Evidence for a Direct Link between Glutathione Biosynthesis and Stress Defense Gene Expression in Arabidopsis [W]

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The mutant *regulator of APX2 1-1* (*rax1-1*) was identified in *Arabidopsis thaliana* that constitutively expressed normally photooxidative stress-inducible *ASCORBATE PEROXIDASE2* (*APX2*) and had ≥50% lowered foliar glutathione levels. Mapping revealed that *rax1-1* is an allele of γ-*GLUTAMYLCYSTEINE SYNTHETASE 1* (*GSH1*), which encodes chloroplastic γ-glutamylcysteine synthetase, the controlling step of glutathione biosynthesis. By comparison of *rax1-1* with the *GSH1* mutant *cadmium hypersensitive 2*, the expression of 32 stress-responsive genes was shown to be responsive to changed glutathione metabolism. Under photo-oxidative stress conditions, the expression of a wider set of defense-related genes was altered in the mutants. In wild-type plants, glutathione metabolism may play a key role in determining the degree of expression of defense genes controlled by several signaling pathways both before and during stress. This control may reflect the physiological state of the plant at the time of the onset of an environmental challenge and suggests that changes in glutathione metabolism may be one means of integrating the function of several signaling pathways.

INTRODUCTION

Plants exposed to environmental stress show diminished photosynthetic metabolism and increased photoreduction of molecular oxygen (O_2), photorespiration, and dissipation of excitation energy at photosystem II (Asada, 1999; Ort and Baker, 2002). Although these processes are protective, they lead to increased formation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), singlet O_2, and hydrogen peroxide (H_2O_2; Asada, 1999). The prevention of oxidative stress is achieved by a network of low molecular weight antioxidants, enzymes that keep them reduced, and ROS-scavenging enzymes (Karpinski et al., 1997; Asada, 1999). The accumulation of ROS also activates defense gene expression as part of protective responses to both biotic and abiotic stimuli (Karpinski et al., 1999; Grant and Loake, 2000; Fryer et al., 2003; op den Camp et al., 2003). Transcript profiling studies, in some cases using transgenic plants altered in expression of antioxidant enzymes, indicate that coordinated regulation of a network of

defense and antioxidant systems occurs (Willekens et al., 1997; Rossel et al., 2002; Pneuli et al., 2003).

A key component of the antioxidant network is the thiol glutathione, which is synthesized from its constituent amino acids, L-Glu, L-Cys, and Gly, in an ATP-dependent two-step pathway catalyzed by the enzymes γ-glutamylcysteine synthetase ([γ-ECS]; EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3), respectively (Noctor et al., 2002). To date, two genes, one coding for plastidial γ-ECS (*GSH1*) and one putative cytosolic glutathione synthetase (*GSH2*), have been identified in *Arabidopsis thaliana* and many other plant species (May and Leaver, 1993;; Rawlins et al., 1995; Cobbett et al., 1998; Noctor et al., 2002). Overexpression or inhibition of *GSH1* expression causes Arabidopsis to have enhanced or depressed levels of glutathione, respectively (Cobbett et al., 1998; Xiang and Oliver, 1998; Vernoux et al., 2000). In keeping with studies on γ-ECS from other organisms, the plant enzyme is considered to be a key regulatory step in glutathione biosynthesis and may be controlled at the level of enzyme activity, synthesis of protein and mRNA (Xiang and Oliver, 1998; Noctor et al., 2002).

Glutathione, primarily in its reduced form (GSH), is present at concentrations of 2 to 3 mM in various plant tissues (Creissen et al., 1999; Meyer and Fricker, 2002; Noctor et al., 2002). Because glutathione is a major cellular antioxidant, it is regarded as a determinant of cellular redox state and may indirectly have an influence on many fundamental cellular processes (Cooper et al., 2002; Noctor et al., 2002; Schafer and Buettner, 2001). Glutathione can engage in thiol-disulphide exchange reactions

that may be a key process in linking the regulation of gene expression to the redox state of cells or specific subcellular compartments (Schafer and Buettner, 2001; Noctor et al., 2002). In plants, the number of regulatory processes that are known to be potentially influenced by the levels or redox state of cellular glutathione pools is small. The regulation of plastid gene expression by the redox state of the glutathione pool provide the best studied examples to date. These include the translation of *rbcL* mRNA, the processing of specific plastid-encoded transcripts, and the modulation of RNA polymerase by a redox-sensitive protein kinase (Irihimovitch and Shapira, 2000; Pfannschmidt, 2003). Not many examples exist that have indicated the possibility of glutathione redox-mediated control of nuclear-located defense gene expression. Glutathione may activate the regulatory proteins NPR1 and possibly protein phosphatase 2C (ABI2), important in salicylic acid (SA) and abscisic acid (ABA) signaling, respectively (Meinhard et al., 2002; Mou et al., 2003). Earlier studies in which glutathione was fed to cells or leaves has been shown to both induce and suppress expression of a range of defense genes (Wingsle and Karpinski, 1996; Karpinski et al., 1997, 2000; Wingate et al., 1988; Loyall et al., 2000). However, given the many aspects of cellular metabolism that glutathione is engaged in (Noctor et al., 2002), such feeding data do not constitute evidence for a direct role in the regulation of antioxidant defense genes.

Under nonstress conditions, *ASCORBATE PEROXIDASE2* (*APX2*), which encodes a component of the antioxidant network, is expressed at extremely low levels. However, when leaves are subjected to excess light or wounding that induces photooxidative stress, the expression of the gene is rapidly induced in bundle sheath tissue (Karpinski et al., 1997, 1999; Fryer et al., 2003; Chang et al., 2004). In this study, it is shown that the mutant *regulator of APX2 1-1* (*rax1-1*), which expresses *APX2* in the absence of excess light or wounding stress, is a lesion in *GSH1*. Thus, a direct link was established between expression of an antioxidant defense gene under steady state (nonstressed conditions) and glutathione. Further analysis of gene expression showed that only stress defense genes were affected by glutathione, ruling out wider effects of this mutation on cellular metabolism. Comparison of expression profiles under high light stress and wounding in *rax1-1* and *cadmium hypersensitive 2-1* (*cad2-1*, another mutant in *GSH1*; Cobbett et al., 1998) revealed unexpected differences despite similar gluthathione levels. From these data, it is deduced that both glutathione metabolism and levels can be considered to influence the poising of cellular defences before their induction as well as the actual response to an external stress.

RESULTS

Isolation and Initial Characterization of Mutants Expressing *APX2* under Nonstress Conditions

An Arabidopsis (Columbia-0 [Col-0]) line transformed with an excess light stress-inducible *APX2* promoter-*LUCIFERASE* gene fusion (*APX2LUC*; Karpinski et al., 1999) was chemically mutagenized and used to screen for *APX2* expression in the absence of excess light stress (see Methods). After screening, two mutants were identified that had a stable, heritable luciferase-positive phenotype (example in Figure 1A). The mutant lines were visually indistinguishable from wild-type plants at all stages of their life cycle under both long (18-h photoperiod) and short (8-h photoperiod) day conditions. All data presented here are from selfed progeny of the fifth backcrossed generation. We have assigned only a single allele number to the mutants and refer to them in this article as *rax1-1*. This is because subsequent sequencing of the mutant loci revealed that the lesion was the same in both mutants (see below).

Level and Distribution of *APX2* Expression in *rax1-1*

In contrast with wild-type plants under nonstress conditions, *rax1-1* plants had detectable levels of *APX2* expression, although this was always less than the levels detected in excess light stressed wild-type plants (Figures 1B and 2A). Based on in vitro determinations of luciferase activity, *rax1-1* had two orders of magnitude greater luciferase activity than the basal activity in nonstressed wild-type plants (Col-0/*APX2LUC* = 167 ± 21 counts per second [cps] mg protein^{-1} [±SE; $n = 43$] versus Col-0/*rax1-1*/*APX2LUC* = 38,676 ± 4354 cps mg protein^{-1} [±SE; $n = 37$]). However, the expression of *APX2LUC* in the mutant under low light conditions was only 7% that of excess light-stressed wild-type plants (Col-0/*APX2LUC* + excess light = 55,1576 ± 123,298 cps mg protein^{-1} [±SE; $n = 6$]).

In long day–grown nonstressed *rax1-1* plants, *APX2LUC* expression was first detected in vivo in the apical region of soil-grown plants at 10 d after germination (Figure 1A, panel 10d). This was followed by a continued increase in detectable luciferase activity in the petioles, midribs, leaf veins, and apex up to the maximum number of nine fully expanded leaves (up to 28 d; Figure 1A, panels 16d and 17d). During these times, expression of *APX2LUC* was not subject to a diurnal rhythm in either short day– or long day–grown plants (data not shown). However, luciferase activity was never detected over the whole of the vascular tissue in any leaf under nonstress conditions, and some leaves failed to express detectable activity (Figure 1A). Thereafter, luciferase activity declined such that it could not be imaged in senescing leaves, although luciferase activity was observed at the axis between cauline leaves and the stem of flower bolts in plants from 28 d onward (bolts >6 cm in length; Figure 1A, panel 32d). *APX2LUC* activity in *rax1-1* was never detected in roots, flowers, siliques, seeds, young plants (more than six true leaves), and cotyledons (data not shown). In situ hybridizations on transverse sections of *rax1-1* leaves showed expression of the gene to be limited to the vasculature (see Supplemental Figure 1S online), identical to the expression pattern of *APX2* in excess light-stressed wild-type plants (Fryer et al., 2003).

Depressed Glutathione Levels in *rax1-1*

Under nonstress conditions, there was no effect of *rax1-1* on the foliar levels of H$_2$O$_2$, lipid peroxidation products, and the levels or redox state of ascorbate and photosynthetic electron transport (data not shown). However, *rax1-1* had 20 to 50% of the foliar level of glutathione compared with wild-type plants over a range
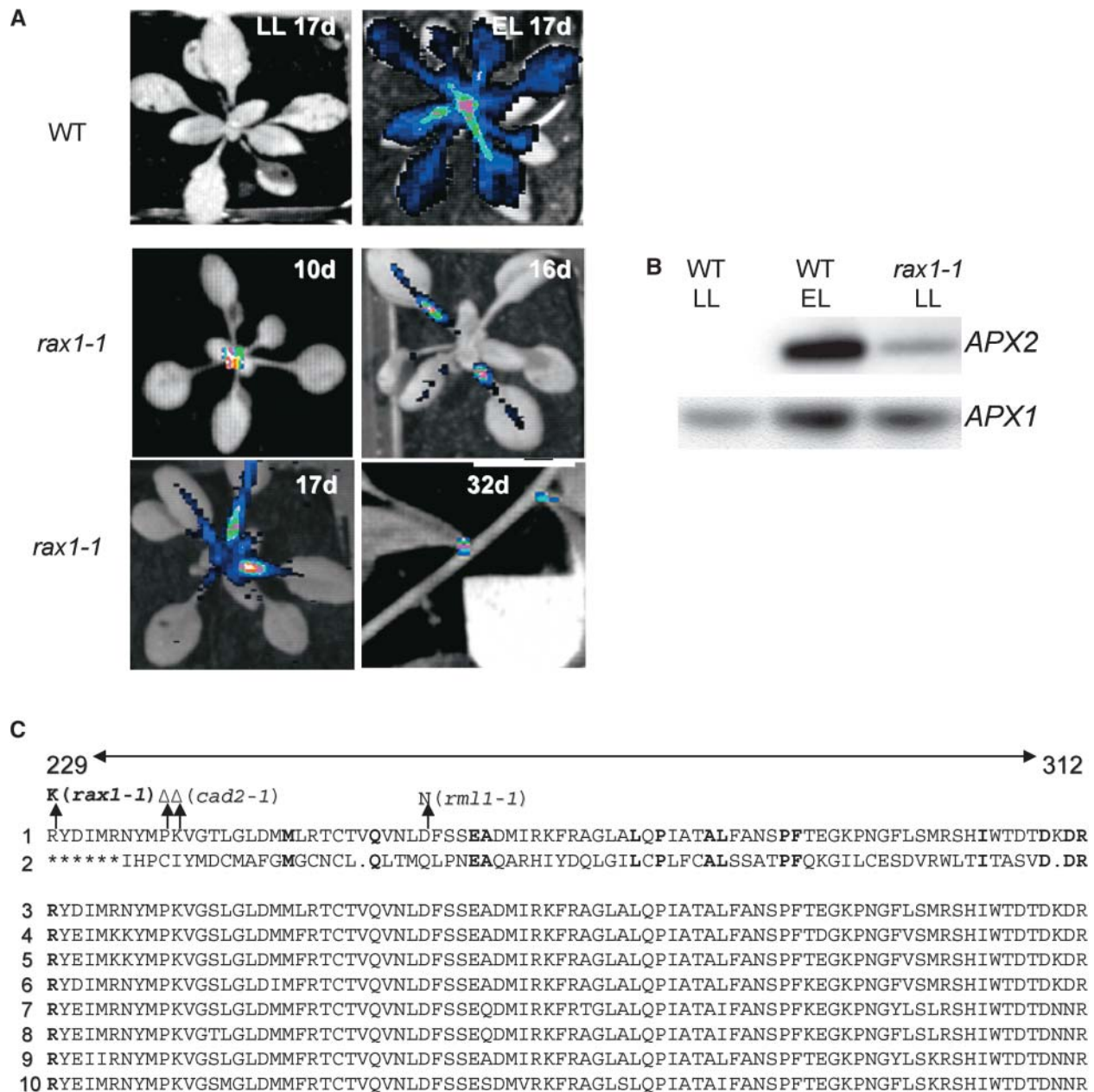
Figure 1. Constitutive *APX2* Expression in *rax1-1*, a Lesion in *GSH1*.

(A) Typical false color image from a CCD camera of luciferase activity in a long day/low light–grown, 17-d-old wild-type *APX2LUC* Arabidopsis rosette before and after exposure to a 10-fold excess light stress for 45 min (LL 17d and EL 17d, respectively) and in long day–grown *APX2LUC/rax1-1* plants at 10, 16, 17, and 32 d after germination. The background image of rosettes was taken when the plants were first placed under the camera, and the luciferase image was taken, after 3 min in the dark, for one minute with an aperture setting of 1.8.

(B) PCR-based detection of *APX2* transcript under nonstress conditions in *rax1-1*. Rapid amplification of cDNA 3′ends (3′RACE) PCR-amplified *APX2* and *APX1* cDNA, equivalent to 3 µg of total RNA, was separated by agarose gel electrophoresis, blotted, and hybridized to ^{32}P-labeled gene-specific probes. In the lane with wild-type excess light (EL) *APX2*-specific PCR products 100-fold less volume was loaded as for the low light (LL) samples from wild-type or *rax1-1* plants. The RNA was pooled from three separate plants harvested on two occasions ($n = 6$). Detection of *APX1* was used here as a control for the PCR.

(C) Alignment of the derived amino acid sequences of γ-ECS residues 229 to 312 from Arabidopsis (1) with that from trypanosome (2) and eight other plant species (3 to 10). This region includes the putative catalytic domain as defined by Leuder and Phillips (1996). The alignment between Arabidopsis and trypanosome with conserved residues in bold is from the same article. The asterisks indicate where the trypanosome sequence shows no homology with those from rat, yeast, and nematode. The *rax1-1* (R229K) mutation is shown as well as *cad2-1* (deletion P238, K239; Cobbett et al., 1998) and *rml1-1* (D259N; Vernoux et al., 2000). The plant γ-ECS sequences are from Indian mustard (3; Y10848), *Medicago truncatula* (4; AF041340), pea (*Pisum sativum*) (5; AF128455), dwarf bean (*Phaseolus vulgaris*) (6; AF128454), rice (*Oryza sativa*) (7; AJ508916), maize (*Zea mays*) (8; AJ302783), tomato (*Lycopersicon esculentum*) (9; AF017983), and onion (*Allium cepa*) (10; AF401621).

Table 1. Thiol Determinations in the Wild Type (Col-0/*APX2LUC*), *rax1-1*, and *cad2-1*

Thiol	Wild Type	*rax1-1*	*cad2-1*
Glutathione	263 ± 88 ($n = 11$)	85 ± 14 ($n = 21$)	73 ± 10 ($n = 12$)
γ-EC	2.8 ± 0.4 ($n = 6$)	2.9 ± 0.5 ($n = 6$)	n.d. ($n = 12$)
Cys	60 ± 10 ($n = 9$)	48 ± 9 ($n = 20$)	82 ± 5 ($n = 4$)
Glutathione (leaf)	345 ± 58 ($n = 3$)	88 ± 45 ($n = 6$)	85 ± 39 ($n = 3$)
Glutathione (petiole)	278 ± 40 ($n = 3$)	70 ± 15 ($n = 6$)	93 ± 50 ($n = 3$)
Glutathione 10 dpg LD	282 ± 28 ($n = 6$)	87 ± 52 ($n = 6$)	–
Glutathione 14 dpg LD	281 ± 21 ($n = 6$)	119 ± 18 ($n = 6$)	–
Glutathione 18 dpg LD	256 ± 48 ($n = 6$)	60 ± 10 ($n = 6$)	–
Glutathione 20 dpg LD	377 ± 79 ($n = 6$)	68 ± 20 ($n = 6$)	–

Levels of various thiols were determined in 5-week-old plants grown in short day (8-h photoperiod) conditions. Glutathione refers to total glutathione content (i.e., GSH + GSSG). Plants kept under long day conditions (LD) were at the same developmental stages (dpg is days postgermination) as those shown in Figure 1A. All foliar thiol contents are calculated in nmols g fresh weight^{-1}. The assays were conducted on acid extracts of leaves by derivitization with monobromobimane and separation of fluorescent thiol conjugates by reverse-phase HPLC (see Methods). n.d., not detected; –, not determined.

of plant ages, irrespective of the photoperiod (Table 1). There was no effect on glutathione redox states at any of the stages of vegetative growth (data not shown). By contrast, foliar levels of Cys and γ-glutamylcysteine (γ-EC) were similar in *rax1-1* and wild-type plants (Table 1). The levels of glutathione in *rax1-1* were similar to that reported for *cad2-1*. However, in contrast with *rax1-1* and wild-type plants, γ-EC was not detectable, and Cys levels were 37 to 70% higher in *cad2-1* (Table 1). As in *cad2-1* (Cobbett et al., 1998), γ-ECS activity was lower in *rax1-1* compared with wild-type plants (*rax1-1*: 3.3 nmols γ-EC formed min^{-1} g fwt^{-1} [SD ± 0.3; $n = 3$]; Col-0/*APX2LUC* 13.7 nmols γ-EC formed min^{-1} g fwt^{-1} [SD ± 2.5; $n = 3$])

Mapping and Identification of the *rax1-1* Locus

The *rax1-1* phenotype segregated as a single recessive Mendelian trait when crossed with Col-0 (observed, 327 wild type, 73 *rax1-1* phenotype; expected, 325 wild type, 75 *rax1-1* phenotype, $n = 400$, $\chi^2 = 0.066$, based on cosegregation of a recessive *rax1-1* locus and a *APX2LUC* dominant transgene [13:3]). Mapping of the *rax1-1* mutation using 48 F2 progeny displaying the mutant phenotype showed it was on chromosome 4 located between the *FCA6* locus and BAC clone F28M11. This interval also harbors *GSH1* (At4g23100). Because *rax1-1* plants had lower levels of glutathione and γ-ECS activity, similar to *cad2-1* (Table 1), we reasoned that *rax1-1* could also be a *GSH1* mutation. Therefore, the genes and cDNAs encoding *GSH1* from both mutant types were sequenced. For both mutant types at the time considered to be different alleles of the same locus,

sequencing revealed a point mutation (G to A) at coordinate 12,105,389 of the chromosome 4 sequence (The Arabidopsis Information Resource [TAIR], www.arabidopsis.org, November 2003). This coordinate is equivalent to coordinate 2883 of the sequence published by Cobbett et al. (1998) (EMBL: AF068299). This mutation was in exon 6 of *GSH1* and resulted in an Arg (R229) to Lys (K) change in the γ-ECS sequence (Figure 1C). This Arg residue is conserved in the putative γ-ECS sequences from eight plant species (Figure 1C). In addition to the point mutation at coordinate 12,105,389, one of the mutant types had an additional point mutation (A to G) at coordinate 12,105,405. This second mutation produced no change in the sequence of γ-ECS (data not shown) but provided confirmation that both mutants arose independently from the screening but were the same allele.

To confirm that *rax1-1* was a *GSH1* mutant allele, crosses were done with *cad2-1* plants and also a transgenic line expressing a *GSH1* cDNA under the control of the *Cauliflower mosaic virus* 35S promoter (Xiang et al., 2001). The *rax1-1* and *cad2-1* mutants failed to complement one another because glutathione levels were not restored to wild-type levels in F1 progeny arising from a *cad2-1* × *rax1-1* cross (Table 2). Equally, the *rax1-1* phenotype was abolished in homozygous *rax1-1* individuals also harboring the 35S:*GSH1* cDNA fusion in that glutathione levels were restored to wild-type levels (Table 2), and this was associated with a loss of luciferase activity from *APX2LUC* (data not shown). Therefore, we concluded that the lesion in *GSH1* is the cause for the *rax1-1* phenotype.

Expression of Genes and Enzymes of the Antioxidant Network in *rax1-1* and *cad2-1*

In contrast with nonstressed *rax1-1* plants (Figures 1A and 1B), *APX2* expression was not detected in nonstressed *cad2-1* plants (Figure 2A). In a survey of the expression of 25 antioxidant defense genes in nonstressed *rax1-1* plants, four additional genes with increased transcript levels were found (*APX1*, *FSD1*, *DHAR1*, and

Table 2. Complementation Analysis of *rax1-1* with *cad2-1* and *35S-GSH1*

Genotype (Col-0)	Glutathione (nmols g fwt^{-1})	n
cad2-1:rax1-1:APX2LUC	145 ± 6 (118)	10
rax1-1:APX2LUC	123 ± 14 (100)	7
cad2-1	103 ± 10 (84)	3
APX2LUC	246 ± 44 (200)	6
35S:*GSH1:APX2LUC*	378 ± 19 (307)	3
35S:*GSH1:rax1-1:APX2LUC*	247 ± 9 (201)	6

Total glutathione content in leaves from F1 progeny arising from a cross between *cad2-1* and *rax1-1* parents compared with the wild type (Col-0/*APX2LUC*) and the *rax1-1* and *cad2-1* parents. Glutathione was determined as in Table 1. The numbers in parentheses are the values relative to *rax1-1/APX2LUC*. The presence of both the *rax1-1* and *cad2-1* mutation in each selected hybrid was confirmed as correct by sequencing of the PCR-amplified *GSH1* gene over both mutations (see Figure 1 and Methods). Similarly, the presence of the *35S-GSH1* transgene (Xiang et al., 2001) was confirmed by PCR between the *Cauliflower mosaic virus* 35S promoter and the *GSH1* coding sequence.
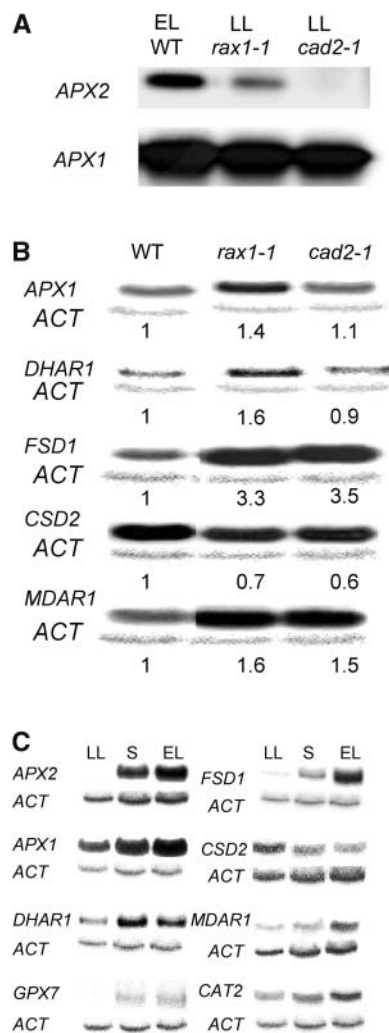
Figure 2. Comparison of Antioxidant Defense Gene Expression in *rax1-1* and *cad2-1*.

(A) PCR-based detection of *APX2* transcript under nonstress conditions in *rax1-1* and *cad2-1*. 3′RACE PCR-amplified *APX2* and *APX1* cDNAs were detected by autoradiography as in the legend of Figure 1B. The wild-type control (EL WT) is from 10-fold excess light (EL) stressed plants, and loading of the *APX2*-specific product was 1% that of the volume loaded for the mutants. The *APX1* is shown here as a PCR control for all cDNA samples. Each sample is pooled from six plants. LL, low light.

(B) Steady state levels of transcripts of antioxidant defense genes in nonstressed wild type, *cad2-1*, and *rax1-1*. RNA gel blots loaded with 20 μg of total RNA were hybridized to specific probes and washed at high stringency, and images were developed using a phosphoimager (see Methods). The RNA preparations of *rax1-1* and *cad2-1* were the same as those in **(A)**. The numbers below each row are values of signal relative to the wild type from this experiment, calculated from densitometer readings derived from the digitized images (Fryer et al., 2003). The band below each group is actin mRNA used as a loading control (see Methods).

(C) *rax1-1*–responsive gene expression in excess light–exposed and systemic leaves. Steady state levels of transcripts encoded by antioxidant defense genes in the wild type (*n* = 3) partially exposed to excess

MDAR1) whereas one, *CSD1*, had reduced transcript levels (see Supplemental Figure 2S online). This initial comparison was extended to determine if the levels of these five transcripts were altered in nonstressed *cad2-1* as well as *rax1-1* (Figure 2B). Transcript levels of both *APX1* and *DHAR1* were elevated in *rax1-1* but not *cad2-1* compared with wild-type plants, whereas those of *FSD1*, *CSD2*, and *MDAR1* were altered in *cad2-1* in the same way as in *rax1-1* (Figure 2B; see Supplemental Figure 2S online). Interestingly, these five *rax1-1* affected genes, like *APX2*, responded in the same way in exposed and systemic leaves of partially excess light-stressed wild-type rosettes (Figure 2C). Of the 19 genes whose transcript levels were not affected in *rax1-1* under nonstressed conditions, most were not induced in their expression by excess light stress, the only exceptions being those encoded by *CAT2* and *GPX7* (Figure 2C).

To determine if the observed transcript changes resulted in changes in the activities of ROS-scavenging enzymes, the activities of Fe- and Cu/Zn- amd Mn-superoxide dismutase (SOD) isoforms, monodehydroascorbate reductase (MDAR), and cytosolic ascorbate peroxidase ([APX]; Figure 3) were determined in the nonstressed mutant and wild-type plants. Whereas total SOD activity was not significantly changed in *rax1-1* and *cad2-1* compared with wild-type plants, the proportion of Fe-SOD activity was increased from 27% of the total in wild-type plants to ∼45% in both mutants (Figure 3A; P < 0.01). Similarly, Cu/Zn SOD activity was a lower proportion of total activity in both mutants (40%; P < 0.01 for *rax1-1* and P < 0.05 for *cad2-1*) compared with wild-type plants (60%). Total MDAR activity was elevated above the wild-type level in both mutants (Figure 3B), whereas an increase in the activity of cytosolic APX was observed only in *rax1-1*, but not *cad2-1*, compared with the wild type (Figure 3C). These data on enzyme activities broadly agreed with the observed changes in transcript levels.

Impact on the Arabidopsis Transcriptome of *rax1-1* and *cad2-1*

To determine if the effects of the *GSH1* mutations in *rax1-1* and *cad2-1* under nonstressed conditions were limited to genes associated with defense against oxidative stress, analysis of the levels of transcripts encoded by ∼8500 Arabidopsis genes was performed using microarrays (Zhu and Wang, 2000). A stringent analysis of the data (see Methods) cut the number of genes whose expression could be compared with wild-type plants down to 3743 and 3734 for *rax1-1* and *cad2-1*, respectively. Pairwise comparisons between these reduced data sets from wild-type plants were conducted in turn with those from *cad2-1* and *rax1-1*. Only genes that showed more than twofold differences in signal intensities between mutants and the wild type were classified as differentially expressed and analyzed further.

light (15-fold). RNA was prepared from directly exposed leaves (EL) and nonexposed leaves that responded systemically to this stress (S; Karpinski et al., 1999), compared with low light nonstressed controls (LL). The amounts of RNA used and the processing of the blots was as in **(B)**.
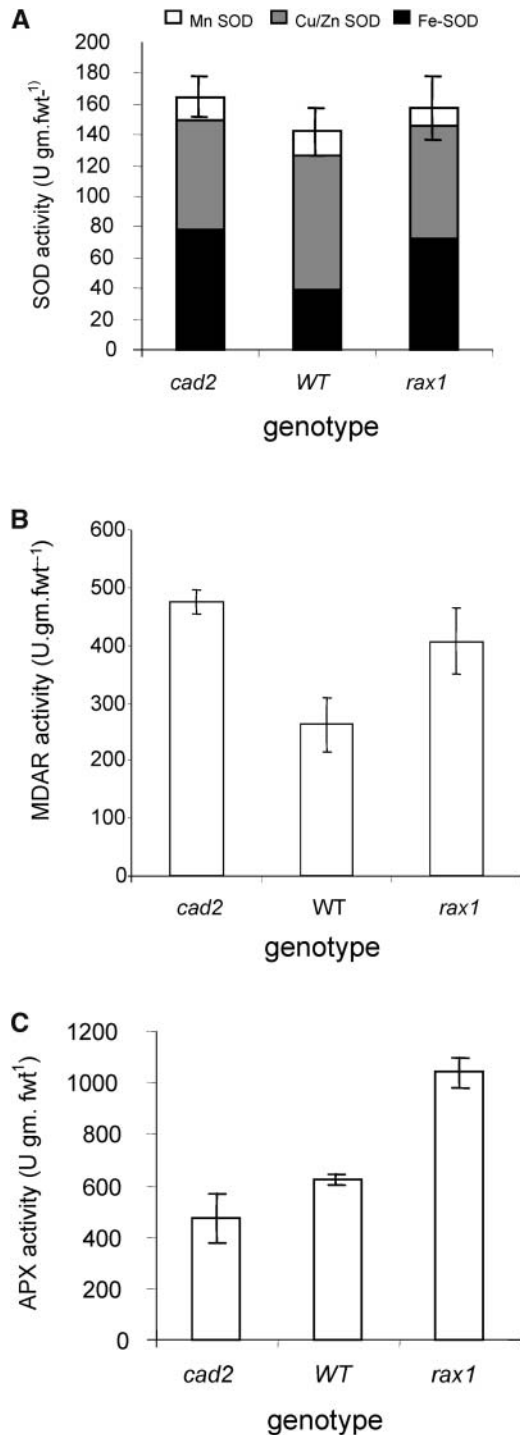
Figure 3. Antioxidant Enzyme Activities Are Altered in Nonstressed *rax1-1* and *cad2-1* Plants.

Enzyme activities in cell-free extracts of leaves pooled from six plants, combined from two separate analyses. The assays were performed according to Jimenez et al. (1997).
(A) Mn-, Cu/Zn-, and Fe-SOD.
(B) MDAR.
(C) APX.

We reasoned that genes whose expression was affected by a depressed level of foliar glutathione would be affected in the same way in *rax1-1* and *cad2-1*, and these were observed (At1g65400, At4g02330, At2g05380, At4g39640, At4g21830, At2g24850, At5g24780, At5g24770, At2g14610, At3g57260, At2g43570, At2g48110, and At4g35580; Table 3). More surprisingly, two further groups could be discerned: seven genes whose expression was only altered in *rax1-1* (At2g40300, At5g01600, At2g29580, At3g51860, At2g32880, At5g66400, and At2g46680; Table 3) and 206 genes with altered transcript level only in *cad2-1* (see Supplemental Table 1S online). The transcript levels of five of the genes listed in Table 3 were analyzed by a quantitative RT-PCR (qRT-PCR) procedure. This showed that the patterns of expression of these genes in the mutants versus wild-type plants was the same as observed in the microarray experiment, although the absolute values differed between the two methods (Table 3, numbers in parentheses).

A randomized sample of genes from both mutant data sets compared with the wild type confirmed that the difference in numbers between the pairwise comparisons was not because of the intrinsic variation in the experiment (see Methods). It should be noted that all of the genes encoding components of the antioxidant network fell outside the microarray analysis either because their levels of expression were too low to score or the changes were less than twofold.

Expression of *APX2* and Other Defense Genes in *rax1-1* and *cad2-1* Leaves Subjected to Stress

When *rax1-1* plants were subjected to excess light stress or wounding, induction of *APX2* transcript was much more pronounced than in control plants (Figures 4A and 4B). qRT-PCR confirmed that *APX2* expression after excess light stress was twofold to threefold higher in *rax1-1* compared with wild-type plants and also in *cad2-1* plants (Figure 5B). For *cad2-1*, this was despite the unaffected very low level of this transcript in low light conditions (Figure 2A). Furthermore, *APX2* transcript levels in *rax1-1*, after a 1-h exposure to fivefold excess light stress, were still detectable 1 h after the stress, when levels in the wild-type plants had returned to below the level of detection (Figure 5A).

As with the *rax1-1*–induced antioxidant defense genes (Figure 2C), the mRNA levels of the five genes selected from the microarray experiment (Table 3) were all elevated in excess light-stressed wild-type plants (Figure 5A). To ensure that qRT-PCR and RNA gel blots produced comparable results, *CSD2* and *FSD1* transcript levels were included in the qRT-PCR assay (Figures 2C and 5A). When mRNA levels for the selected genes were compared from mutants and the wild type exposed to excess light, all the genes showed altered expression patterns in the mutants although no common pattern of responses was apparent (Figure 5B). The expression of six genes that had been identified as excess light stress responsive (U. Bechtold, C. Gapper, and P.M. Mullineaux, unpublished data) but did not have altered levels in the nonstressed *rax1-1* (Figure 5A) were assessed for their expression in excess light-stressed mutants compared with wild-type plants (Figure 2B). These showed altered transcript levels in the mutants compared with the wild

Table 3. Transcript Levels of Genes Whose Expression, When Compared with Nonstressed Wild-Type Plants, Was Affected in Nonstressed *rax1-1* Alone or in Both *rax1-1* and *cad2-1*

Affymetrix Probe Set	Locus Name	Signal in *rax1-1*/Signal in Wild Type	*t* Test	Signal in *cad2-1*/Signal in Wild Type	*t* Test
Higher Transcript Levels in *rax1-1*					
16031_at	**At5g01600**	3.44	0.113	0.95	0.375
18933_at	**At2g40300**	2.15 (4.6)	0.097	1.08 (1.1)	0.153
15115_f_at	At2g05380	2.65	0.010	2.52	0.000
19577_at	At1g65400	2.36	0.001	3.15	0.024
19267_s_at	At4g02330	2.33	0.010	3.25	0.059
13255_i_at	At4g39640	2.16	0.045	3.17	0.023
Lower Transcript Levels in *rax1-1*					
12521_at	**At3g51860**	0.28	0.036	0.74	0.189
16038_s_at	**At5g66400**	0.28 (0.45)	0.171	1.42 (1.85)	0.349
19646_at	**At2g46680**	0.36	0.104	1.41	0.208
12212_at	At3g52070	0.46	0.133	n.d.	–
15750_at	At2g03980	0.46	0.016	0.52	0.017
19213_at	**At2g29580**	0.48	0.010	1.08	0.278
19901_at	**At2g32880**	0.48	0.039	1.05	0.373
13706_at	At2g18700	0.48	0.091	0.51	0.099
18969_g_at	At5g57550	0.49	0.046	0.55	0.076
13146_s_at	At4g35750	0.50	0.070	0.58	0.086
12622_at	At1g23100	0.50	0.114	n.d.	–
17840_at	At2g43570	0.35 (0.82)	0.106	0.38 (0.18)	0.115
19675_at	At4g35580	0.40	0.198	0.39	0.195
13212_s_at	At3g57260	0.43 (0.18)	0.093	0.44 (0.08)	0.094
17008_at	At2g24850	0.44	0.028	0.21	0.017
14635_s_at	At2g14610	0.44	0.088	0.45	0.093
15141_s_at	At5g24770	0.45	0.076	0.29	0.052
15125_f_at	At5g24780	0.47	0.058	0.20	0.030
18224_s_at	At4g21830	0.50 (0.8)	0.141	n.d. (0.3)	–
12244_s_at	At2g48110	0.50	0.177	0.48	0.167

The data were obtained from microarray experiments using a total of three independent preparations of RNA per line prepared from plants grown in controlled environment conditions (see Methods). Locus identifiers in bold indicate genes with altered expression only in *rax1-1*. The numbers in parentheses are the same comparisons conducted using a qRT-PCR procedure for that gene with two replicate RNA preparations independent of those used for the microarray experiments. n.d., not detected; –, not determined.

type, but as with the *rax1-1/cad 2-1*–responsive group from low light plants, no common pattern was observed.

Whole Plant Responses to Excess Light Stress and Bacterial Infection

The exposure of *rax1-1* or *cad2-1* plants to excess light did not produce any difference, when compared with parental plants, in any chlorophyll *a* fluorescence parameter used to measure different aspects of the efficiency of photosynthetic electron transport (see Supplemental Figure 3S online). Active photosynthetic electron transport is required for *APX2* expression in excess light-stressed wild-type plants, but this was not the reason for the elevated *APX2* expression in excess light stressed *rax1-1* plants (Karpinski et al., 1997, 1999; Fryer et al., 2003). Whereas H_2O_2 levels increased in excess light-stressed plants, the mutants showed no differences compared with the wild type (data not shown). Similarly, the pattern of change in both glutathione redox state and levels in response to excess light

was not different from the wild type, although the mutants always had lowered glutathione levels (data not shown). The decreased levels of transcripts involved in resistance to pathogens (Table 3) prompted us to test if the response to infection was compromised in the mutants. Both *rax1-1* and *cad2-1* showed weaker resistance to avirulent *Pseudomonas syringae* infection (Figure 6).

DISCUSSION

A lowered level of glutathione and/or its redox state have been noted in diverse plant species in response to long-term exposure to a range of abiotic stresses (Rauser et al., 1991; De Vos et al., 1992; Walker and McKersie, 1993; Chaumont et al., 1995; Kampfenkel et al., 1995; Iturbe-Ormataexe et al., 1998; Brüggemann et al., 1999; Hernandez et al., 2000; Nikiforova et al., 2003). These lowered glutathione levels were accompanied by changes in many components of the antioxidant/ROS-scavenging network. Thus, despite the emphasis often given to
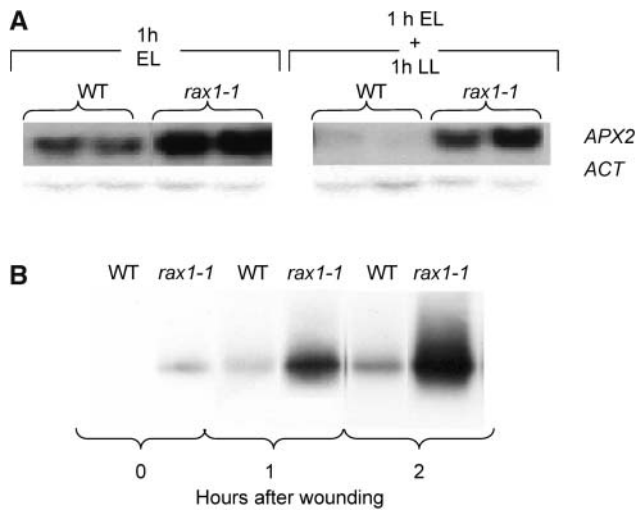
Figure 4. Induction of *APX2* in Stressed *rax1-1* Plants.

(A) Autoradiograph of RNA gel blot of *APX2* transcript levels in two pools ($n = 3$ for each pool) of *rax1-1* and wild-type plants after exposure to a fivefold excess light stress for 1 h (1h EL). The same number of excess-light stressed plants were left for another 1 h under ambient light conditions before harvesting (1h EL + 1h LL). Actin mRNA (*ACT*) is used here as a loading control. The protocols for producing the RNA gel blot autoradiographs were as described in the legend of Figure 2B.
(B) Induction of *APX2* expression in wounded wild-type and *rax1-1* plants. Autoradiograph of *APX2* cDNA amplified by 3′RACE PCR (see Methods), blotted, and hybridized to a gene-specific probe. Under the same conditions, *APX3* transcript was constant in all samples (data not shown). Samples were taken before wounding (0 h) and then at 1 and 2 h after wounding. RNA was pooled from three wounded leaves from six plants.

glutathione (May et al., 1996; Allan and Fluhr, 1997; Noctor et al., 2002), there is no compelling evidence from these comparative studies that glutathione or any other single antioxidant could play a unique role in the regulation of antioxidant defenses. The isolation of *rax1-1* by screening for *APX2* expression under normal growth conditions and demonstrating that it is a lesion in *GSH1* with reduced glutathione levels is an important observation. The way this mutant was identified provides direct evidence that glutathione metabolism and the expression of other components of plant antioxidant defenses are tightly linked.

The increased oxidation or lowered levels of cellular glutathione pools caused by different environmental conditions may constitute a signal that is mimicked by the permanently lowered levels of glutathione in *rax1-1* and *cad2-1* (Table 1). This is because the glutathione redox couple ($2GSH \leftrightarrow GSSG + 2H^+$), unlike those of other antioxidants, is such that its redox potential can be determined by the concentration of GSH as well as the GSH/GSSG ratio (Schafer and Buettner, 2001; Pfannschmidt, 2003). This means that the GSH concentration alone can have an influence on cellular redox state and possibly influence redox-sensitive regulators (Schafer and Buettner, 2001; Noctor et al., 2002). This concept is sometimes referred to as redox tone (Cooper et al., 2002). Therefore, we reasoned that the level of glutathione common to *cad2-1* and *rax1-1* might permit identi-

fication of genes sensitive to cellular redox state. This was the case for the transcript levels and enzyme activity of three (out of 25) antioxidant defense genes surveyed (those encoding Fe- and Cu/Zn-SOD and MDAR; Figures 2 and 3) and a further 16 genes (out of 3743) from a microarray analysis (Table 3).

Surprisingly, the lowered level of glutathione per se did not provide an explanation for all of our observations. It was possible to identify genes whose transcript levels were specifically affected in *rax1-1* or *cad2-1* (Figure 2, Table 3; see Supplemental Table 1S online). The expression of 10 genes, including three
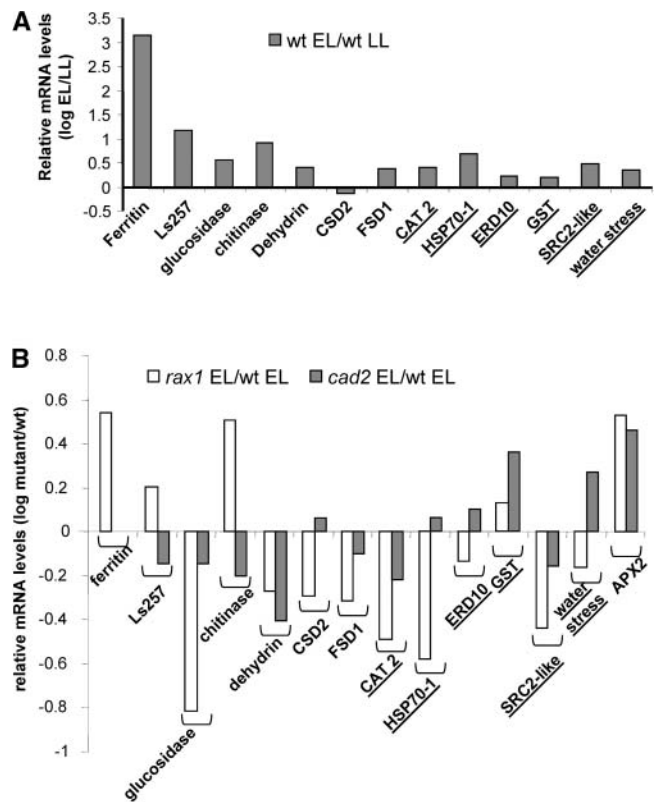

Figure 5. Excess Light Stress Responses of *APX2* and Other Stress-Responsive Genes in Wild-Type and Mutant Plants.

(A) Transcript levels of stress defense genes in light-stressed (fivefold excess light for 45 minutes) wild-type plants relative to those in low light. Genes with altered expression in nonstressed *rax1-1* and *cad2-1* (Table 3) are shown. These are compared with light stress–responsive genes (underlined) whose transcripts were not affected in the mutants under nonstress conditions. All measurements were made using qRT-PCR (see Methods) using three determinations from each of two separate experiments ($n = 6$). The mean threshold cycle (Ct) values used to calculate the log ratio of excess light (EL)/low light (LL) transcript levels had standard deviations from the mean for each cDNA no greater than ±10%. The locus identifiers for the genes are given in Supplemental Table 2S online.
(B) Transcript levels of stress defense genes after fivefold excess light exposure for 45 min in *cad2-1* and *rax1-1* relative to the wild type. The same genes were assayed by qRT-PCR as in **(A)** in two separate sets of determinations. The mean threshold cycle (Ct) values used to calculate the log ratio of mutant/wild-type transcript levels had standard deviations from the mean for each cDNA no greater than ±5%.
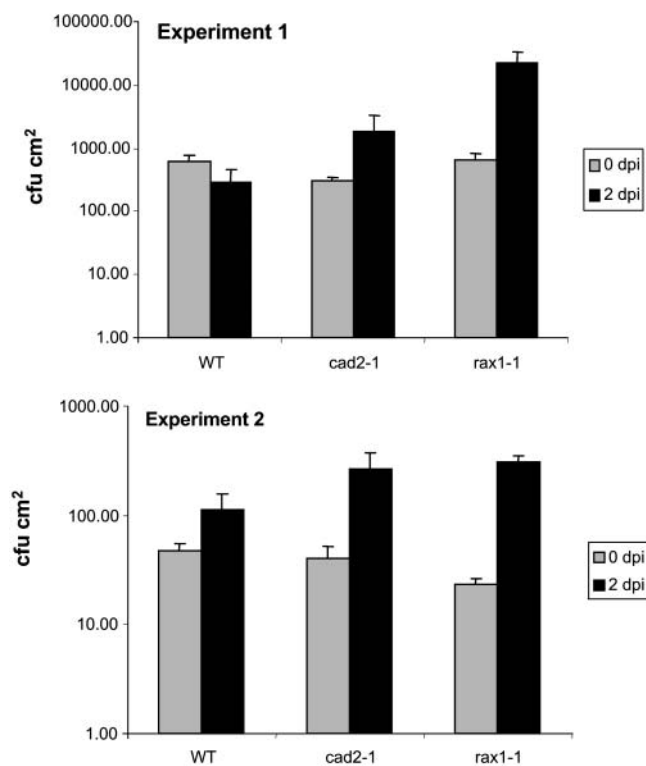
Figure 6. Growth of Avirulent Bacteria in *rax1-1* and *cad2-1* Plants.

In two experiments on separate batches of plants, avirulent *P. syringae* pv tomato DC3000*avrRpm1* was vacuum infiltrated into whole rosettes at day 0 (0 d postinoculation [dpi]). Note that two different inoculation densities were used in experiments 1 and 2. Bacteria were recovered from leaves 0 and 2 d (dpi) later and the increase in colony-forming units (cfu) per cm² determined. The data shown are mean values ($\pm$ SE, $n = 3$).

antioxidant genes, most notably *APX2* itself, but also *DHAR1* and *APX1*, were affected in *rax1-1* but not in *cad2-1* (Figure 2, Table 3). The *rax1-1* mutant had near wild-type levels of γ-EC, Cys (Table 1), Ser, Gly, and Glu (Ball, 2001), thus ruling out the levels of these compounds as possible additional effectors of expression of this group of genes. The discrepancy between *rax1-1* and *cad2-1* in the levels of thiol intermediates (Table 1) suggests that some other aspect of glutathione biosynthesis, besides its level, may be contributing to the regulation of defense gene expression in these plants. The distinct *cad2-1* group of genes did not permit further analysis of this problem because the mutant had too many variables, such as elevated Cys levels, undetectable levels of γ-EC (Table 1), and possibly perturbed amino acid metabolism. *root meristemless 1* (*rml1*) is a mutant allele of *GSH1* (Figure 1C) that has ~5% of wild-type glutathione levels and is severely defective in root growth (Vernoux et al., 2000). However, transgenic lines harboring antisense *GSH1* display a similar reduced level of glutathione to *rml1* and yet do not have such a severe phenotype (Xiang et al., 2001), perhaps reflecting subtle effects of altered glutathione metabolism on gene expression.

The differences in stress defense gene expression profiles between *rax1-1* and *cad2-1* may also suggest that the different lesions could have affected regulatory as well as catalytic

functions of γ-ECS. Inspection of the position of the lesions in the *GSH1* mutants adds some support to this notion. Both the *cad2-1* and *rml1-1* lesions lie within a putative catalytic domain of γ-ECS, as defined by a block of distant homology to trypanosome γ-ECS (Figure 1C; Leuder and Phillips, 1996). By contrast, the *rax1-1* lesion lies outside this domain (Figure 1C), which may suggest that the effect of this mutation may be on a function not directly associated with the catalytic function of the enzyme. It should be noted that γ-ECS from plants shows a high degree of sequence conservation over the full length of the mature polypeptide (Noctor et al., 2002), which suggests that functions apart from the catalytic activity might be important in this enzyme.

Further evidence that glutathione biosynthesis, or the action of specific regions of *GSH1*, may influence stress protective gene expression in subtle ways comes from the results of our screen of M2 plants equivalent to 17,000 M1 individuals. If a perturbation in ROS homeostasis was solely responsible for enhanced *APX2* expression, then it would have been expected to have recovered mutants that had diminished levels of other foliar antioxidants (e.g., similar to *vtc-1* and *npq1-2*; Conklin et al., 1996; Niyogi et al., 1998). Furthermore, if depressed glutathione levels alone were the main influence on *APX2* expression, we would have expected to, but did not recover, a wider range of *GSH1* mutant alleles (for example, equivalent to *cad2-1*). In support of these conclusions, neither *vtc1-1*, *npq1-2*, or *cad2-1* under nonstress conditions showed detectable *APX2* expression (Figure 2A; Ball, 2001). In addition, *rax1-1* did not show any increase in total foliar H_2O_2 levels or increases specifically in vascular tissue that would have explained the increased expression of *APX2* (see Results; Fryer et al., 2003).

Under Nonstress Conditions, Glutathione Metabolism Affects a Group of Genes Functionally Clustered around Stress Defense

All of the known genes that showed altered expression in the microarray comparison between nonstessed *rax1-1* and the wild type have been reported in the literature to be also responsive to stress, important as host factors in disease, or implicated in stress defenses in plants (Table 4; 24/26). Two more genes (At2g29580 and At4g35750) have homologs that have been implicated in stress responses in animal cells (Table 4). Interestingly, 8 of the 17 genes with depressed levels of transcripts in *rax1-1* compared with the wild type (Table 3) have been shown to be altered in their expression by either ABA (three; Table 4), SA (two; Table 4), or jasmonic acid ([JA]; three; Table 4). In the more extensive *cad2-1* specific group, a preliminary inspection of the annotation suggests that a high proportion of the affected genes may encode defense-related functions (see Supplemental Table 2S online). JA has been shown to influence glutathione metabolism by elevating transcript levels of γ-ECS, glutathione synthetase, and glutathione reductase (Xiang and Oliver, 1998). SA treatment of leaves leads to an increase in glutathione levels often ahead of induction of defense gene expression. This may influence the redox state of NPR1, a glutathione-sensitive regulatory protein in the SA signaling pathway in Arabidopsis (Mou et al., 2003). Confirmation of this observation comes from the increased growth of avirulent *P. syringae* in *rax1-1* and *cad2-1*

Table 4. The *rax1-1* and *cad2-1/rax1-1*–Affected Genes All Encode Stress Defense-Related Functions

Locus Name	Annotation	Stress Response or Function	Reference
At2g40300	Putative ferritin	Induced by high light, ozone, Fe-induced oxidative stress, and nitric oxide	Murgia et al. (2001), (2002)
At5g01600	Ferritin 1 precursor	Induced by high light, ozone, Fe-induced oxidative stress, and nitric oxide	Murgia et al. (2001), (2002)
At1g65400	Putative lectin	Chitin induced	Zhang et al. (2002)
At4g02330	Putative pectinesterase	Cell wall protein interacts with viral movement proteins	Chen et al. (2000)
At4g39640	Putative γ-glutamyltransferase	Glutathione catabolism	Storozhenko et al. (2002)
At2g29580	RNA recognition motif–containing RNA binding protein	RNA stabilization during heat shock	Gallouzi et al. (2001)
At2g03980	Putative Gly-Asp-Ser-Leu family-motif lipase/hydrolase; pFAM domain	Lipid breakdown response to pathogens and wounding	Schaller (2001)
At5g57550	Xyloglucan endotransglycosylase	Induced by touch, darkness, cold, heat, and brassinosteroids	Iliev et al. (2002)
At4g35750	Putative E1B 19K/Bcl2 interactor Nip2	Regulator of apoptosis/cell death	Han et al. (1998); Hoeberichts and Woltering (2003)
At2g18700	Putative trehalose-6-phosphate synthase	Trehalose synthesis for tolerance to drought, stress, and cold	Wingler (2002); Jang et al. (2003)
At2g46680	Homeodomain transcription factor ATHB-7	Induced by drought and ABA	Söderman et al. (1996)
At1g23100	10-kD chaperonin	Subunit of HSP60, induced by heat shock	Koumoto et al. (1996)
At3g51860	CAX1 Ca^{2+}/H^+ antiporter	Calcium signaling, stress adaptation	Hirschi (1999)
At5g66400	Dehydrin RAB18-like protein	Induced by drought and ABA	Welin et al. (1994)
At2g24850	Putative tyrosine aminotransferase	Tocopherol biosynthesis. Induced by JA, wounding, high light, UV, and oxidative stress	Lopukhina et al. (2001); Sandorf and Holländer-Czytko (2002)
At5g24780	Vegetative storage protein 1	Induced by wounding, herbivory, and JA	Berger et al. (2002); Utsugi et al. (1998)
At5g24770	Vegetative storage protein 2	Induced by wounding, herbivory, copper, ozone, senescence, and JA	Utsugi et al. (1998); Berger et al. (2002); Mira et al. (2002)
At2g14610	PR-1–like protein	Induced in hypersensitive response, pathogen infection, and by SA	Rogers and Ausubel (1997); Cameron et al. (1999)
At3g57260	Glucan endo-1,3-β-glucosidase	Induced in hypersensitive response and pathogen infection. Downregulated by ABA	Rezzonico et al. (1998)
At2g43570	Class IV chitinase	Induced by pathogens and SA	de A Gerhardt et al. (1997); Park et al. (2003)
At4g35580	NAM/CUC2-like protein	Petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2 domain transcription factor. Induced by fungal infection and wounding	Collinge and Boller (2001); Duval et al. (2002)

plants, indicating that lowered glutathione levels are weakening these plants' defense capability (Figure 6). Thus, glutathione levels or its redox state does impact on stress defenses at the whole plant level.

The Influence of Glutathione during Photooxidative Stress

In wild-type plants, *APX2* expression was only detected after excess light stress or, to a smaller degree, after wounding (Figure 4; Karpinski et al., 1997; Chang et al., 2004). In *rax1-1*, increased induction of *APX2* expression over that of wild-type plants was observed in response to excess light and wounding (Figures 4 and 5B) and in *cad2-1* after excess light stress (Figure 5B). Redox changes in photosynthetic electron transport and accumulation of H_2O_2 in vascular tissue have been identified as prerequisites for *APX2* induction in wild-type plants (Karpinski et al., 1999; Fryer et al., 2003). However, neither in mesophyll nor vascular tissue of light stressed *rax1-1* and *cad2-1* plants were these

parameters observed to differ from those of wild-type plants (see Supplemental Figure 3S online) nor was the pattern of change in glutathione levels different between mutants and the wild type subjected to excess light stress (data not shown). These data indicate that the mutants were no more affected by photooxidative stress at the whole plant level than Col-0; therefore, other signals must be the cause for enhanced *APX2* expression. The expression of a further 12 stress-associated genes was skewed in excess light stressed mutants compared with the wild type (Figure 5B). These included six genes influenced by *rax1-1* and *cad2-1* under ambient conditions and six that were not. These data suggest that both the levels and biosynthesis of glutathione act in several different ways to influence stress defense mechanisms according to the physiological state of the plant.

Conclusion

Glutathione levels and its biosynthesis can profoundly affect the poising of stress defenses in plants, and this is achieved without any direct impact on cellular ROS levels. The direct interaction of glutathione or γ-ECS with one or more regulatory proteins may be how this control is achieved. In bacteria, yeast, animal, and plant cells, at least two mechanisms have been described by which glutathione can modulate the activity of signaling proteins independently of ROS levels. There are the so-called thiol switches, in which glutathione changes the redox state of thiol-groups of proteins that act as redox cofactors, altering either the activity or redox state of regulatory proteins, and secondly by *S*-glutathiolation of regulatory proteins with direct conjugation of glutathione to target Cys residues (Klatt and Lamas, 2000; Cooper et al., 2002; Delaunay et al., 2002; Mou et al., 2003; Paget and Buttner, 2003).

METHODS

Plant Material, Growth Conditions, and Stress Treatments

Arabidopsis thaliana plants were grown in controlled environment conditions under short and long days (8-h and 16-h photoperiods, respectively) as previously described (Bechtold et al., 2004). The *APX2LUC* transformant (Col-0) has been described previously (Karpinski et al., 1999). The *35S-GSH1* transgenic Arabidopsis line and *cad2-1* were kind gifts from D.J. Oliver, Iowa State University, and C. Cobbett, University of Melbourne, Australia, respectively.

Plants were subjected to either complete or partial exposure to 5- to 15-fold excess light or wounding, as described previously (Karpinski et al., 1997, 1999; Chang et al., 2004). Vacuum infiltration of whole rosettes with avirulent *Pseudomonas syringae* pv tomato DC3000*avrRpm1* was performed as described by Muskett et al. (2002).

Mutagenesis, Screening, and Mapping of *rax1-1*

APX2LUC seed were mutagenized in batches of 10,000 using 0.5% (v/v) ethyl methanesulfonate for 8 h. Ethyl methanesulfonate–treated seed had a germination frequency of 70%. Mutagenesis efficiency was confirmed in M1 plants by analyzing the frequency of chlorophyll deficiency in developing embryos from 100 siliques harvested from plants of each seed batch at 10 to 12 d postanthesis. M2 seeds were collected from independent pools of 500 M1 plants. M2 plants (500) from each pool were grown for 16 d under long day conditions, sprayed with 1 mM D (-)- luciferin

(Karpinski et al., 1999), and assayed for their luciferase activity in 12-well microtitre dishes using a Victor Multilabel Counter 1420 workstation (Wallac, Finland). Plants were scored positive if one of the six readings was >100 cps (background was 30 to 40 cps). Positive plants (120) were repotted and grown for seed production. A repeat assay was done on 12 M3 progeny from each positive M2 rosette. Only phenotypically normal plants were kept to confirm the phenotype in the M3 generation. Inheritance of the phenotype was determined by following segregation after two subsequent backcrosses. The only two mutants from a total of 65 pools of M1 plants with a stably heritable, recessive phenotype were backcrossed to Col-0 a further three more times.

For the mapping of *rax1*, the strategy described by Jander et al. (2002) was adopted. DNA from 48 F2 progeny from a cross between the mutants and Arabidopsis Landsberg *erecta* that showed the luciferase positive, low glutathione phenotypes, were analyzed for the segregation of polymorphisms using custom-designed fluorescently tagged INDEL markers separated on an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA). Identification of *GSH1* on the map used information from TAIR (www.arabidopsis.org). Amplification and sequence determination of *GSH1* and its full-length cDNA, from both versions of the *rax1-1* mutation, was conducted using specific primers derived from the wild-type sequence. The John Innes Centre Genome Laboratory (www.jicgenomelab.co.uk) conducted both the sequencing and the mapping. The presence of both the *rax1-1* and *cad2-1* mutations in individual plants was confirmed by sequencing PCR products obtained with the following primer pair: 5′-ACCAACTATCTACGG-3′ and 5′-CATACCA-GAGTTATAAGGTGGG-3′ (5′ at 2572 and 3386 in EMBL accession number AF068299).

Imaging and Measurement of Luciferase Activity and H$_2$O$_2$ Accumulation

The imaging and in vitro determination of luciferase activity was as described previously (Karpinski et al., 1999; Chang et al., 2004). The visual detection of H$_2$O$_2$ was performed as described by Fryer et al. (2002). Total foliar H$_2$O$_2$ was determined as described by Creissen et al. (1999), except that 100 mg of Arabidopsis leaf tissue per 1 mL of extraction medium was used.

Preparation of RNA and RNA Gel Blots

Total RNA was isolated from leaves as described previously (Bechtold et al., 2004; Chang et al., 2004). A minimum of three RNA preparations each from a separate plant was made per time point. Preparation of RNA gel blots and the probes for *APX1* (At1g07890.1), *APX2* (At3g09640.1), and *APX3* (At4g35000.1) have been described previously (Karpinski et al., 1997). Other probes used in the RNA gel blots or DNA gel blots were from ESTs obtained from the ABRC (Cleveland, OH). The inserts were amplified by PCR using universal and reverse primers. These ESTs used are as follows: *APX4* (At1g77490.1, AA042529), *APX5* (At4g08390.1, T41685), *CAT1* (At1g70630.1, T43867), *CAT2* (At4g35090, T41566), *CAT3* (At1g20620.1, N38125), *CSD1* (At1g08830.1, T42186), *CSD2* (At2g28190.1, H36758), *CSD3* (At5g18100.1, T88473), *DHAR1* (At1g19570.1, H37392), *DHAR2* (At1g75270, N37468), *DHAR3* (At5g16710.1, H37601), *FSD1* (At4g25100.2, AA042744), *FSD2* (At5g51100.1, Y12641), *GOR1* (At3g54660.1, D89620), *GOR2* (At3g24170.1, U37697), *GPX1* (At2g925080, AJ000469), *GPX7* (At4g31875.1, NL049607), *GSH1* (At4g23100.1, R84030), *GSH2* (At5g27380.1, T43120), *MDAR1* (At1g63940.2, T04550), *MDAR2* (At5g03630.1, R64883), and *MSD1* (At3g10920.1, T20828). The pea (*Pisum sativum*) *ACTIN* probe used to check RNA loadings was recovered by PCR from a full-length cDNA inserted the plasmid pBluescript SK+ (X67666) using T3 and T7 primers.

PCR Procedures for Analysis of RNA

The blotting and probing of 3′RACE products (Frohman et al., 1988) was used to analyze *APX2* and *APX1* expression as previously described (Bechtold et al., 2004; Chang et al., 2004). qRT-PCR was performed using a cybergreen fluorescence-based assay kit (DyNAmo SBYR Green qPCR kit; Finnzymes Oy, Espoo, Finland) according to the manufacturer's instructions. The PCR reactions and calculation were performed on an Opticon 2 real-time PCR machine using the manufacturer's software (Genetics Research Instrumentation, Braintree, UK). All mRNA levels were calculated from threshold cycle values and as relative to controls and normalized with respect to actin transcript levels according to Gruber et al. (2001). The primers used in qRT-PCR are given in Supplemental Table 2S online.

Microarray Analysis

Nine Affymetrix 8K GeneChips (Affymetrix, Santa Clara, CA; Zhu and Wang, 2000) were hybridized with target cRNA prepared from each of three independent RNA preparations for mutants and wild-type control. The plants were grown in a randomized block design mixing up plants for each of *cad2-1*, *rax1-1*, and the wild type (Col-0/*APX2LUC*). Each RNA preparation was pooled from eight 5-week-old short day–grown plants. RNA quality was assessed by running 1 μL of each RNA sample on Agilent RNA6000nano LabChips (Agilent Technology 2100 Bioanalyzer version A.01.20 SI211; Agilent Technologies, Palo Alto, CA). Affymetrix GeneChip array expression profiling was performed at the John Innes Centre Genome Lab, http://www.jicgenomelab.co.uk. All protocols used can be found in the Affymetrix Expression Analysis Technical Manual II (Affymetrix Manual II; http://www.affymetrix.com/support/technical/manuals.affx). After reading, the initial microarray data files were analyzed using Affymetrix software Microarray Suite (version 5.0). This included calculation of absolute values and normalization of the data with respect to internal standards. The data were then imported into Microsoft Excel, and three pairwise comparisons were performed of *cad2-1* versus the wild type, *rax1-1* versus the wild type, and a comparison of two randomly mixed samples. According to the detection call given to each probe set in each experiment (P, present; M, marginal; A, absent), the six data sets were classified and those having 6 P; 5 P + 1 M; 4 P + 2 M; 5 P + 1 A were accepted; all the others were discarded. The probe sets were sorted according to the differences in expression, and genes with less than a twofold difference were rejected. For each retained probe set, average signal in mutant, average signal in the wild type, fold change, and *t* test values were determined. The randomized test produced a list of 41 upregulated and four downregulated genes. The best *t* test values resulting from the randomized test were between 10 and 1000 times larger than those obtained in the mutant versus the wild type analyses. The identification of genes corresponding to the probe sets was performed using identifiers downloaded as a text file (affy8k-2002-12-23.txt) from the TAIR Web site (www.arabidopsis.org). Further analysis of the data used the following Web-based resources: The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), The Institute for Genomic Research (www.tigr.org), the Affymetrix Web site, including Netaff-X tools (www.affymetrix.com), the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/dgenome.html), and the Gene Ontology Consortium (www.geneontology.org/index.shtml).

Thiol Determinations

The determination of glutathione, γ-EC, and Cys contents as both oxidized and reduced forms was performed as previously described (Creissen et al., 1999; Bechtold et al., 2004).

Enzyme Assays

SOD, MDAR, and APX activity determinations were performed on Arabidopsis cell-free leaf extracts using the protocols of Jimenez et al. (1997). The activity of γ-ECS was determined in cell-free foliar extracts of Arabidopsis, prepared from 100 mg of leaf material per 1 mL of extraction buffer, essentially as described by Creissen et al. (1999). All data were calculated on a fresh weight basis.

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