditions. Knockout-*Apx1* plants were characterized by suppressed growth and development, altered stomatal responses, and augmented induction of heat shock proteins during light stress. The inactivation of *Apx1* resulted in the induction of several transcripts encoding signal transduction proteins. These were not previously linked to H_2O_2 signaling during stress and may belong to a signal transduction pathway specifically involved in H_2O_2 sensing during normal metabolism. Surprisingly, the expression of transcripts encoding H_2O_2 scavenging enzymes, such as catalase or glutathione peroxidase, was not elevated in knockout-*Apx1* plants. The expression of catalase, two typical plant peroxidases, and several different heat shock proteins was however elevated in knockout-*Apx1* plants during light stress. Our results demonstrate that *in planta* accumulation of H_2O_2 can suppress plant growth and development, interfere with different physiological processes, and enhance the response of plants to abiotic stress conditions. Our findings also suggest that at least part of the induction of heat shock proteins during light stress in *Arabidopsis* is mediated by H_2O_2 that is scavenged by APX1.

Keywords: *Arabidopsis*, ascorbate peroxidase, DNA array, hydrogen peroxide, oxidative stress, signal transduction.

Introduction

Hydrogen peroxide (H_2O_2) is a signaling molecule involved in the control of key biological processes, such as programmed cell death (PCD), abiotic stress responses, hormonal signaling, and pathogen defense (Dat *et al.*, 2000; Hirt, 2000; Kovtun *et al.*, 2000; Mittler, 2002; Mullineaux and Karpinski, 2002; Pei *et al.*, 2000). It is formed in cells by the direct transfer of two electrons to O_2, mediated by enzymes such as glycolate or glucose oxidase, or by the dismutation of superoxide (O_2^-) to H_2O_2, catalyzed by superoxide dismutases (SODs). Under controlled growth conditions, the production of O_2^- by photosynthetic cells is estimated at a constant rate of 250 μmol mg^{-1} chl h^{-1}, and the steady-state level of H_2O_2, produced by SODs and other cellular sources, at 0.5 μmol g^{-1} fresh weight (Asada and Takahashi, 1987; Polle, 2001). These rates reflect the background level of H_2O_2 production in photosynthetic tissues in the light. In the dark, or in non-photosynthetic tissues, H_2O_2 is mainly formed by the dismutation of O_2^-, produced by leakage of electrons from electron transfer carriers in the mitochondria or microsomes, or by fatty acid oxidation (Corpas *et al.*, 2001; Davidson and Schiestl, 2001).

In response to pathogen infection, the rate of H_2O_2 production is dramatically enhanced in cells as a result of the activation of O_2^--producing enzymes, such as NADPH oxidases, and the dismutation of O_2^- to H_2O_2 by SODs (Hammond-Kosack and Jones, 1996; Mittler *et al.*, 1999). H_2O_2 is also formed during pathogen infection by the enzymatic activity of amine oxidases and cell wall-bound

peroxidases (Allan and Fluhr, 1997; Vranova *et al.*, 2002). Under these conditions, H_2O_2 activates different signal transduction pathways essential for programmed cell death and pathogen defense (Hammond-Kosack and Jones, 1996). During abiotic stresses, H_2O_2 is formed by NADPH oxidases (Cazale *et al.*, 1999; Knight and Knight, 2001; Pastori and Foyer, 2002), by specific cellular pathways associated with stress metabolism, such as the photorespiratory pathway (Corpas *et al.* 2001), and by the uncoupling of metabolic reactions that result in the leakage of electrons from different electron carriers to the reduction of O_2 (Asada, 1999; Dat *et al.*, 2000; Mittler, 2002). The enhanced production of H_2O_2 via these routes is also thought to act as a signal that activates defense mechanisms and mediates the acclimation or hardening of plants to extreme environments (Bowler and Fluhr, 2000).

Because H_2O_2 is toxic and yet participates in key signaling events, plant cells require different cellular mechanisms that regulate their intracellular level of H_2O_2. These include ascorbate peroxidase (APX; Asada, 1999), catalase (CAT; Willekens *et al.*, 1997), and glutathione peroxidase (GPX; Roxas *et al.*, 1997). In addition, the balance between SODs and APX, catalase, or glutathione peroxidase activities is considered to be crucial for determining the steady-state level of O_2^- and H_2O_2. This balance, together with sequestering of metal ions such as iron and copper, is thought to be important to prevent the formation of the highly toxic $HO^{\cdot}$ via the metal-dependent Haber–Weiss or the Fenton reactions (Asada and Takahashi, 1987; Bowler *et al.*, 1991; Haber and Weiss, 1934). Antioxidants such as ascorbic acid and glutathione, found at very high concentrations in chloroplasts and other cellular compartments, are also crucial for the defense of plants against oxidative stress (Noctor and Foyer, 1998). Consequently, mutants with suppressed ascorbic acid levels (Conklin *et al.*, 1996) and transgenic plants with suppressed H_2O_2-scavenging enzymes (Mittler *et al.*, 1999; Orvar and Ellis, 1997; Willekens *et al.*, 1997) are hypersensitive to abiotic stress conditions and pathogen attack. In addition, overexpression of O_2^-- and H_2O_2-scavenging enzymes was found to increase the tolerance of plants to abiotic stress conditions (Allen, 1995).

Recent studies identified a number of signal transduction components involved in the detection of H_2O_2 and the activation of defense mechanisms in plants. These include: a two-component histidine kinase, a receptor-like protein kinase, the MAPKKK, AtANP1 (also the NtNPK1), and the MAPKs, AtMPK3/6 and Ntp46MAPK (Czernic *et al.*, 1999; Desikan *et al.*, 2001; Kovtun *et al.*, 2000; Samuel *et al.*, 2000; Vranova *et al.*, 2002). In addition, calmodulin has been implicated in H_2O_2 signaling (Desikan *et al.*, 2001; Harding *et al.*, 1997). However, our current knowledge of the H_2O_2 signal transduction pathway of plants is very limited (Mittler, 2002; Pastori and Foyer, 2002). Moreover, it is solely based upon the studies in which stress conditions or H_2O_2 was externally applied to plants to activate the H_2O_2 signal transduction pathway (Desikan *et al.*, 2001; Kovtun *et al.*, 2000). Because stress treatments or external H_2O_2 application may activate additional signal transduction pathways such as pathogen-response pathways or general stress-response pathways, these treatments may complicate the analysis of the H_2O_2 signal transduction pathway of plants.

To study the response of plants to elevated *in planta* levels of H_2O_2 in the absence of abiotic stress, pathogens, or oxidants, we introduced a lesion in the H_2O_2 scavenging machinery of plants and studied plants growing under optimal conditions. Under these conditions, no external stress is imposed on plants; instead, H_2O_2 levels within cells are specifically elevated because the expression of an H_2O_2-metabolizing enzyme is disrupted. For our study, we chose to inactivate the gene encoding the major cytosolic isoform of ascorbate peroxidase (*Apx1*; Mittler and Zilinskas, 1992). In contrast to all other isoforms of APX, APX1 is highly responsive to various biotic and abiotic stresses, and is considered to play an important role in H_2O_2 scavenging in plants (Asada, 1999; Mittler, 2002; Shigeoka *et al.*, 2002).

Results

Molecular characterization of Apx1*-deficient* Arabidopsis *plants*

As shown in Figure 1, we isolated an *Arabidopsis* line (cv. WS) containing a T-DNA insert in the second exon of *Apx1.* This line was isolated from the Wisconsin T-DNA collection (Sussman *et al.*, 2000). In contrast to tobacco plants expressing an antisense construct to APX1 (Mittler *et al.*, 1999; Orvar *et al.*, 1997), knockout-*Apx1 Arabidopsis* plants had a late flowering and delayed development phenotype, suggesting that the lack of *Apx1* affected plant development in *Arabidopsis* (Figure 1b; Table 1). This phenotype was enhanced in plants grown under long days (18 h light cycle) or constant light, and was almost completely abolished in plants grown under short days (8 h light cycle; data not shown). Protein and RNA blots performed on wild-type and knockout-*Apx1* plants confirmed that homozygote knockout-*Apx1* plants did not contain detectable levels of APX1 protein or RNA (Figure 1c). Activity measurements indicated that total APX activity in knockout-*Apx1* plants was suppressed by 70% compared to that of wild types (data not shown).

To avoid complications resulting from developmental differences between plants, we conducted all our comparisons between wild-type and knockout-*Apx1* plants with 14–17-day-old plants that were developmentally indistinguishable (all growing under optimal growth conditions, i.e. 21°C, 18 h or constant light cycle, 100 μmol m^{-2} sec^{-1},

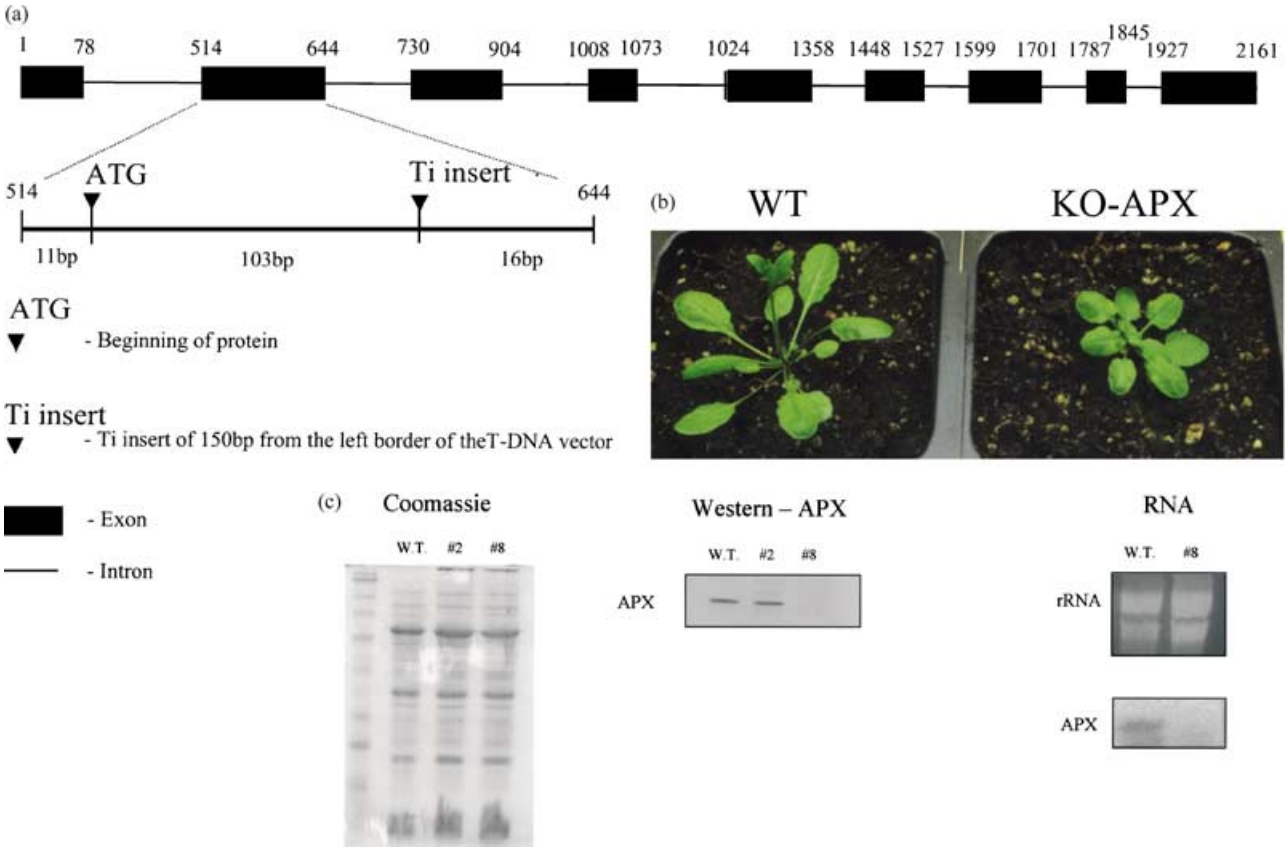

Figure 1. Characterization of APX1-deficient plants.
(a) A map showing the site of T-DNA integration into the *Apx1* gene.
(b) A photograph of 17-day-old wild-type (WT) and knockout-*Apx1* (KO-APX) plants grown under constant light (100 μmol m^{-2} sec^{-1}).
(c) A Coomassie-stained protein gel (left), a protein gel blot performed with APX1-specific antibodies (middle), and an RNA blot (right), showing that knockout-*Apx1* (KO-APX) plants do not contain APX1 mRNA or protein. Methods and experimental protocols are described in Experimental procedures. #2, heterozygous; #8, homozygous.

and a relative humidity of 70%). We performed all our assays in triplicates using a minimum of 60 plants per treatment (three replicates of at least 20 plants each) and repeated all experiments at least thrice.

*Physiological characterization of knockout-*Apx1 *plants*

For the physiological characterization of knockout-*Apx1* plants, we placed wild-type and knockout-*Apx1* plants in the dark for 30 min and shifted them back to light. We then measured the photosynthetic activity and stomatal conductance at different intervals using a Li-Cor LI-6400 apparatus. We also sprayed light-grown wild-type and knockout-*Apx1* plants with a solution of abscisic acid (ABA; 50 μM) and measured the rate of stomatal closure. As shown in Figure 2, knockout-*Apx1* plants had a lower rate of maximal photosynthetic activity (about 60% of wild-type activity, Figure 2a) and altered stomatal responses (Figure 2b). Thus, compared to wild-type plants that opened their stomata upon a shift from dark to light, the stomata of knockout-*Apx1* plants were almost non-responsive to this

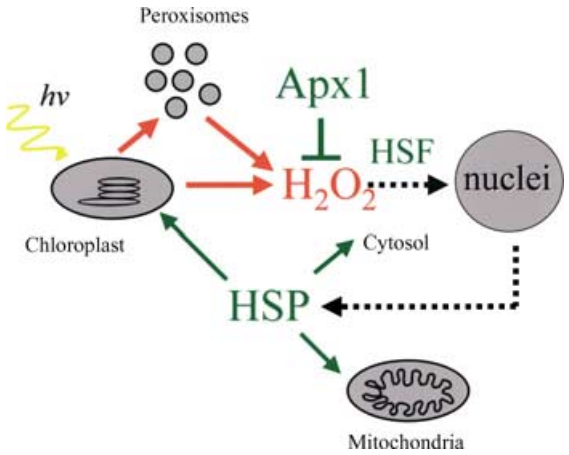

Figure 7. A model showing the involvement of APX1 in the induction of heat shock proteins (HSPs) during light stress in *Arabidopsis*.
Light (*hv*) is shown to enhance the production of H_2O_2 in the chloroplast and peroxisomes (red arrows). H_2O_2 then diffuses into the cytosol where it is scavenged by APX1. H_2O_2 not scavenged by APX1 activates a signal transduction pathway (dashed arrows) that results in the induction of chloroplastic, mitochondrial, and cytosolic heat shock proteins, HSPs (green arrows). In the absence of APX1 (knockout-*Apx1* plants), this response is augmented (Figures 4 and 5; Table 5).

Table 1 Developmental time course of wild-type (WT) and knockout-*Apx1* plants

Time (days)	WT	Knockout-*Apx1*
0	End of vernalization	End of vernalization
4	First two cotyledons	First two cotyledons
10	First two true leaves	First two true leaves
17	Five true leaves and bolting 2.5 cm	Seven true leaves
19	Inflorescence stem 6 cm	Eight true leaves, starting to bolt
23	Inflorescence stem 13–15 cm	70% Inflorescence stem 0.75 cm 30% Inflorescence stem 5 cm
26	Inflorescence stem 20–22 cm, has siliques	25% Inflorescence stem 15 cm 12.5% Inflorescence stem 8 cm 12.5% Inflorescence stem 5 cm 50% Inflorescence stem 1 cm
27	Inflorescence stem 24 cm, has siliques	25% Inflorescence stem 18 cm, has siliques 12.5% Inflorescence stem 11 cm 12.5% Inflorescence stem 8 cm 50% Inflorescence stem 2–4 cm

Eighty wild-type and knockout-*Apx1* plants were scored. Unless otherwise stated, more than 95% of the plants are as described. Plants were grown at 21–22°C, constant light 100 μmol m^{-2} sec^{-1}, and a relative humidity of 70%. This analysis was repeated thrice with similar results.

treatment (Figure 2b). The response of knockout-*Apx1* plants to abscisic acid application was, however, similar to that of wild types (Figure 2c).

DNA array and biochemical analysis of knockout-Apx1 *plants*

To study changes in gene expression resulting from the lack of APX1 in *Arabidopsis* plants (grown under optimal conditions), we performed a DNA array analysis using Affymetrix chips (8200 gene chips). Our results expressed as mean and standard error for three different measurements are shown in Tables 2–4. Table 2 shows the expression level of different O_2^-- and H_2O_2-scavenging enzymes in knockout-*Apx1* plants compared to wild-type plants. Surprisingly, we did not detect an increase in the expression level of the other APX isozymes (including APX4 and APX5, not shown in the table) in knockout-*Apx1* plants. Moreover, in contrast to tobacco plants expressing an antisense construct to APX1 in which catalase and CuZnSOD expression was elevated (Rizhsky *et al.*, 2002a), the expression of catalase and at least one isozyme of CuZnSOD was suppressed in knockout-*Apx1* plants.

In Tables 3 and 4, we have included transcripts with a known or putative function, elevated (Table 3) or suppressed (Table 4), 0.5 (log2)-fold or higher, in knockout-*Apx1* plants compared to wild-type plants. The different transcripts have been grouped on the basis of their putative

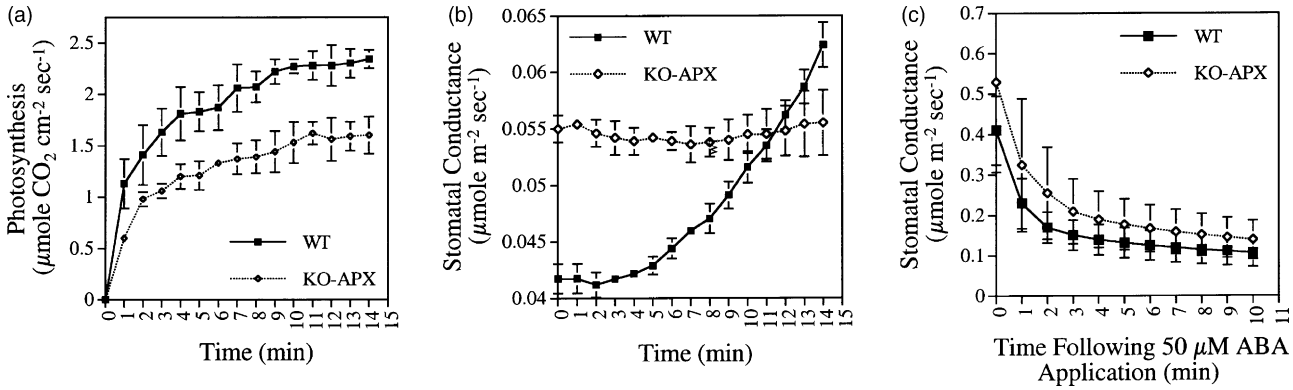

Figure 2. Suppressed photosynthetic activity and abnormal guard cell responses in knockout-*Apx1* (KO-APX) plants.
(a) A graph showing the suppression of photosynthetic activity in KO-APX plants compared to WT plants.
(b) A graph showing the abnormal guard cell response of KO-APX plants upon shift of plants from dark to light. In (a) and (b) plants were placed in the dark for 30 min and then transferred to light (100 μmol m^{-2} sec^{-1}). Photosynthesis (a) and guard cell responses (b) were recorded simultaneously every minute, starting immediately upon the transfer of plants to light.
(c) A graph showing the closure of stomata in WT and KO-APX plants upon ABA application in the light. Additional methods and experimental protocols are described in Experimental procedures. Data shown is mean and standard deviation of three independent measurements.

Table 2 Expression levels of different transcripts involved in O_2^- and H_2O_2 scavenging in wild-type (WT) and knockout-*Apx1* plants, in the absence of stress

	Expression level compared to WT (% of control ± SD)	
Transcript	WT	Knockout-*Apx1*
CuZnSOD (chl)	**100 ± 6.2**	**48 ± 1.9**
CuZnSOD (cyt)	100 ± 16	128 ± 16
FeSOD	100 ± 11	95 ± 18
APX1	**100 ± 4.2**	**2 ± 0.01**
APX2	nd	nd
APX3	100 ± 8	87 ± 2.3
APX (tyl)	100 ± 6.9	72 ± 8.8
APX (str)	100 ± 16	71 ± 14
MDAR	100 ± 7.2	60 ± 9
GR1	100 ± 7	68 ± 11
GR2	100 ± 16	117 ± 12
CAT1	**100 ± 4.1**	**67 ± 1.6**
CAT	**100 ± 7.2**	**74 ± 2**
GPX1	100 ± 7	118 ± 13
GPX2	100 ± 5	102 ± 4.4
GPX (phospholipid)	100 ± 1.5	105 ± 3

Results, presented as percentage of control compared to WT plants, are expressed as average ± SD of three independent measurements each performed with a different chip. RNA was extracted from 120 to 150 WT and knockout-*Apx1* plants and hybridized to Affymetrix chips (8200 gene chip) as described in Experimental procedures. Transcripts indicated in bold are significantly different between WT and knockout-*Apx1* ($P = 0.01$) plants. Plants were grown at 21–22°C, constant light 100 μmol m^{-2} sec^{-1}, and a relative humidity of 70%. Abbreviations used: APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; GPX, glutathione peroxidase; MDAR, monodehydroascorbate reductase; nd, not detected; SD, standard deviation; SOD, superoxide dismutase; str, stroma; tyl, thylakoid; chl, chlorophyll; cyt, cytochrome.

function into: signal transduction components, transcription factors, defense and detoxification transcripts, and cellular organization, metabolism and biogenesis transcripts. In a separate column, we also indicate whether these transcripts are also induced by other stresses.

Concurring with our working hypothesis that the disruption of *Apx1* will result in an internal oxidative stress to plants (growing under controlled conditions), the expression of a number of genes that were previously associated with oxidative stress was elevated in knockout-*Apx1* plants (Table 3). These included the pathogenesis-related protein-1 (PR-1), blue-copper-binding protein, glutathione-S-transferase (GST6), ferritin, and the zinc finger protein ZAT12 (Desikan *et al.*, 2001). The expression of a number of transcripts encoding signal transduction components and transcription factors was also elevated in knockout-*Apx1* plants compared to wild-type plants. Some of these were previously associated with other stresses whereas others were not (to the best of our searching ability). Some of these transcription factors and signaling transcripts, elevated in knockout-*Apx1* plants, may belong to the H_2O_2 signal transduction pathway of plants. In addition to the signal transduction and transcription factor transcripts, the expression of a number of defense, cellular organization, and metabolic transcripts was altered in knockout-*Apx1* plants. These may serve as putative downstream transcripts regulated by the H_2O_2 signal transduction pathway in response to the absence of APX1.

To confirm the results obtained by the DNA chip analysis, we performed RNA blots with RNA from wild-type and knockout-*Apx1* plants and probed these with some of the transcripts shown in Table 3. As shown in Figure 3(a), the results from the RNA blots were in good agreement with our DNA chip analysis.

We also measured the level of reduced glutathione (GSH) and H_2O_2 in leaf extracts from wild-type and knockout-*Apx1* plants. The level of these compounds can serve as a good measure of the degree of oxidative stress encountered by plants (Mittler, 2002; Noctor and Foyer, 1998). As shown in Figure 3(b), knockout-*Apx1* plants had higher levels of reduced glutathione and H_2O_2 compared to wild-type plants.

Table 4 summarizes the transcripts suppressed in knockout-*Apx1* plants grown under optimal growth conditions. In addition to chloroplastic CuZnSOD and catalase, a number of transcripts encoding signal transduction enzymes and transcription factors were suppressed in knockout-*Apx1* plants. The suppression of these may be linked to the H_2O_2 signal transduction pathway. Additional tables showing the expression level of transcripts with unknown function, elevated or suppressed in knockout-*Apx1* plants, can be found as Supplementary Material to this article.

Light stress of knockout-Apx1 *plants*

To further characterize knockout-*Apx1* plants and to examine their response to a mild abiotic stress, we subjected wild-type and knockout-*Apx1* plants to a moderate light stress of 425 μmol m^{-2} sec^{-1}. As shown in Figure 4, this light stress resulted in the induction of APX1 and ELIP (early light inducible protein) in wild-type plants, and the induction of ELIP in knockout-*Apx1*. Interestingly, the induction of transcripts encoding the 70 kDa heat shock protein (HSP70), 1 h following the application of light stress, was much higher in knockout-*Apx1* plants compared to that in wild-type plants (Figure 4). The conditions for light stress used in our experiment did not result in any apparent cell death in wild-type or knockout-*Apx1* plants (not shown), or the induction of APX2, previously reported to be induced during high light stress (2000 μmol m^{-2} sec^{-1}; Karpinski *et al.*, 1999; Figure 4; also measured by the DNA chips and by RT-PCR, not shown; see Figure 6 for a positive control for APX2 induction). The induction of APX1 in wild-type plants during light stress (425 μmol m^{-2} sec^{-1}; Figure 4)

Table 3 Transcripts elevated in knockout-*Apx1* (KO-APX) plants compared to wild-type (WT) plants in the absence of stress or pathogen infection

	Average log2 fold	SE	1 h light stress signal log2 fold	Other stresses	GenBank number	Description
Signal transduction transcripts elevated in KO-APX plants						
15616_s_at	1.80	0.16	(+) 1.5		L04999	Serine threonine kinase (pro25)
12497_at	1.67	0.21	(+) 1.3	W	F20M17.8	Putative receptor-like kinase
17917_s_at	1.63	0.05	nc		T3K9.14	Calcium-binding protein (CaBP-22)
13177_at	1.63	0.25	nc	P	T20K18.70	Growth factor like protein
12353_at	1.43	0.05	(+) 0.5		F13M22.21	Receptor-like kinase
18003_at	1.30	0.08	nc		AF188334	Toll/interleukin-1 receptor-like
17499_s_at	1.27	0.19	(+) 0.8		AF107726	Cyclic nucleotide gated channel
19848_s_at	1.23	0.12	nc		T3K9.13	Calmodulin-like protein
19433_at	1.17	0.26	(+) 1.1	W	F21P8.160	Serine/threonine kinase
13022_at	1.10	0.16	nc	C/D/S	F21H2.4	Protein phosphatase type 2C
16952_s_at	0.90	0.08	nc	W	F13H10.4	Calmodulin-like protein
17114_s_at	0.67	0.09	nc		D21840	MAP kinase (MPK4)
17991_g_at	0.53	0.05	nc	W	AF178075	Calmodulin 9 (CAM9)
Transcription factors transcripts elevated in KO-APX plants						
15779_g_at	1.60	0.29	nc	W	X98676	ZAT7, zinc finger protein
20382_s_at	1.50	0.50	nc	S	T9D9.6	WRKY-type DNA-binding
13271_g_at	1.33	0.47	nc	Hs/W	U68561	Heat shock transcription factor 21
13015_s_at	1.23	0.21	(−) 0.9	P/H/W	X98673	ZAT12, zinc finger protein
18217 _g_at	0.87	0.26	(− 1)	S	X95573	Salt-tolerance zinc finger protein
13273_at	0.87	0.24	nc	Hs/W	U68017	Heat shock transcription factor 4
15203_s_at	0.70	0.08	nc	S(−)/W	AB013887	RAV2, novel DNA-binding protein
16064_s_at	0.70	0.16	(−) 0.7	H	AB008106	Ethylene responsive factor 4
18600_at	0.63	0.17	nc	S/W	L76926	Putative zinc finger protein
20032_at	0.53	0.05	nc		F17L21.4	Putative squamosa promoter-binding
Defense and detoxification transcripts elevated in KO-APX plants						
19178_at	2.67	0.12	nc	P/H	Y18227	Blue copper binding-like protein
14635_s_at	2.50	0.36	(−) 1.6	P/H	M90508	PR-1-like
13212_s_at	2.27	0.53	(−) 0.5	P/H/W	M90509	Beta-1,3-glucanase
16031_at	1.77	0.05	(+) 1.3	D/C/I	X94248	Ferritin
14636_s_at	1.67	0.25	(−) 1	P/H/W	M90510	Thaumatin-like
16439_at	1.50	0.08	nc	P	X72022	CXc750 pathogen-inducible
14111_s_at	1.43	0.63	nc		F18A5.290	Putative disease resistance protein
18604_at	1.37	0.12	nc	H	AF069298	100aa heat, auxin, ethylene induced
19181_s_at	1.37	0.12	(+) 0.4	D/C/O	AF053065	Late embryogenesis abundant (21)
12764_f_at	1.37	0.05	nc	W	T17M13.10	Putative glutathione S-transferase
16054_s_at	1.13	0.09	(−) 0.5	W	Y14251	Glutathine-S-transferase (GST11)
13695_at	1.10	0.22	nc	P/W	AF021346	Disease resistance protein (NDR1)
15118_s_at	1.00	0.28	nc	W	L11601	Glutathione S-transferase
17119_s_at	0.83	0.09	nc	W	AF132212	OPDA-reductase
16981_s_at	0.70	0.14	(−) 1.2		U35829	Thioredoxin h (TRX5)
13186_g_at	0.70	0.08	nc	D	Z35475	Thioredoxin
12802_at	0.70	0.00	nc	D/C/W/H	T19K4.170	DnaJ-like protein

19426_s_at	0.67	0.21	nc	W	AF098964	Disease resistance protein RPP1
13189_s_at	0.63	0.05	(−) 1.1		Z35476	(TOUL) thioredoxin
16009_s_at	0.63	0.05	(+) 0.5	P/H/W	F17A22.12	Glutathione S-transferase (GST6)
16060_at	0.53	0.05	nc	D	D89051	ERD6 early dehydration-induced
16001_at	0.53	0.05	nc		AF035385	SEN5, senescence-associated
16081_s_at	0.50	0.08	nc	D/C	AF141659	AtHVA22a, ABA/stress induced
13785_at	0.50	0.08	(−) 0.6	C/W	F14N22.20	Cold-regulated, cor15b
19665_at	0.47	0.05	(−) 0.4		X95585	DAD-1, apoptosis suppressor
Cellular organization, metabolism and biogenesis transcripts elevated in KO-APX plants						
14704_s_at	3.03	0.05	(+) 0.9		F7H1.2	Putat. retroelement pol polyprotein
18567_at	2.63	1.32	nc		F14M4.4	Putat. alcohol dehydrogenase
17008_at	2.60	0.57	nc		F27C12.23	Putat. tyrosine aminotransferase
19121_at	1.43	0.37	(+) 1.1		AF055847	AIR1 auxin-inducible
17832_at	1.43	0.21	nc	LO	U94998	Non-symbiotic hemoglobin (AHB1)
19118_s_at	1.40	0.49	(+) 1.7		AF098630	Cell wall-PM disconn. protein
16702_at	1.40	0.45	nc		T16F16.15	Putative phloem-specific lectin
20369_s_at	1.10	0.08	(+) 0.6		A44314	Ammonium transport (AMT1)
13617_at	1.07	0.24	nc		F14M13.10	Mito. dicarboxylate carrier
19961_at	1.03	0.17	nc	W	F20M13.240	Glycine-rich 2 (GRP2)
15085_at	0.83	0.12	nc		F7H19.200	UDP-galactose transporter
20446_s_at	0.80	0.14	nc		T25N20.21	Putative glucan synthase
13091_r_at	0.77	0.26	(−) 0.3		T14P1.12	Putative transport SEC61 beta
16014_at	0.77	0.05	nc		U11766	GAST1, gibberellin-regulated
17002_at	0.67	0.12	nc	D/P/H	AJ238804	Lipid transfer protein
19450_at	0.67	0.12	nc	W	X75365	SUC1, sucrose-proton symporter
15115_f_at	0.67	0.05	nc		AF104330	Glycine-rich protein (GRP3S)
14116_at	0.67	0.09	nc		AF077407	Hexose transporter-like
16950_s_at	0.63	0.25	nc		Z26753	Sec61 beta-subunit
18673_at	0.60	0.08	nc		D79218	Non-coding RNA, cyt-repressed
17882_at	0.57	0.12	nc		F13M23.60	SEC61 GAMMA
19999_s_at	0.57	0.09	nc		AB017977	cAMP phosphodiesterase
15452_at	0.57	0.09	(−) 0.3		Z49859	Copper transporter
16940_g_at	0.57	0.12	nc		F25I18.15	Putative synaptobrevin
14567_at	0.53	0.05	nc		F16M14.6	Putative acetyltransferase
20190_at	0.53	0.12	nc	D/C	T24I21.7	Nodulin-like protein
13093_at	0.50	0.14	nc		T24C20.20	B12D-like, seed development
19969_at	0.50	0.08	nc		F9L1.5	Ubiquinol-cyt-*c*-reductase

Results (mean and SE) are presented as fold induction (log2) over WT expression. Two accession numbers are given to each transcript, Affymetrix (left) and Genebank (right). The known or putative function of each transcript is also given (right column). The induction/suppression of each transcript following a 1 h light stress, expressed as fold (log2) over WT, is indicated in a separate column (+, induction; −, suppression; nc, no change). The induction of each transcript by different stressful conditions (C, cold; D, drought; H, hydrogen peroxide; Hs, heat shock; I, excess iron; LO, low oxygen; O, high oxygen; P, pathogen; S, salt; W, wounding) is indicated in the other stresses column. This information was collected from various sources as indicated as Supplementary Material. RNA preparation and analysis by Affymetrix chips (*Arabidopsis* 8200 gene chip) are described in Experimental procedures and as Supplementary Material.

Table 4 Transcripts suppressed in knockout-*Apx1* (KO-APX) plants compared to wild-type (WT) plants in the absence of stress or pathogen infection

	Average signal log2 fold	SE	1 h light stress signal log2 ratio	Other stresses	GenBank number	Description
Signal transduction transcripts suppressed in KO-APX plants						
13587_at	0.63	0.26	nc		F21M11.2	Acid phosphatase
16124_s_at	0.57	0.17	nc		AF053366	Blue light photoreceptor PHR2
15135_s_at	0.60	0.22	(+) 0.6		U01955	Laminin receptor-like protein
Transcription factors transcripts suppressed in KO-APX plants						
19887_s_at	0.60	0.00	(+) 0.4		U75599	Leucine zipper protein
15676_at	0.53	0.34	nc		AF138743	Zinc finger protein 1 (zfn1)
14723_f_at	0.50	0.08	(−) 0.6		AF003096	AP2 domain protein RAP2.3
Defense and detoxification transcripts suppressed in KO-APX plants						
16429_at	3.73	0.19	(−) 5.6	H/O/W	U63815	Ascorbate peroxidase (APX1)
18755_at	2.30	0.80	nc		F14M19.60	Pathogenesis-related protein
15581_s_at	1.07	0.09	nc	O(−)	AF061519	Plastid Cu/Zn SOD
15606_at	1.20	0.16	nc		AF061517	Plastid Cu/Zn SOD chaperone (CCS1)
15776_at	0.90	0.14	nc	D/W	D10703	Dehydration-induced RD22
17196_at	0.83	0.12	(−) 0.7		F16A16.110	Proline-rich APG-like protein
16629_at	0.77	0.05	(−) 1	W	AF087932	Hydroperoxide lyase (HPL1)
17018_s_at	0.63	0.05	(+) 0.4	W(−)	U18929	Cytochrome P450 monooxygenase
12752_s_at	0.60	0.16	(+) 0.3		T8O5.170	Peroxidase prxr1
13154_s_at	0.57	0.12	(−) 1	W	F18O19.30	Putative endochitinase
14856_at	1.03	0.76	nc	W	F13P17.32	Putative cytochrome P450
20442_i_at	0.50	0.16	(+) 0.5	W	F3O9.21	Putative cytochrome P450
13218_s_at	0.57	0.12	(+) 0.5	H/W(−)	AF021937	Catalase 3 (CAT3)
Cellular organization, metabolism and biogenesis transcripts suppressed in KO-APX plants						
16575_s_at	2.27	0.26	nc		L40954	Oleosin
18859_at	1.63	0.75	nc		F8A5.29	Putative clathrin coat assembly protein
20227_s_at	1.37	0.21	(−) 2.2		AB027252	f-AtMBP myrosinase binding protein
16991_at	1.03	0.21	(−) 0.4		L73G19.10	Fibrillarin-like protein
18215_at	0.93	0.25	nc		Z97335	Selenium-binding protein like
17572_s_at	0.90	0.22	nc		AF083036	Ammonium transporter
12381_at	0.87	0.17	nc		AB003522	Beta subunit of coupling factor one
15186_s_at	0.87	0.25	nc	H	AF023167	Adenosine-5-phosphosulfate red. (APSR)
13120_at	0.83	0.05	nc		T9A14.50	Extensin-like protein
17517_at	0.77	0.09	nc		L41245	Thionin (Thi2.2)
12412_at	0.77	0.05	(+) 0.4		T1J8.6	*S*-adenosylmethionine synthetase
20640_s_at	0.77	0.12	(+) 0.3		S45911	Glyceraldehyde-3-pho.-dehyd. (GapB)
18696_s_at	0.73	0.19	nc	H	U96045	APS reductase (PRH)
13099_s_at	0.73	0.19	(+) 0.9		T22J18.12	Sucrose-proton symporter (SUC2)
12844_s_at	0.73	0.33	(−) 0.7		X16077	18S rRNA gene
15141_s_at	0.73	0.21	(−) 1.4		D85191	Vegetative storage protein
15182_at	0.70	0.16	nc		D85339	Hydroxypyruvate reductase
13588_at	0.70	0.08	(+) 0.4		F28A23.40	Phosphoglycerate dehydrogenase

16504_at	0.70	0.08	nc		Z97335	Hydroxymethyltransferase
15990_at	0.67	0.09	nc		S74719	Sedoheptulose-1,7-bisphosphatase
15992_s_at	0.67	0.09	(+) 0.3		X16432	Elongation factor 1-alpha
13682_s_at	0.63	0.33	nc		F14P13.17	Lycopene beta cyclase
15627_at	0.63	0.33	(+) 0.4		U80186	Pyruvate dehydrogenase E1 beta
16431_at	0.63	0.09	(−) 0.5		T6A23.27	Nonspecific lipid transfer protein
18439_s_at	0.63	0.09	(+) 0.4		X97484	Putative phosphate transporter
16994_at	0.63	0.05	nc		F6E13.25	60S ribosomal protein L7
17394_s_at	0.60	0.08	nc		M64115	Glyceraldehyde-3-pho. dehyd. (B)
18484_at	0.60	0.08	(+) 0.4		T28I19.40	Squalene epoxidase-like
15642_at	0.57	0.12	nc		AF129511	Very long chain fatty acid condensing
14645_at	0.57	0.05	(+) 0.5	D/S	D13043	Thiol protease
16926_at	0.57	0.05	nc		Z97343	Ribosomal protein
16997_at	0.57	0.17	nc		X75162	BBC1 protein, cell division
15145_s_at	0.57	0.05	nc		D64155	Possible aldehyde decarbonylase
17374_at	0.57	0.12	nc		F17L21.20	Putative 60S ribosomal protein L17
16106_at	0.53	0.05	nc		U77381	WD-40 repeat protein (AtArcA)
16508_at	0.53	0.05	nc		X94626	AATP2-plastidic ATP/ADP transporter
18683_s_at	0.53	0.12	nc	C	L27158	Omega-3 fatty acid desaturase
15837_at	0.50	0.08	(+) 0.4		T27A16.27	Putative thiamin biosynthesis protein
17386_at	0.50	0.08	nc	W	F26H11.10	Putative proline-rich protein
20117_at	0.50	0.08	(+) 0.5		Z97341	Putative oligopeptide transporter

Results (mean and SE) are presented as fold induction (log2) over WT expression. Two accession numbers are given to each transcript, Affymetrix (left) and GenBank (right). The known or putative function of each transcript is also given (right column). The induction/suppression of each transcript following a 1 h light stress, expressed as fold (log2) over WT, is indicated in a separate column (+, induction; −, suppression; nc, no change). The induction of each transcript by different stressful conditions (C, cold; D, drought; H, hydrogen peroxide; Hs, heat shock; I, excess iron; LO, low oxygen; O, high oxygen; P, pathogen; S, salt; W, wounding) is indicated in the other stresses column. This information was collected from various sources as indicated as Supplementary Material. RNA preparation and analysis by Affymetrix chips (*Arabidopsis* 8200 gene chip) are described in Experimental procedures and as Supplementary Material.

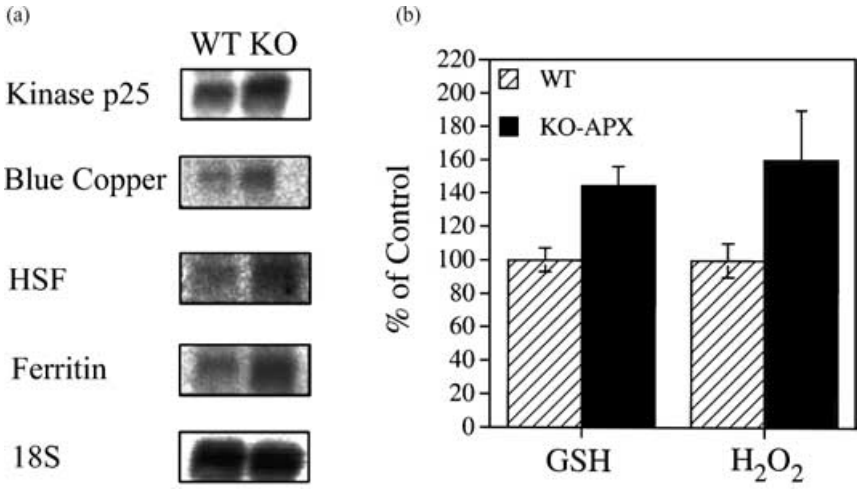

Figure 3. Enhanced expression of transcripts associated with oxidative stress and elevated levels of reduced glutathione (GSH) and H_2O_2 in knockout-*Apx1* (KO-APX) plants.
(a) RNA gel blots showing the enhanced expression of different transcripts associated with oxidative stress in KO-APX plants.
(b) A graph showing the elevated levels of GSH and H_2O_2 in KO-APX plants compared to WT plants. Methods for RNA gel blots and biochemical analysis are described in Experimental procedures.
Data shown in (b) is mean and standard deviation of three independent measurements. HSF, heat shock transcription factor 21.

suggested that this level of light stress enhanced the production of H_2O_2 in *Arabidopsis*.

To extend our analysis of gene expression during light stress in knockout-*Apx1* plants, we performed an Affymetrix chip analysis comparing wild-type and knockout-*Apx1* plants subjected to a 1 h light stress (8200 gene chip; Table 5, 1 h light stress column in Table 3, Figure 4). Our analysis revealed some interesting differences between the response of wild-type and knockout-*Apx1* plants to this stress. Thus, the induction of at least 16 transcripts encoding different heat shock proteins and two transcripts encoding putative heat shock transcription factors (HSFs) by light stress was much higher in knockout-*Apx1* plants compared to that in wild-type plants (indicated in bold in Table 5; see also Figure 4).

Analysis of gene expression performed on wild-type and knockout-*Apx1* plants at 0, 1, and 48 h of light stress (425 μmol m^{-2} sec^{-1}), using DNA chips, revealed that the induction of the two putative heat shock transcription factors and the different heat shock proteins was transient and did not continue after plants acclimated to the light stress treatment (Figure 5). This result was in agreement with the RNA blots for HSP70 shown in Figure 4.

Light stress in knockout-*Apx1* plants resulted in the induction of transcripts encoding two typical plant peroxidases and a catalase (indicated by an asterisk (*) in Table 5), suggesting that these might be involved in the removal of H_2O_2 during light stress in knockout-*Apx1* plants. In contrast to the transient induction of the heat shock-associated transcripts at 1 h light stress (Figures 4 and 5), the induction of these transcripts was elevated in knockout-*Apx1* plants at early and late time points, suggesting that the removal of H_2O_2 by peroxidases and catalase was critical in these plants during all stages of plant acclimation to light stress (Figure 5). Analysis of the expression pattern of cyclophilin indicated that this transcript had a very different expression profile between wild-type and knockout-*Apx1* plants during light stress (Figure 5c).

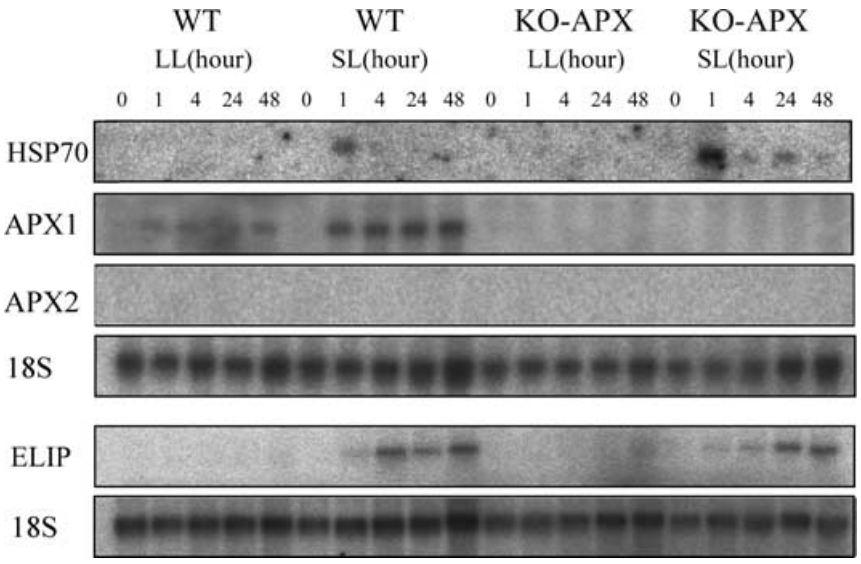

Figure 4. Enhanced expression of heat shock protein HSP70 in knockout-*Apx1* (KO-APX) plants during light stress.
RNA gel blots were performed with RNA obtained from control and light stress (425 μmol m^{-2} sec^{-1})-treated WT and KO-APX plants. The induction of HSP70 is shown to be augmented in KO-APX plants 1 h following light stress application compared to WT plants. 18S rRNA was used to control for RNA loading. Methods and experimental protocols are described in Experimental procedures. ELIP, early light inducible protein; HSP, heat shock protein; LL, low light (100 μmol m^{-2} sec^{-1}); SL, strong light (425 μmol m^{-2} sec^{-1}).

Table 5 Transcripts differentially induced in knockout-*Apx1* (KO-APX) plants 1 h following light stress compared to wild-type (WT) plants subjected to the same treatment

	Fold log2	Gene accession number	Description
Signal transduction transcripts elevated in KO-APX plants following a 1 h light stress			
17553_at	2.2	AF084570	KBP12 interacting protein (FIP37)
18178_s_at	1.5	U95973	Phosphatidylinositol-4-phosphate 5-kinase
17445_at	1.1	F9D16.40	Phosphatase like protein
18847_at	1	F16F14.7	Putative purple acid phosphatase precursor
Transcription factors transcripts elevated in KO-APX plants following a 1 h light stress			
12431_at	1.5	T19L18.4	**Putative heat shock transcription factor**
20342_at	1.3	F4F15.20	**Putative heat shock transcription factor**
20659_at	1.2	U90439	Putative CCCH-type zinc finger protein
14479_at	1.1	TPA14.90	EF-Hand containing protein-like
13533_at	1	F8K7.13	Similar to SWI/SNF complex regulator
Defense and detoxification transcripts elevated in KO-APX plants following a 1 h light stress			
13278_f_at	4.7	Y14070	**Heat shock protein 17.6 A**
12434_at	4.2	F9C22.6	**Cyclophilin-like protein**
13275_f_at	3.9	X17293	**17.4 kDa heat shock protein**
13282_s_at	3.3	U72958	**HSP23.6- mitochondrial**
13279_at	3.2	X63443	**HSP17.6-II**
15954_at	2.6	U72155	Beta-glucosidase (psr3.2)
20323_at	2.2	F16P2.12	**Putative small heat shock protein**
13285_at	2.1	M62984	**Heat shock protein 83**
13284_at	2.1	AJ002551	**Heat shock protein 70**
15404_at	1.8	F15I1.13	**HSP20/alpha crystallin family**
15172_s_at	1.6	D84414	**Luminal binding protein (BiP)**
16916_s_at	1.5	X77199	**Heat shock cognate 70–2**
17815_at	1.5	Z97342	Disease resistance RPP5 like
15985_at	1.4	X98808	(*) peroxidase ATP3a
16466_s_at	1.4	Y08903	**HSC70-G7**
13287_at	1.3	Z70314	**Heat shock protein**
17942_s_at	1.3	X98322	(*) peroxidase, prxr10
13558_s_at	1.2	T1J8.9	Putative ABC transporter
13641_at	1.2	F17M5.60	Putative NBS/LRR disease resistance
18497_at	1.2	F22D22.13	**70 kDa heat shock protein**
13274_at	1.1	+ U13949	**Heat shock protein AtHSP101**
13552_at	1.1	F18O19.28	Putative endochitinase
16905_s_at	1.1	M4E13.140	(*) catalase
13283_at	1	Z11547	**Mitochondrial chaperonin HSP60**
Cellular organization, metabolism and biogenesis transcripts elevated in KO-APX plants following a 1 h light stress			
13291_at	3.4	U18413	IAA11 gene (auxin induced)
15609_s_at	3.2	D34630	Acetyl-CoA carboxylase
14390_at	2.8	F8K7.5	Similar to POS5 from *Saccharomyces cerevisiae*
18698_s_at	2.2	X17528	Mitochondrial citrate synthetase.
13656_at	2	T7B11.13	Unknown, similar to bacterial tolB proteins
16489_at	1.9	X67421	extA extensin gene
12846_s_at	1.5	X52631	DNA for rRNA intergenic region
15169_s_at	1.5	AF061286	Gamma-adaptin 1
14057_at	1.5	F10M23.310	Putative aconitase
17627_at	1.5	F23M19.8	Similar to FAB1 protein from *S. cerevisiae*
17674_at	1.5	T32G6.2	Putative U4/U6 small nuclear ribonucleoprotein
16541_s_at	1.3	AB023423	AST91 mRNA for sulfate transporter
15793_at	1.3	T19F6.22	FtsH protease
15192_at	1.1	D17582	Putative sugar transport protein, ERD1
17581_g_at	1.1	AF105064	GIGANTEA (GI)
15015_at	1.1	T14P1.16	Putat. mitoch. translation elongation factor G
17473_at	1.1	Z18242	Calnexin homolog.
20354_s_at	1.1	T12M4.19	Putative SF2/ASF splicing modulator
12975_at	1.1	F24H14.14	Putative spliceosome-associated protein
18760_at	1.1	F21M12.1	Putative leucyl-tRNA synthetase
18823_s_at	1.1	F17M5.20	WD-repeat-like protein

Table 5 continued

	Fold log2	Gene accession number	Description
18826_at	1.1	F22D22.29	Putative glucan synthase
13205_at	1	U37587	Cell division cycle protein (CDC48)
15689_at	1	U40341	Carbamoyl phosphate synthetase large chain
17091_s_at	1	U40269	Origin recognition complex largest subunit
17570_g_at	1	AF066080	Sihydrolipoamide *S*-acetyltransferase
13126_g_at	1	T27E13.15	Ubiquitin activating enzyme 1 (UBA1)
17857_at	1	T3P4.5	Cytoplasmic aconitate hydratase
12606_at	1	AF075598	Putative fibrillin

Results are presented as fold induction (log2) over WT expression. Two accession numbers are given to each transcript, Affymetrix (left) and Genebank (right). The known or putative function of each transcript is also given (right column). Transcripts associated with the heat shock response are indicated in bold. Transcripts associated with H_2O_2 removal are indicated by an (*). RNA preparation and analysis by Affymetrix chips (*Arabidopsis* 8200 gene chip) are described in Experimental procedures and as Supplementary Material.

Heat shock of knockout-Apx1 *plants*

To further test the involvement of APX1 in the induction of heat shock proteins in *Arabidopsis*, we subjected wild-type and knockout-*Apx1* plants to a heat shock treatment (37°C, 5 h; 100 μmol m^{-2} sec^{-1}). *Apx1* contains a functional heat shock factor-binding element in its promoter (Storozhenko *et al.*, 1998) and is induced by heat shock (Mittler and Zilinskas, 1992; Rizhsky *et al.*, 2002b; see also Figure 6a). However, in contrast to the differences observed in the induction of heat shock proteins between wild-type and knockout-*Apx1* plants during light stress (Table 5; Figures 4 and 5), we could not find a similar difference in the induction of heat shock proteins between wild-type and knockout-*Apx1* plants during heat shock.

Under our experimental conditions, APX2 was not induced during light stress (425 μmol m^{-2} sec^{-1}; Figure 4; also tested by RT-PCR and DNA chips; not shown) or heat shock (Figure 6a; also tested by RT-PCR; not shown). This finding was in contrast to a previous report on the induction of APX2 during heat shock (Panchuk *et al.*, 2002). As shown in Figure 6(b), APX2 was however induced during high light stress, i.e. 2000 μmol m^{-2} sec^{-1} (see also Karpinski *et al.*, 1999), suggesting that this isoform of APX is very specialized.

Discussion

Compensation for Apx1 *deficiency in* Arabidopsis

Studying plants that lack H_2O_2 scavenging genes may result in the identification of alternative enzymes or pathways that compensate for the loss of H_2O_2 removal activity (Mittler, 2002). Tobacco plants with suppressed APX1 expression contained elevated levels of transcripts encoding cytosolic CuZnSOD, catalase, and glutathione reductase to compensate for the loss of APX1 (Rizhsky *et al.*, 2002a). Interestingly, the response of *Arabidopsis* plants to *Apx1* deficiency was different. Under normal growth conditions, we could not detect an induction of catalase, CuZnSOD, or glutathione reductase (Table 2). Furthermore, we did not detect an increase in the expression of the cytosolic APX isozymes, APX2 (Figures 4 and 5) and APX3 (Table 2), or the chloroplastic APX isozymes (Table 2). In contrast, we found that the expression of chloroplastic CuZnSOD was suppressed in knockout-*Apx1* plants (Table 2). This response may result from the relatively high sensitivity of this isozyme to H_2O_2 (Scioli and Zilinskas, 1988), or may represent an attempt by knockout-*Apx1* plants to reduce the level of H_2O_2 produced in chloroplasts. If the latter is correct, a new question may arise, i.e. how is superoxide formation suppressed in the chloroplasts of knockout-*Apx1* plants?

The reason(s) underlining the differences between the response of tobacco and *Arabidopsis* to *Apx1* deficiency is unknown. It is possible that in contrast to *Arabidopsis*, tobacco with its tetraploid genome is able to compensate for *Apx1* deficiency in a more efficient manner. Tobacco was found to have a high degree of plasticity in response to APX1, catalase, or APX1 + catalase deficiency, and was able to compensate for the loss of APX1 in a manner that prevented the accumulation of H_2O_2 in cells (Rizhsky *et al.*, 2002a). In contrast, *Arabidopsis*, that did not induce alternative H_2O_2 scavenging enzymes, was unable to prevent the accumulation of H_2O_2 (Figure 3) and had a delayed growth and flowering phenotype (Figure 1; Table 1).

An essential component of the response of *Arabidopsis* to the lack of APX1, possibly resulting from the accumulation of H_2O_2, appeared to be the enhanced expression of transcripts encoding the iron-binding protein ferritin, and a copper-binding protein (blue-copper-binding protein; Table 3). These may be critical for sequestering of free iron and copper ions and preventing the formation of hydroxyl radicals. Although the expression of catalase and other H_2O_2-scavenging enzymes was not elevated in knockout-*Apx1* plants under low-light conditions (100 μmol m^{-2} sec^{-1};

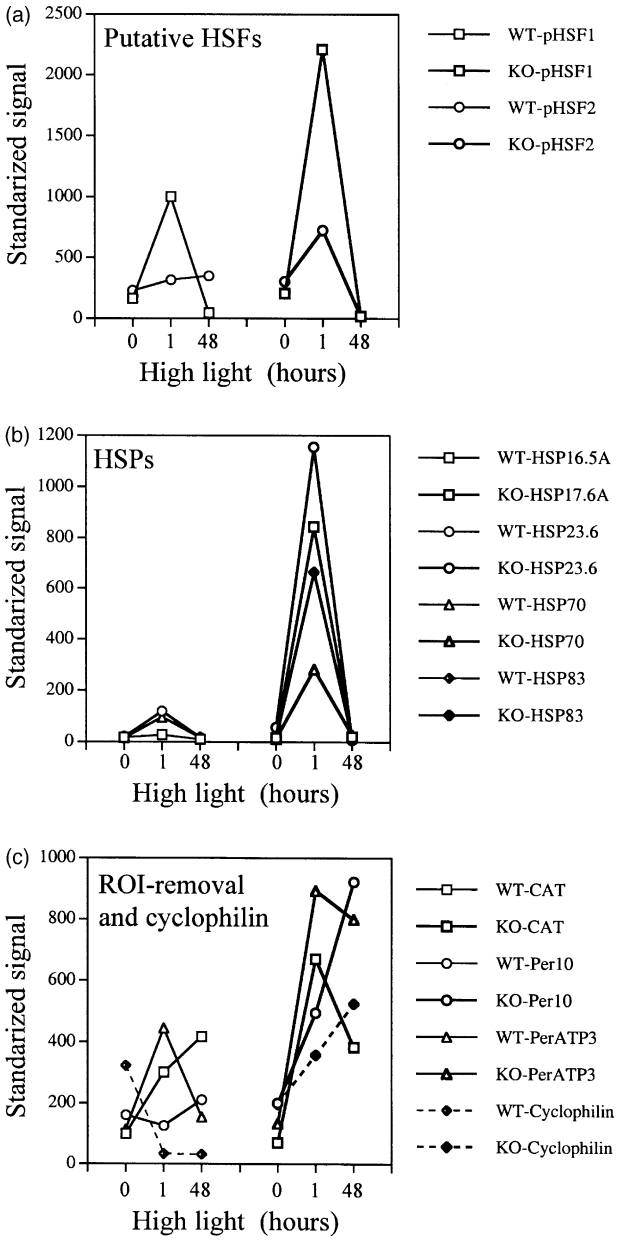

Figure 5. Analysis of gene expression during light stress in knockout-*Apx1* (KO-APX) plants.
Wild-type (WT) and KO-APX plants were subjected to light stress (425 μmol m^{-2} sec^{-1}) for 0, 1, and 48 h, and changes in transcript levels were assayed by DNA arrays (chips). (a) and (b) show the changes in expression of the two putative heat shock transcription factors (HSFs) and heat shock proteins (HSPs), and (c) shows the changes in expression of catalase (CAT), two peroxidases, per10 (peroxidase prxr10) and perATP3 (peroxidase ATP3a), involved in H_2O_2 removal, and cyclophilin. The fold change between the induction of these transcripts in WT and KO-APX plants is also shown in Table 5. Protocols for RNA isolation and DNA chip analysis are described in Experimental procedures and as Supplementary Material.

Table 2), the treatment of knockout-*Apx1* plants with high light (425 μmol m^{-2} sec^{-1}) resulted in the induction of catalase and at least two different peroxidases (prxr10 and ATP3a; Table 5). Typical plant peroxidases were not considered to play an important role in H_2O_2 scavenging in plants (Asada and Takahashi, 1987). The finding that at least two typical plant peroxidases are induced in knockout-*Apx1* plants during light stress may, however, change this concept, especially because some typical peroxidases can use ascorbic acid as their reducing substrate (Asada and Takahashi, 1987; Mittler and Zilinskas, 1992). Further studies are required to examine the possibility that prxr10 and ATP3a are involved in H_2O_2 removal in plants.

*Signal transduction transcripts elevated in knockout-*Apx1 *plants*

The majority of signal transduction transcripts induced in knockout-*Apx1* plants (Tables 3 and 5) were not reported to be involved in the H_2O_2 signal transduction pathway (Czernic *et al.*, 1999; Desikan *et al.*, 2001; Kovtun *et al.*, 2000; Samuel *et al.*, 2000; Vranova *et al.*, 2002). As was previously suggested (Bowler and Fluhr, 2000; Knight and Knight, 2001), calcium appears to play a central role in H_2O_2 signaling in plants. Thus, we found that the expression of transcripts encoding at least four different calcium-binding proteins, two calmodulin-like proteins, CaBP22 and calmodulin 9, was elevated in knockout-*Apx1* plants. Interestingly, the expression of transcripts encoding a cyclic nucleotide-gated channel (CNGC4), possibly involved in calcium signaling and stomatal responses, was upregulated in knockout-*Apx1* plants (Table 3). Because knockout-*Apx1* plants were impaired in their stomatal responses (Figure 2b), it is possible that the abnormal expression of this cyclic nucleotide-gated channel is linked to these alterations in knockout-*Apx1* plants. Hydrogen peroxide was suggested to play a central role in ABA-mediated stomatal closure by directly activating a calcium channel in guard cells (Pei *et al.*, 2000). Using whole plants deficient in APX1 (Figure 1) and containing higher than normal levels of H_2O_2 (Figure 3b), we found that H_2O_2 may also be involved in the opening of guard cells during a shift of plants from dark to light (Figure 2b). Alternatively, the enhanced levels of H_2O_2 in knockout-*Apx1* plants may have prevented the opening of stomata because they induced stomatal closure as proposed by Pei *et al.* (2000). We are currently studying how *Apx1* deficiency affects ion currents in intact guard cells of knockout-*Apx1* plants.

A number of putative receptor-like kinases were upregulated in knockout-*Apx1* plants (Table 3). We did not, however, detect an enhanced expression of a two-component histidine kinase, previously suggested to play a role in H_2O_2 sensing (Desikan *et al.*, 2001). It is also not clear whether the receptor-like kinases shown in Table 3 are involved in H_2O_2 sensing. However, because their expression is elevated in plants containing higher than normal levels of H_2O_2 (Figure 3b), such a role is possible. The expression of at

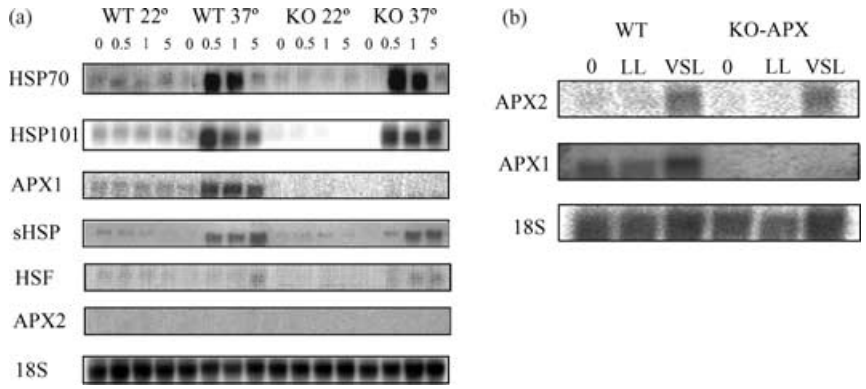

Figure 6. The induction of heat shock proteins (HSPs) during heat shock is not affected in knockout-*Apx1* (KO-APX) plants.
(a) RNA gel blots performed with RNA obtained from control and heat shock (37°C)-treated WT and KO-APX plants showing that the induction of heat shock proteins in knockout-*Apx1* plants during heat shock is not altered.
(b) A control RNA gel blot performed with RNA obtained from WT and KO-APX plants subjected to high light stress (2000 μmol m^{-2} sec^{-1}) showing the induction of APX2 under these conditions in WT and KO-APX plants. Methods and experimental protocols are described in Experimental procedures. KO, KO-APX; sHSP, small HSP; VSL, very strong light (2000 μmol m^{-2} sec^{-1}). The probe used for the detection of HSF was a non-specific HSF probe.

least two serine/threonine kinases and a protein phosphatase 2C (PP2C) was enhanced in knockout-*Apx1* plants, suggesting that different protein phosphorylation reactions are involved in H_2O_2 sensing. Protein phosphatase 2C was previously linked to different abiotic stress conditions including drought, cold, and salt stresses (Rodriguez, 1998). The induction of this transcript in knockout-*Apx1* plants in the absence of any external stresses may suggest that protein phosphatase 2C induction during different environmental stresses is mediated at least in part by H_2O_2. Interestingly, we did not detect an enhanced expression of MAPK3 or MAPK6, previously linked to H_2O_2 sensing in plants (Kovtun *et al.*, 2000). Instead, we detected an increase in the expression level of MAPK4 (Table 1).

Many of the transcription factors elevated in knockout-*Apx1* plants are also induced during biotic or abiotic stresses (Table 3). Based on our findings and at least one additional report (Desikan *et al.*, 2001), the ZAT zinc finger family of transcription factors may be linked to H_2O_2 responses. Other transcription factors involved in H_2O_2 sensing are WRKY, heat shock transcription factors, and ethylene response factors (Table 3; Mittler, 2002). Because the APX1 promoter contains at least one functional heat shock transcription factor (HSF) binding site (Storozhenko *et al.*, 1998), the induction of HSF4 and HSF21 in knockout-*Apx1* (Table 3) plants may suggest that these factors are involved in H_2O_2 signaling in plants.

A link between the heat shock response and H_2O_2 accumulation during light stress in Arabidopsis

We identified a link between H_2O_2 and the induction of heat shock proteins during light stress in *Arabidopsis* (Figures 4 and 5; Table 5). It was previously reported that in *Arabidopsis*, high light stress results in the induction of the cytosolic APX isozymes, APX1 and APX2 (Mullineaux and Karpinski, 2002; see also Figure 4 for APX1). However, the significance of this induction was not entirely clear because H_2O_2 is predominantly produced in the chloroplast and peroxisomes during high light stress. Because H_2O_2 can be transported through aquaporins (Henzier and Steudle, 2000), and because H_2O_2 was shown to leak from isolated chloroplasts treated with high light (Asada *et al.*, 1974), it was postulated that the induction of APX1 and APX2 protects the cytosol and other cellular compartments during high light stress (Mittler, 2002; Mullineaux and Karpinski, 2002). Here, we show that in the absence of APX1, light stress in *Arabidopsis* results in the augmented induction of heat shock proteins (Figures 4 and 5; Table 5), suggesting that H_2O_2 produced during light stress in *Arabidopsis* diffuses into the cytosol and activates a signal transduction pathway that enhances the expression of heat shock proteins in the different cellular compartments. In the absence of APX1 this induction is much stronger because H_2O_2 that leaks into the cytosol is not scavenged. This model, shown in Figure 7, suggests that at least part of the induction of heat shock proteins during light stress in *Arabidopsis* is mediated by H_2O_2 that is scavenged by APX1. In contrast, the induction of heat shock proteins during heat shock may be mediated by a different pathway that does not involve APX1 (Figure 6).

Growth suppression as a result of H_2O_2 accumulation

The cause of inhibition of plant growth and flowering time in *Apx1*-deficient *Arabidopsis* (Figure 1; Table 1) is unknown. It is possible that the enhanced levels of H_2O_2 in these plants (Figure 3) affected the expression of transcription factors involved in the regulation of plant growth and flowering. Alternatively, it is possible (however, unli-

kely; Asada and Takahashi, 1987) that APX1, similar to certain typical peroxidases, is involved in the biodegradation of auxin. Because the growth suppression of knockout-*Apx1* plants was dependent upon day length and light intensity (not shown), it is reasonable to assume that this effect was directly linked to H_2O_2 accumulation in cells and not to an effect of APX1 activity on the level of auxin. Furthermore, a similar developmental effect was not observed in tobacco plants expressing an antisense construct to APX1. These plants induced alternative H_2O_2 scavenging pathways and did not contain elevated levels of H_2O_2 (Rizhsky *et al*., 2002a). Although our chip analysis revealed that the expression of GIGANTEA, a gene involved in the determination of flowering time in *Arabidopsis* (Fowler *et al*., 1999), was elevated during light stress in knockout-*Apx1* plants (Table 5), it is not clear whether this gene is directly involved in the suppression of flowering time in knockout-*Apx1* plants. More research is needed to establish a link between the accumulation of H_2O_2 in cells during normal conditions or stress, and the inhibition of growth and flowering time of plants. The knockout-*Apx1* plants described in this report may provide an entry point into these studies because they may mimic the effect of different environmental stresses on plant growth and development through a known substrate, i.e. H_2O_2.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana (cv. WS) plants were grown in growth chambers (Percival E-30HB and Conviron E7-2) under controlled conditions: 21–22°C, 18 h or constant light cycle, 100 μmol m^{-2} sec^{-1}, and a relative humidity of 70%. Knockout *Arabidopsis* plants (cv. WS) containing a T-DNA insert in *Apx1* were obtained from the *Arabidopsis* knockout facility at the University of Wisconsin-Madison according to the knockout facility recommended protocols (http://www.biotech.wisc.edu/Arabidopsis/) using the following DNA primers: JL-202 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′ and APXI 5′-TTTTCCCATCTATATACCACCAACCCTAA-3′. The selected knockout-*Apx1* plants plants were out-crossed and selfed to check for segregation and to obtain a pure homozygote line as recommended by Sussman *et al*. (2000). Confirmations of APX1 deficiency and segregation analysis were performed by PCR, genomic DNA blots, and RNA and protein blots.

Stress treatments

Light stress was performed by increasing the light intensity from 100 to 425 μmol m^{-2} sec^{-1}. Controlled plants were kept at 100 μmol m^{-2} sec^{-1}. All other growth parameters were maintained constant. At different times (0, 1, 4, 24, and 48 h), plants were sampled for RNA analysis. Heat shock was performed by changing the temperature from 22 to 37°C. Controlled plants were kept at 22°C. All other growth parameters were maintained constant. At different times (0, 0.5, 1, and 5 h), plants were sampled for RNA analysis. All experiments were performed in parallel on wild-type and knockout-*Apx1* plants (each in triplicates).

Molecular, physiological, and biochemical analysis

RNA and protein were isolated and analyzed by RNA and protein blots as previously described (Pnueli *et al*., 2002). A ribosomal 18S rRNA probe or ethidium bromide staining was used to control for RNA loading. Coomassie Blue R-250 staining of protein gels was used to control for protein loading. Photosynthesis, stomatal conductance, and dark respiration were measured with a Li-Cor LI-6400 apparatus as described by Rizhsky *et al*. (2002b) using the *Arabidopsis* leaf chamber (Li-Cor, Lincoln, NE, USA). These measurements were performed on plants kept in the dark for 30 min and shifted to light (100 or 200 μmol m^{-2} sec^{-1}) for 15 min. To induce stomatal closure by ABA, a solution of 50 μM ABA was sprayed on plants in light (100 μmol m^{-2} sec^{-1}). Following a 1 min incubation, leaves were clamped and the decrease in stomatal conductance was measured with LI-6400 as described above. Reduced glutathione was determined by HPLC as described in Xiang and Oliver (1998), H_2O_2 was assayed as described by Rizhsky *et al*. (2002a), and APX activity was measured according to Mittler and Zilinskas (1992).

DNA chip analysis

In three independent experiments, RNA was isolated from 40 to 50 wild-type or knockout-*Apx1* plants (a total of 120–150 plants per line), grown under controlled conditions as described above, or subjected to light stress. This RNA was pooled and used to perform the chip analysis. Each of the different pools of wild-type or knockout-*Apx1* plants, grown under non-stressful conditions, was assayed by three different chips. Each of the different pools of wild-type or knockout-*Apx1* plants, subjected to 0, 1, and 48 h light stress, was assayed by one chip. Affymetrix chip analysis (*Arabidopsis* 8200 gene chip; Affymetrix, Santa Clara, CA, USA) was performed at the University of Iowa DNA facility (http://dna-9.int-med.uiowa.edu/microarrays.htm). Conditions for RNA isolation, labeling, hybridization, and data analysis are described as Supplementary Material (in accordance with MIAME recommendations). A comparative analysis of samples was performed with the GeneChip mining tool V 5.0 and the Silicon Genetics GeneSpring V 5.1. Some of the comparison results were confirmed by RNA blots.

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Supplementary Material

Files showing the expression level of transcripts with unknown function, elevated or suppressed in knock-out *Apx1* plants, as well as files with Affymetrix chip data, are available to download from the following website: http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1715/TPJ1715sm.htm

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