Identification of *PAD2* as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis

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Summary

The Arabidopsis *pad2-1* mutant belongs to a series of non-allelic camalexin-deficient mutants. It was originally described as showing enhanced susceptibility to virulent strains of *Pseudomonas syringae* and was later shown to be hyper-susceptible to the oomycete pathogen *Phytophthora brassicae* (formerly *P. porri*). Surprisingly, in both pathosystems, the disease susceptibility of *pad2-1* was not caused by the camalexin deficiency, suggesting additional roles of PAD2 in disease resistance. The susceptibility of *pad2-1* to *P. brassicae* was used to map the mutation to the gene At4g23100, which encodes γ-glutamylcysteine synthetase (γ-ECS, GSH1). GSH1 catalyzes the first committed step of glutathione (GSH) biosynthesis. The *pad2-1* mutation caused an S to N transition at amino acid position 298 close to the active center. The conclusion that *PAD2* encodes GSH1 is supported by several lines of evidence: (i) *pad2-1* mutants contained only about 22% of wild-type amounts of GSH, (ii) genetic complementation of *pad2-1* with wild-type *GSH1* cDNA restored GSH production, accumulation of camalexin in response to *P. syringae* and resistance to *P. brassicae* and *P. syringae*, (iii) another GSH1 mutant, *cad2-1*, showed *pad2*-like phenotypes, and (iv) feeding of GSH to excised leaves of *pad2-1* restored camalexin production and resistance to *P. brassicae*. Inoculation of Col-0 with *P. brassicae* caused a coordinated increase in the transcript abundance of *GSH1* and *GSH2*, the gene encoding the second enzyme in GSH biosynthesis, and resulted in enhanced foliar GSH accumulation. The *pad2-1* mutant showed enhanced susceptibility to additional pathogens, suggesting an important general role of GSH in disease resistance of Arabidopsis.

Keywords: camalexin, *Phytophthora brassicae*, *Pseudomonas syringae*.

Introduction

The plant stress hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play important roles in the establishment of disease resistance in many plant–pathogen interactions (Dong, 1998; Glazebrook, 2005). In contrast, the disease resistance of Arabidopsis to *Phytophthora brassicae* and of tomato (*Lycopersicon esculentum*) to *Phytophthora infestans* was found to be largely independent of known stress hormone signaling pathways (Roetschi *et al.*, 2001; Si-Ammour *et al.*, 2003; Smart *et al.*, 2003). The disease resistance of the resistant Arabidopsis accession Col-0 to *P. brassicae* was maintained in mutants deficient in SA-, JA- or ET-dependent signaling. However, the phytoalexin-deficient mutant *pad2-1* (Glazebrook and Ausubel, 1994) was found to be hyper-susceptible to *P. brassicae*. The *pad2-1* mutant was originally described as being partially camalexin-deficient and showing increased susceptibility to the bacterial pathogen *P. syringae* (Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1997). Interestingly, the camalexin deficiency of *pad2-1* was not the cause of its enhanced disease susceptibility to either *P. syringae* or *P. brassicae*. A null allele of the cytochrome P450 monoxygenase gene *CYP71B15*, *pad3-1* (Zhou *et al.*, 1999), nearly abolished camalexin synthesis yet had little effect on resistance to these pathogens (Glazebrook and Ausubel, 1994; Roetschi *et al.*, 2001). The *pad2-1* mutant was later shown to be more susceptible to other pathogens as well (Ferrari *et al.*, 2003; Van Wees *et al.*, 2003). Apparently, *PAD2* encodes a gene product with important but unknown functions in the general disease resistance of Arabidopsis.

The hyper-susceptibility of *pad2-1* to *P. brassicae* was exploited for the positional cloning of *PAD2*. We present evidence that *PAD2* encodes γ-glutamylcysteine synthetase (γ-ECS, γ-glutamylcysteine ligase, GSH1), the enzyme that catalyzes the first step of *de novo* GSH biosynthesis. GSH1 is encoded by a single-copy gene (At4g23100) in the Arabidopsis genome. The first *GSH1* cDNA of plants cloned from Arabidopsis encoded a predicted 60 kDa protein that was structurally unrelated to GSH1 of mammals and yeast (May and Leaver, 1994). The tripeptide GSH (γ-L-glutamyl-L-cysteinylglycine) is synthesized in two ATP-dependent reactions. GSH1 (EC 6.3.2.2.) catalyzes the formation of a peptide bond between the γ-carboxyl of L-Glu and the α-amino group of L-Cys. GSH synthetase (GSH2; EC 6.3.2.3.), which is also encoded by a single gene in Arabidopsis (At5g27380; Rawlins *et al.*, 1995), catalyzes the addition of glycine to γ-glutamylcysteine (γ-EC). GSH1 and GSH2 both contain plastidic transit peptides. In Arabidopsis, multiple transcription initiation leads to an exclusive plastidial localization of GSH1, while GSH2 predominantly accumulates in the cytosol (Wachter *et al.*, 2005). GSH synthesis is subject to complex regulation. The expression and enzymatic activity of GSH1 is under multiple controls at the transcriptional, post-transcriptional and post-translational levels (Jez *et al.*, 2004; May *et al.*, 1998b; Xiang and Oliver, 1998).

The identification of *PAD2* as *GSH1* suggested that adequate levels of GSH are important in Arabidopsis for limiting the spread of virulent *P. syringae* and for establishing disease resistance to *P. brassicae*. The ubiquitous thiol tripeptide GSH has been implicated in many different aspects of cellular biochemistry (reviewed in May *et al.*, 1998a; Mullineaux and Rausch, 2005; Noctor, 2006; Noctor and Foyer, 1998; Noctor *et al.*, 1998a,b, 2002). GSH is present in up to millimolar concentrations in plant cells, and functions as a major determinant of cellular redox homeostasis. GSH plays important roles in stress physiology by reducing reactive oxygen species (ROS) via the ascorbate–GSH cycle which consists of three interdependent redox couples: ascorbate/dehydroascorbate, GSH/GSSG and NADPH/NADP (Noctor and Foyer, 1998). As a result of a redox reaction, GSH is oxidized to GSSG, which is recycled back to GSH by a reaction catalyzed by GSH reductase using NAD(P)H as a reducing agent. GSH can function as a modulator of redox-controlled enzymatic reactions and thiol-based regulatory switches (Foyer and Noctor, 2005; Paget and Buttner, 2003), in protein modification via glutathionylation (Dixon *et al.*, 2005; Klatt and Lamas, 2000), and as a co-substrate in conjugation and detoxification processes catalyzed by GSH transferases, GSH peroxidases and glyoxalases (Edwards *et al.*, 2000). As a precursor of phytochelatins, the production of GSH is involved in heavy metal tolerance (Cobbett and Goldsbrough, 2002). In addition to its various roles in cellular protection, GSH serves as a storage and transport form of reduced sulfur (Kopriva and Rennenberg, 2004), participates in the regulation of cell division in root apical meristems (Sanchez-Fernandez *et al.*, 1997), promotes flowering (Ogawa *et al.*, 2004), and plays a role in the nodulation process (Frendo *et al.*, 2005). GSH can engage in thiol–disulfide exchange reactions that may link the regulation of gene expression to the redox state of cells (Baier and Dietz, 2005; Pfannschmidt, 2003). In animals, GSH was demonstrated to be involved in redox-dependent activity changes of transcription (Mihm *et al.*, 1995). Thiol–disulfide status also appears to be important in disease resistance signaling. The reduction of key cysteines on the regulatory protein NPR1 and on the transcription factors TGA1 and TGA4 was shown to be crucial in the SA-dependent activation of the *PR1*-gene encoding pathogenesis-related (PR) protein 1, and changes in the GSH level were suggested to play a role in this process (Després *et al.*, 2003; Mou *et al.*, 2003). Interestingly, GSH treatment has been reported to activate the expression of a number of stress and defense genes (Dron *et al.*, 1988; Loyall *et al.*, 2000; Wingate *et al.*, 1988), including PR genes (Creissen *et al.*, 1999; Gomez *et al.*, 2004; Senda and Ogawa, 2004).

The effect of GSH deficiency on disease resistance has been analyzed previously. The *cad2-1* mutant, which accumulated only about 30% of wild-type amounts of GSH, showed an unaltered disease resistance phenotype to virulent and avirulent strains of *Hyaloperonospora parasitica* and virulent and avirulent strains of *P. syringae* pv. *tomato*, respectively (May *et al.*, 1996a). Thus, GSH was concluded to be of minor importance for plant disease resistance. In contrast, *cad2-1* and *rax1-1*, an Arabidopsis GSH1 mutant with similar GSH content as *cad2-1*, were shown to be more susceptible to avirulent strains of *P. syringae* (Ball *et al.*, 2004). The identification of *pad2-1* as a GSH-deficient mutant demonstrates that adequate levels of GSH are important for the accumulation of resistance-related compounds (Glazebrook and Ausubel, 1994; Roetschi *et al.*, 2001) and for the establishment of disease resistance to many pathogens.

Results

Positional cloning identifies PAD2 as a γ-glutamylcysteine synthetase

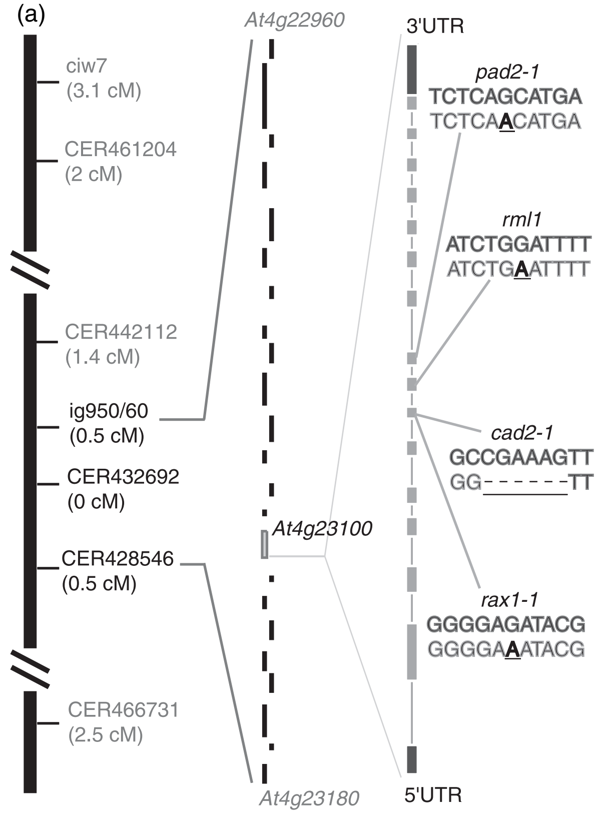
The hyper-susceptibility of *pad2-1* to *P. brassicae* was used for positional cloning of the *PAD2* gene. The segregation of disease susceptibility was analyzed in reciprocal crosses of *pad2-1* with the resistant Arabidopsis accession

Wassilewskija (Ws). All plants of the F_1 progeny were resistant, while 24% of the F_2 progeny derived from self-pollination of the F_1 progeny were susceptible. This is consistent with the 3:1 ratio expected for segregation of a recessive allele of a single nuclear gene ($\chi^2 = 0.60$, $P < 0.05$). Bulk segregant analysis confirmed the location of *PAD2* on chromosome 4 (Glazebrook and Ausubel, 1994). *P. brassicae* susceptibility co-segregated with the SSLP marker *ciw7* on the lower arm of chromosome 4 (data not shown). To refine the position of *PAD2*, a mapping population from the *pad2-1* × Ws cross was established, and 412 F_2 chromosomes were analyzed with SSLP and CAPS markers. The results are summarized in Figure 1(a). The markers Ig950/60 and CER428546, showing very low recombination frequencies, were found to flank the *pad2-1* mutation. These markers defined a region of about 110 kb containing 23 annotated ORFs. The 23 candidate genes were PCR-amplified from *pad2-1* DNA and sequenced. Comparison with the Col-0 wild-type sequence identified a single G to A nucleotide transition at position 1697 from the start codon of the gene At4g23100. This mutation resulted in replacement of a serine by an asparagine residue at position 298 in the 522 amino-acid protein (Figure 1b). At4g23100 encodes γ-glutamylcysteine synthetase (GSH1), which catalyzes the first dedicated step of GSH biosynthesis (May and Leaver, 1994). Mutant alleles of *GSH1* have been characterized previously. They include *cadmium-sensitive 2-1* (*cad2-1*; Cobbett *et al.*, 1998), *root meristemless 1* (*rml1*; Vernoux *et al.*, 2000) and *regulator of APX2* 1–1 (*rax1-1*; Ball *et al.*, 2004). Figure 1(b) shows the position and nature of the four mutations in a region considered to be the catalytic domain of GSH1. An additional *PAD2* allele (*eds47* = *pad2-2*) was identified based on complementation tests (Glazebrook *et al.*, 1996). Sequencing of the *GSH1* gene of the *pad2-2* mutant revealed that the *pad2-2* mutation was identical to that in *pad2-1*.

The GSH1 *mutation in the* pad2-1 *mutant causes reduced GSH and increased cysteine accumulation*

The identification of *PAD2* as *GSH1* led to the question of whether *pad2-1* is deficient in GSH. GSH and cysteine levels were determined in Col-0 and *pad2-1* by HPLC using homoglutathione (which is not present in Arabidopsis) as an internal standard. Figure 2(b) shows that foliar GSH levels in *pad2-1* were reduced to about 21% of the level in wild-type plants. However, *pad2-1* contained about five times more of the GSH1 substrate cysteine. These results demonstrate that the mutation in *GSH1* interfered with GSH accumulation in *pad2-1*. Real-time RT-PCR revealed that Col-0 and *pad2-1* contained very similar transcript levels of *GSH1* and *GSH2* (Figure 2a), indicating that the GSH deficiency of the *pad2-1* mutant was not based on differential accumulation of *GSH1* or *GSH2* transcripts.

(a)



(b)

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WT      IPIMPKGRYDIMRNYMPKVGTLGLDMMLRTCTVQVNLDFS
mutant  IPIMPKGKYDIMRNYM--LGTLGLDMMLRTCTVQVNLNFS
               rax1-1   cad2-1              rml1

WT      SEADMIRKFRAGLALQPIATALFANSPFTEGKPNGFLSMR
mutant  SEADMIRKFRAGLALQPIATALFANSPFTEGKPNGFLNMR
                                           pad2-1
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Figure 1. Positional cloning of *PAD2*.
(a) Genetic map of the *PAD2* region at the bottom of chromosome 4 of Arabidopsis (left). CAPS and SSLP markers were used to map *PAD2* to a region flanked by Ig950/60 and CER428546. The recombination frequency for each marker is indicated in brackets. The defined region included 23 predicted genes. Sequencing identified a point mutation in gene At4g23100 that encodes GSH1. Right side: gene structure of *GSH1* and position and nature of the *pad2-1* mutation in relation to other known *GSH1* mutants. Mutations are underlined in the second line of each comparison. Deletions in *cad2-1* are indicated by dashes. The *rax1-1* and *cad2-1* mutations are located in exon 6, *rml1* in exon 7 and *pad2-1* in exon 8.
(b) Partial amino acid sequence of GSH1 of Arabidopsis (At4g23100; positions 221–300) compared with other mutant alleles of GSH1. The line labeled 'mutant' shows the mutations of the known GSH1 mutants: *rax1-1* (R228K; Ball *et al.*, 2004), *cad2-1* (deletion of P237, K238 and V239L; Cobbett *et al.*, 1998), *rml1* (D258N; Vernoux *et al.*, 2000) and *pad2-1* (S298N; this paper). The region includes the putative catalytic domain defined by Lueder and Phillips (1996). The cysteine residue highlighted in black at position 251 is thought to be part of the active site of GSH1.

To confirm that the multiple phenotypes of *pad2-1* were caused by the point mutation in *GSH1*, the *GSH1* wild-type cDNA was expressed under the control of the CaMV 35S

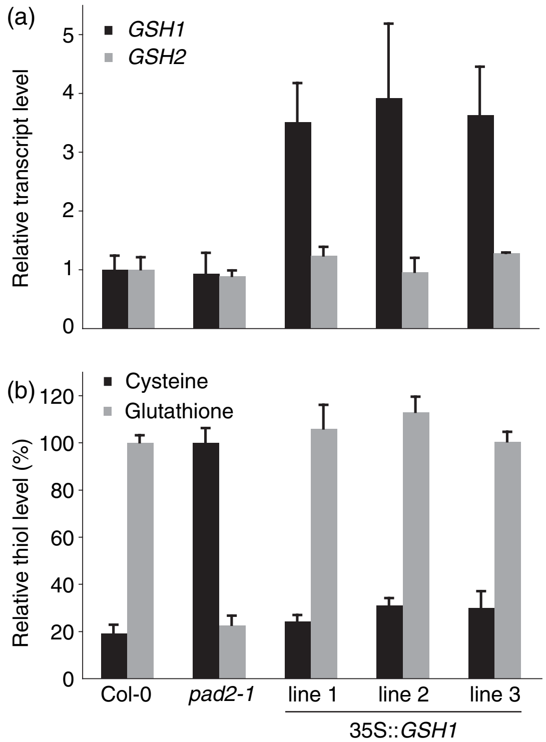

Figure 2. Complementation of the glutathione deficiency of *pad2-1* by overexpression of the wild-type *GSH1* cDNA.
(a) *GSH1* and *GSH2* transcript levels in Col-0, *pad2-1* and three complemented lines of *pad2-1* (35S::*GSH1*). Transcript levels were determined by real-time RT-PCR and are given relative to Col-0. The mean and SE from two experiments are reported. The relative transcript levels of *GSH1* in the three complemented lines were significantly higher than in Col-0 ($P < 0.001$, *t*-test).
(b) GSH and cysteine content of Col-0, *pad2-1* and three complemented lines of *pad2-1* (35S::*GSH1*). The values are given relative to Col-0 for GSH or *pad2-1* for cysteine: 100% equals 304 nmol g^{-1} FW for GSH and 93 nmol g^{-1} FW for cysteine. Leaves of 7-week-old plants (four leaves of 6–8 plants) were analyzed. The results show means and SE of three independent experiments. The GSH content in the three complemented lines was not significantly higher than in Col-0.

promoter in transgenic *pad2-1* plants. Figure 2(a) shows for three independent transgenic lines, that expression of the wild-type *GSH1* cDNA in *pad2-1* caused a greater than threefold increase in *GSH1* transcripts. Genetic complementation restored GSH accumulation of *pad2-1*, but did not lead to overaccumulation of GSH. The three complemented lines contained GSH and cysteine levels similar to wild-type plants (Figure 2b). None of the 36 transgenic lines analyzed contained >1.5 times the wild-type amount of GSH. The expression of *GSH2*, encoding the second enzyme of GSH biosynthesis, was very similar in Col-0, *pad2-1* and the complemented lines. The results from real-time RT-PCR experiments and RNA blot analysis were consistent (data not shown).

GSH content has been linked to several developmental roles in plants, so *pad2-1* was analyzed for some growth parameters. Analysis of the leaf number and leaf size of 20 plants per genotype at 13, 25 and 44 days after germination did not reveal a significant difference between the *pad2-1* mutant and Col-0. Seed production was identical in *pad2-1* and Col-0. Apparently, the reduced GSH content of *pad2-1* was sufficient for normal fitness under optimal growth conditions.

Complementation of camalexin deficiency and disease susceptibility of pad2-1

In order to establish a link between the mutation in *GSH1*, the GSH deficiency and the other *pad2-1*-related phenotypes, the complemented 35S::*GSH1 pad2-1* lines were tested for resistance to *P. brassicae* and *P. syringae* pv. *maculicola* ES4326 (*Psm* ES4326) and for restoration of camalexin production in response to *Psm* ES4326 infection. Figure 3(a) shows the result of an infection experiment with *P. brassicae*. Three-week-old seedlings of Col-0, *pad2-1* and two complemented 35S::*GSH1 pad2-1* lines were inoculated with zoospores of *P. brassicae* isolate D and analyzed 8 days later. Col-0 proved to be fully resistant, while the *pad2-1* mutant was highly susceptible to infection by *P. brassicae*. The first signs of susceptibility were observed 3 days post-inoculation (dpi) in the form of water-soaked lesions, and most *pad2-1* plants were dead or dying 1-week post-inoculation. In contrast, the complemented 35S::*GSH1 pad2-1* lines were as resistant to *P. brassicae* as Col-0. Similarly, inoculation experiments with *Psm* ES4326 confirmed that *pad2-1* showed enhanced susceptibility in comparison with wild-type, while the complemented lines accumulated a bacterial titer very similar to that in wild-type plants (Figure 3b). Finally, as shown in Figure 3(c), the complemented lines accumulated wild-type amounts of camalexin in response to infection with *Psm* ES4326, while camalexin accumulation in the *pad2-1* mutant was reduced by about 90%. In summary, the constitutive expression of wild-type *GSH1* transcripts complemented the GSH deficiency of *pad2-1*, restored resistance to *P. brassicae*, reduced the enhanced susceptibility to *Psm* ES4326 and restored wild-type levels of camalexin. It was concluded that *PAD2* encodes GSH1, and that all observed phenotypes of *pad2-1* plants are caused by the point mutation identified in *GSH1*.

Physiological complementation of camalexin deficiency and disease susceptibility of pad2-1

The *pad2-1* mutant has several biochemical phenotypes: it contains more cysteine, less γ-glutamylcysteine and GSH, and it has a reduced potential to accumulate phytochelatins. Experiments with excised leaves were performed to test whether feeding of GSH could reverse some of the *pad2-1* phenotypes. Figure 4(a) shows that feeding reduced GSH (5 mM) to excised leaves of *pad2-1* plants prior to inoculation

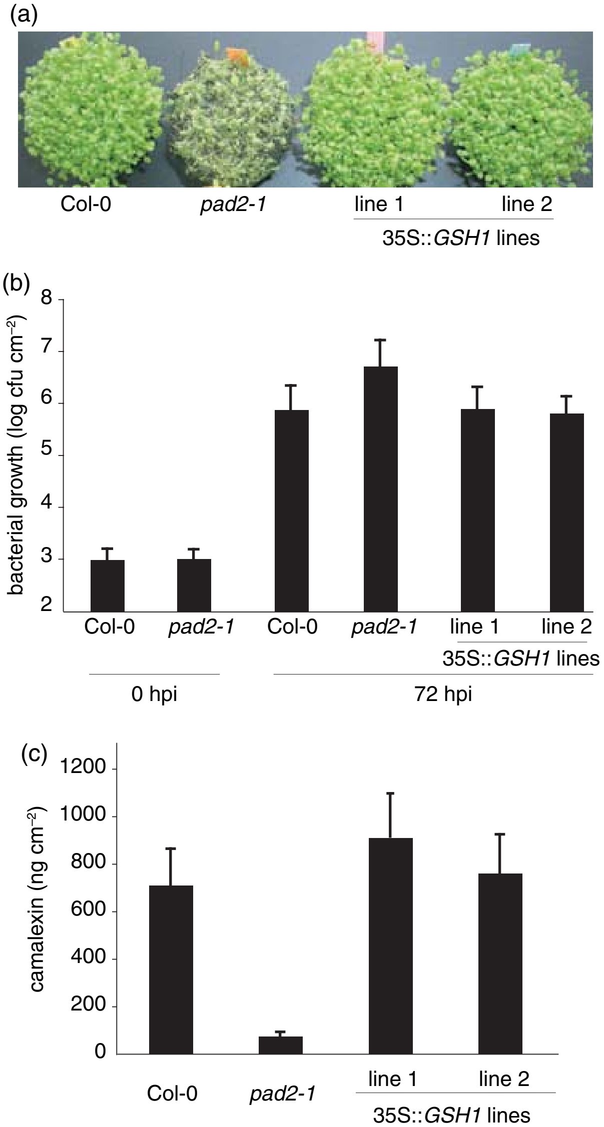

Figure 3. Complementation of camalexin deficiency and disease resistance phenotypes of *pad2-1*.

(a) Disease resistance phenotype of Col-0, *pad2-1* and complemented lines of *pad2-1* (35S*::GSH1*) after zoospore inoculation with *P. brassicase* isolate D. Four-week-old plants were spray-inoculated with a zoospore suspension (150 000 zoospores ml^{-1}) until run-off, and incubated in humid conditions at 18°C for 8 days. The experiment was repeated four times with similar results.

(b) Bacterial titer of Col-0, *pad2-1* and complemented lines of *pad2-1* (*35S::GSH1*). Leaves of 4.5-week-old plants were infiltrated with *Psm* ES4326 and the bacterial titer was determined 0 and 72 h post-inoculation (hpi). Bars represent means and SD of log-transformed data (four replicates at 0 h and 16–20 replicates are 72 h). At 72 hpi, the bacterial titer in *pad2-1* is significantly higher than in the other three genotypes ($P < 0.0001$, Mann–Whitney *U*-test).

(c) Camalexin content of Col-0, *pad2-1* and complemented lines of *pad2-1* (35S*::GSH1*) at 48 hpi with *Psm* ES4326. Bars represent means and SD of eight replicates. Camalexin levels in the 35S::*GSH1* lines were not significantly different from Col-0.

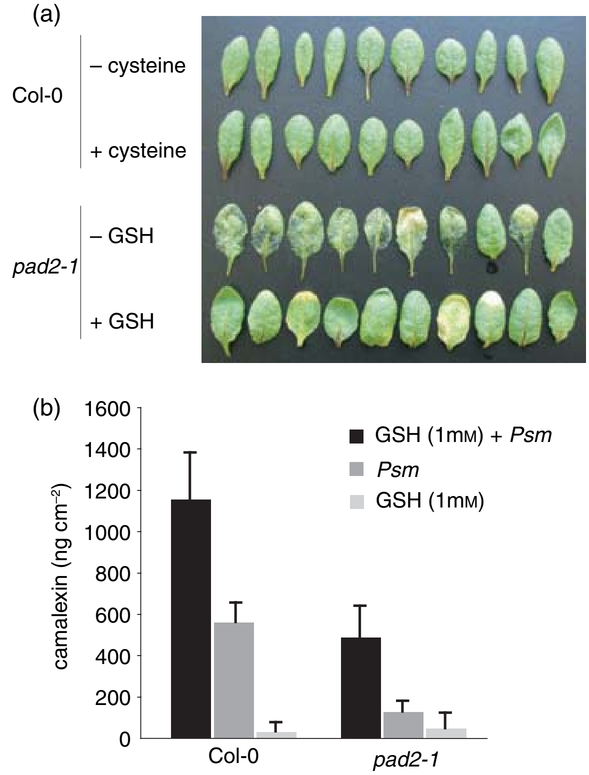

Figure 4. Physiological complementation of *pad2*-phenotypes.

(a) Physiological complementation of disease susceptibility of *pad2-1* to *P. brassicae*. Wild-type Col-0 or *pad2-1* leaves of 5-week-old plants were excised and incubated in either water, 1 mM cysteine or 5 mM GSH for 6 h. The leaves were then spray-inoculated with zoospores of *P. brassicae* isolate D (150 000 zoospores ml^{-1}). The picture was taken at 7 dpi. The experiment was repeated twice with similar results.

(b) Physiological complementation of *Psm*-induced camalexin accumulation by feeding reduced GSH. Leaves of Col-0 and *pad2-1* plants were excised and inoculated with *Psm* ES4326. The petioles were placed into 1.5 ml centrifuge tubes containing water or a solution of 1 mM GSH. Camalexin levels were determined at 40 h post-inoculation (hpi). Bars represent means and SD of eight replicates. The effects of GSH feeding on camalexin accumulation in *Psm*-inoculated Col-0 and *Psm*-inoculated *pad2-1*, respectively, were statistically significant ($P < 0.001$, *t*-test). Similar results were obtained in another replicate experiment.

resulted in increased protection against *P. brassicae*. In three independent experiments, a total of 28 out of 33 water-incubated *pad2-1* leaves were susceptible (85%), while only six of 33 GSH-treated *pad2-1* leaves showed signs of susceptibility (18%). No difference between water- and GSH-treated leaves was seen in uninoculated controls (data not shown). Feeding 1 mM cysteine to excised leaves of Col-0 did not interfere with their resistance to *P. brassicae*. Analysis of thiols demonstrated that feeding 5 mM GSH to cut leaves of *pad2-1* led to a 40-fold increase of GSH at the time of inoculation, to a level 10-fold higher than in untreated Col-0. Feeding 1 mM cysteine to cut leaves of Col-0 led to a 50-fold increase in cysteine and a threefold increase in GSH at the time of inoculation. Figure 4(b) summarizes the effect of feeding GSH to excised leaves on camalexin accumulation. Feeding GSH to *pad2-1* had no effect on camalexin accumulation. Feeding GSH to excised leaves of *pad2-1* inoculated with *Psm* ES4326 enhanced camalexin production fourfold to levels similar to inoculated wild-type plants. Comparable results were obtained by feeding GSSG, while feeding NADPH or NADH was ineffective (data not shown). GSH treatment also led to a doubling of camalexin accumulation in Col-0 in response to *Psm* ES4326. Feeding of cysteine prior to inoculation with *Psm* ES4326 had no significant effect on the disease resistance of the leaves (data not shown). Finally, deficiency in phytochelatin was not the cause of the susceptibility of *pad2-1*. The phytochelatin-deficient mutant *cad1-3* (Howden *et al.*, 1995) was resistant to *P. brassicae* in experiments with zoospore inoculation of 4-week-old seedlings (data not shown). Together, these results support the idea that the *pad2-1* phenotypes were caused by GSH deficiency rather than by elevated cysteine or deficiency in phytochelatin.

Analysis of additional GSH-deficient mutants

Three mutant alleles of *GSH1* of Arabidopsis are already known (Figure 1a). Among these, *rml1* contains only about 3% of the wild-type amounts of GSH and shows severe developmental phenotypes that lead to lethality (Vernoux *et al.*, 2000). No growth phenotypes were reported for *pad2-1*, *cad2-1* or *rax1-1*. The GSH content of the three mutants was compared in order to place *pad2-1* in this allelic series. The direct comparison shown in Figure 5(a) indicated that *pad2-1* contained on average 22% of foliar GSH compared to Col-0, followed by *cad2-1* with 30% and *rax1-1* with 38%. The absolute and relative GSH contents of Col-0 and the *GSH1* mutants were variable among different experiments

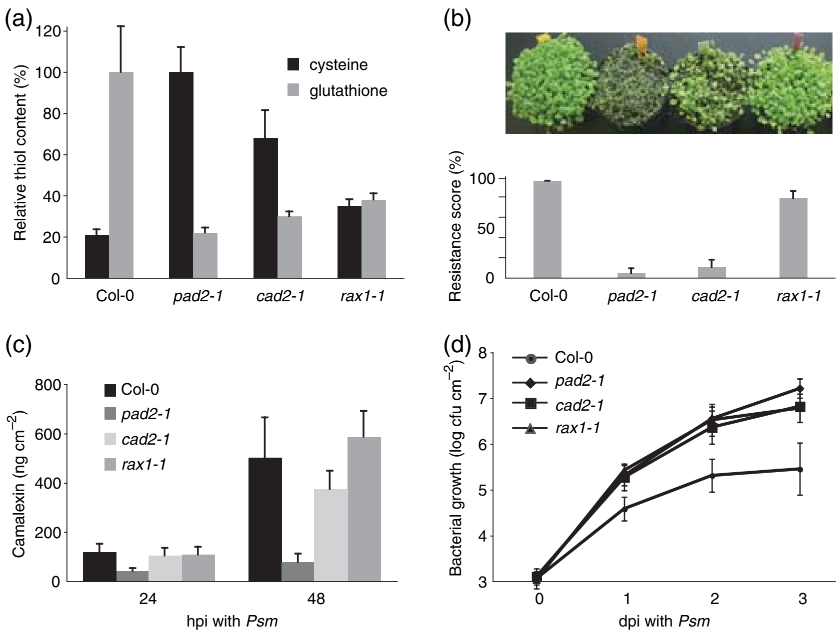

Figure 5. Comparison of Col-0 and the GSH-deficient mutants *pad2-1*, *cad2-1* and *rax1-1* in terms of GSH content, camalexin accumulation and disease resistance to *P. brassicae* and *P. syringae*.

(a) Comparison of foliar GSH and cysteine content. The results represent means and SE of three independent experiments. Four leaves of 6–8 plants each were used per experiment. The 100% value for GSH of Col-0 corresponded to 319 nmol g^{-1} FW. The 100% value for cysteine for *pad2-1* corresponded to 182 nmol g^{-1} FW. The GSH content of the three mutants was significantly different from that of Col-0 ($P < 0.05$, *t*-test). The GSH content of *pad2-1* was significantly different from that of *rax1-1* and *cad2-1* ($P < 0.005$, *t*-test). The GSH content of *cad2-1* was different from that of *rax1-1* at a lower confidence level ($P < 0.06$, *t*-test). The cysteine contents of the three mutants were significantly different from that of Col-0 ($P < 0.04$, *t*-test) and from each other ($P < 0.02$, *t*-test) except for the difference between *pad2-1* and *cad2-1* ($P < 0.08$, *t*-test).

(b) Disease resistance to *P. brassicae*. Top: 3-week-old seedlings were spray-inoculated with zoospores of *P. brassicae* isolate D (150 000 zoospores ml^{-1}) and incubated in a humid chamber for 8 days. The experiment was repeated three times with similar results. Bottom: 7-week-old plants were plug-inoculated with *P. brassicae* isolate HH and incubated in a humid chamber for 6 days. The results represent means and SE of two repetitions. A minimum of five leaves from six plants were used for each experiment. Resistance scores were determined as described in Experimental procedures. The resistance scores of *pad2-1* and *cad2-1* ($P < 0.0001$, *t*-test) and of *rax1-1* ($P = 0.05$, *t*-test) were significantly different from Col-0.

(c) Comparison of camalexin levels 24 and 48 h post-inoculation (hpi) with *Psm* ES4326. Bars represent means and SD of six replicates. Only *pad2-1* is significantly different from Col-0 ($P < 0.0001$, *t*-test). The experiment was repeated with similar results.

(d) Disease resistance to *P. syringae*. Leaves were inoculated with *Psm* ES4326 and the bacterial titer was determined 0, 1, 2 and 3 days post-inoculation (dpi). Data points represent means and SD of log-transformed data from four (0 dpi), eight (1 and 2 dpi) or 16 (3 dpi) replicates. The bacterial titer in Col-0 is significantly lower than for the other three genotypes at 1, 2 and 3 dpi ($P < 0.01$, Mann–Whitney *U*-test). Similar differences at 3 dpi were observed in two more independent experiments.

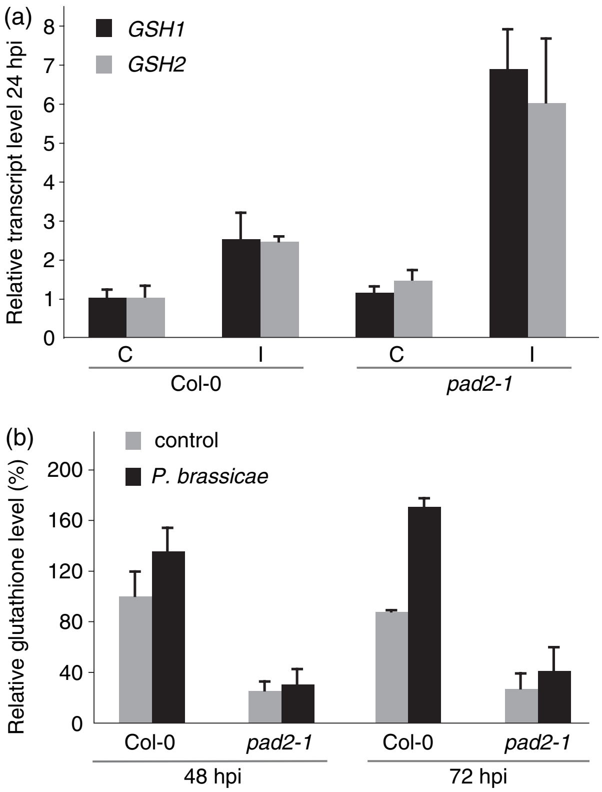

Figure 6. Accumulation of *GSH1* and *GSH2* transcripts and of GSH following inoculation with *P. brassicae*.
Leaves of 7-week-old Col-0 and *pad2-1* plants were plug-inoculated with *P. brassicae* isolate HH. Plugs not containing *P. brassicae* were used as a control treatment.
(a) Relative transcript levels of *GSH1* and *GSH2* at 24 hpi determined by real-time RT-PCR. The transcript level of uninoculated Col-0 was set to 1. Means and SE from two experiments are shown. The increase in transcript levels of *GSH1* and *GSH2* was significant in Col-0 and *pad2-1* ($P < 0.005$, *t*-test).
(b) Relative GSH content determined by HPLC 48 and 72 hpi. The GSH content of Col-0 (176 nmol g^{-1} FW) was set to 100%. The results represent means and SE of two experiments. Three to five leaves from at least six plants were used for each time point. Increases in GSH content were statistically significant for Col-0 only at 72 hpi ($P < 0.05$, *t*-test).

depending on environmental conditions (see Discussion). Figure 5(a) also shows that *pad2-1* accumulated about 5 times, *cad2-1* about 3 times and *rax1-1* 1.7 times more cysteine than Col-0. Inoculation of the three GSH-deficient mutants with *P. brassicae* led to only minor increases in foliar GSH (Figure 6b; data not shown). The three mutants were used to test the effect of varying foliar GSH content on GSH-related phenotypes. Disease resistance against *P. brassicae* was tested using the zoospore inoculation method (Figure 5b, top) and the plug inoculation method (Figure 5b, bottom). In both tests, *pad2-1* proved to be more susceptible than *cad2-1*, while the resistance of *rax1-1* was hardly affected at all. Figure 5(c) shows the results of a comparative analysis of the mutants for their capacity to accumulate camalexin in response to *Psm* ES4326. Camalexin levels in *pad2-1* were 16% of wild-type 48 h post-inoculation (hpi), while camalexin levels in *cad2-1* and *rax1-1* were not significantly different from wild-type. All three mutants showed significantly increased susceptibility to *Psm* ES4326 (Figure 5d).

Accumulation of GSH1 *and* GSH2 *transcripts and GSH in response to* P. brassicae

GSH seems to play an important role in the establishment of disease resistance to *P. brassicae* and other pathogens. Figure 6 analyses *GSH1* and *GSH2* transcript accumulation and the level of foliar thiols in Col-0 and *pad2-1* in response to *P. brassicae*. The abundance of both transcripts increased in Col-0 about 2.5-fold and in *pad2-1* more than sixfold at 24 hpi (Figure 6a). Despite this strong increase in transcript abundance, the GSH levels remained low and the cysteine levels high (data not shown) in inoculated *pad2-1* over a period of 72 hpi. In contrast, the foliar GSH content of Col-0 was increased 1.7-fold at 72 hpi (Figure 6b).

Reduced GSH levels cause susceptibility to other pathogens

The GSH deficiency suggested that the disease susceptibility of *pad2-1* was unlikely to be restricted to *P. brassicae* and virulent strains of *P. syringae*. The disease resistance phenotype of *pad2-1* has been reported in a number of publications (see Table S1 for overview). The *pad2-1* mutant was resistant to *Leptosphaeria maculans*, *Erysiphe orontii* and avirulent isolates of *P. syringae.* Depending on the accession–isolate combination, the *pad2-1* mutation had no or only a weak effect on resistance to *H. parasitica*. In contrast, *pad2-1* was more susceptible to *Botrytis cinerea* (Ferrari *et al*., 2003) and *Alternaria brassicicola* (Van Wees *et al*., 2003). To confirm and extend these results, we tested the disease resistance of *pad2-1* to two necrotrophic pathogens. Figure 7 shows that *pad2-1* was significantly more susceptible than Col-0 to *B. cinerea* or *Plectospaerella cucumerina*. Three days post-inoculation, the lesion size was about 2.7 times larger in *B. cinerea*-inoculated *pad2-1* compared to Col-0. The lesions caused by *P. cucumerina* were about nine times larger in *pad2-1* at 6 dpi. These results confirmed that the GSH deficiency of *pad2-1* interferes with disease resistance against a variety of pathogens.

Discussion

We have used the susceptibility of *pad2-1* to *P. brassicae* to clone the *PAD2* gene based on its map position (Figure 1). Our results indicate that *PAD2* encodes the known metabolic enzyme GSH1, which catalyzes the first dedicated step of GSH biosynthesis (May and Leaver, 1994). Our conclusion is supported by several lines of evidence. First, *pad2-1*

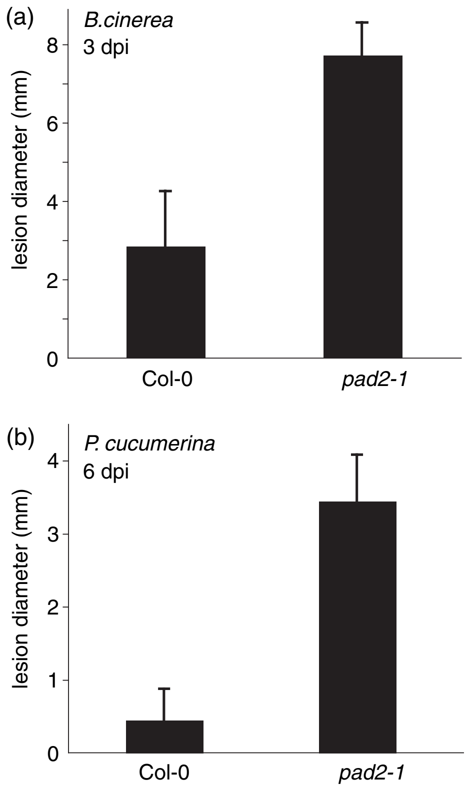

Figure 7. GSH deficiency causes susceptibility to other pathogens.
Disease resistance of Col-0 and *pad2-1* was tested against (a) *B. cinerea* isolate BMM and (b) *P. cucumerina*. Leaves of 7-week-old plants were inoculated as described in Experimental procedures. Disease resistance was scored by measuring the diameter of lesions (3 dpi for *B. cinerea* and 6 dpi for *P. cucumerina*). The results show mean values and SE from two independent experiments. A minimum of three leaves from six plants were used for each treatment. The lesion diameter of *pad2-1* was in both cases significantly different from that of Col-0 ($P < 0.0001$, *t*-test).

contained reduced levels of GSH, and genetic complementation of *pad2-1* with the wild-type *GSH1* cDNA restored GSH content and complemented all *pad2-1*-associated phenotypes (Figures 2 and 3). Second, another GSH-deficient allele of *GSH1*, *cad2-1*, was also more susceptible to *P. brassicae* and *Psm* ES4326 (Figure 5). Finally, feeding of GSH to excised leaves of *pad2-1* led to the restoration of pathogen-induced camalexin synthesis and to increased resistance to *P. brassicae* (Figure 4). The physiological complementation experiments indicated that the *pad2-1* phenotypes were a direct result of GSH deficiency.

Comparison of pad2-1 *with other* GSH1 *mutants*

Three other *GSH1* mutants of Arabidopsis have been described previously. Of these, *rml1* (Vernoux *et al.*, 2000) accumulated about 3% of the wild-type amounts of GSH and showed severe developmental defects. The *cad2-1* mutant was identified as a cadmium-sensitive mutant that accumulated only 15–45% of the wild-type amounts of GSH (Cobbett *et al.*, 1998; Howden *et al.*, 1995). The *rax1-1* mutant, which accumulated 20–50% of wild-type GSH, was identified based on its constitutive expression of photo-oxidative stress-inducible ascorbate peroxidase 2 (Ball *et al.*, 2004). The GSH deficiency apparently did not negatively affect these mutants' growth and fitness under normal growth conditions.

The *pad2-1* mutant contained less GSH than *cad2-1* or *rax1-1*, and showed the most severe phenotypes (Figure 5). It was the most susceptible mutant with regard to infection with *P. brassicae*, and it accumulated much less camalexin in response to *Psm* ES4326 than did *cad2-1* and *rax1-1*. All mutants were similarly susceptible to *Psm* ES4326. Surprisingly, *rax1-1* showed wild-type accumulation of camalexin and resistance to *P. brassicae*. Under our experimental conditions, *rax1-1* contained more GSH (38% of Col-0) than *cad2-1* (30%) and *pad2-1* (22%). The levels of the GSH1 substrate cysteine were highest in *pad2-1* (4.8-fold increase compared to Col-0) and lowest in *rax1-1* (1.7-fold increase compared to Col-0). Cysteine feeding experiments with Col-0 excluded increased cysteine levels as a cause of the observed phenotypes (Figure 4; data not shown). The phenotypic differences also appeared not to be caused by the different capacity of the mutants to accumulate GSH in response to pathogens. All three mutants showed at best only a small increase in GSH in response to inoculation with *P. brassicae* (Figure 6b, data not shown). In conclusion, relatively small differences in foliar GSH appeared to have dramatic but variable effects on the various phenotypes, indicating that the range of GSH concentrations in the various mutants is close to a threshold below which the phenotypic effects become more severe.

Although *pad2-1* consistently showed the lowest GSH content, the absolute as well as the relative level was variable between experiments depending on environmental parameters. Large variations in relative GSH content have been reported for *cad2-1* (15–45%; Cobbett *et al.*, 1998; Howden *et al.*, 1995) and *rax1-1* (20–50%; Ball *et al.*, 2004). GSH biosynthesis is positively regulated by increasing light intensity (Karpinski *et al.*, 2003; Noctor *et al.*, 1998b; Ogawa *et al.*, 2004), while high humidity has a negative effect on GSH accumulation (data not shown; May *et al.*, 1996b). Interestingly, high humidity was also reported to negatively affect hypersensitive cell death and disease resistance (May *et al.*, 1996b; Wang *et al.*, 2005; Zhou *et al.*, 2004).

Regulation of GSH accumulation in response to P. brassicae

Col-0 reacted to inoculation with *P. brassicae* with a coordinated 2.5-fold increase in *GSH1* and *GSH2* transcripts (Figure 6a). Similar results were reported by microarray analysis of the interaction of Arabidopsis with other pathogens (Figure S1; Zimmermann *et al.*, 2004). The transcriptional activation of both biosynthetic genes resulted in a 1.7-fold increase in foliar GSH at 3 dpi with *P. brassicae*. Overexpression of the wild-type *GSH1* cDNA in *pad2-1* transgenics led to a three to fourfold increase in *GSH1* transcript abundance but caused the transgenic plants to accumulate at best 1.5 times more GSH. *GSH1* transcript abundance and GSH accumulation were apparently only loosely connected. Overexpression of *GSH1* in various plant species rarely resulted in more than a twofold increase in foliar GSH (Cobbett *et al.*, 1998; Creissen *et al.*, 1999; Gomez *et al.*, 2004; Noctor *et al.*, 1996, 1998a,b; Xiang *et al.*, 2001).

The low concentration of GSH in unstressed *pad2-1* did not affect the transcript abundance of the GSH biosynthetic genes, indicating that low GSH is not sufficient to cause transcript accumulation (Figure 6a). The expression of *GSH1* and *GSH2* was, however, much more strongly induced in *pad2-1* compared with Col-0. This potentiated induction is unlikely to be caused by a reduced negative feedback of GSH on the rate of transcription of these genes. Feeding of GSH to Arabidopsis had no inhibitory effect on *GSH1* or *GSH2* transcript abundance (Xiang and Oliver, 1998). The biosynthesis of GSH is under complex control involving regulatory steps at the transcriptional and post-transcriptional level (Jez *et al.*, 2004; May *et al.*, 1998b; Xiang and Oliver, 1998). Interestingly, treatment with JA increased the transcript levels of *GSH1* and *GSH2* in Arabidopsis without affecting GSH levels (Xiang and Oliver, 1998). It was postulated that full activation of *GSH1* depended on at least two signals, one that triggers increased transcript accumulation and a second that leads to the post-translational activation of the GSH1 enzyme. A candidate for this second signal is hydrogen peroxide which, when externally applied, led to the accumulation of GSH without an increase in *GSH1* and *GSH2* transcript abundance (May and Leaver, 1993; Xiang and Oliver, 1998). GSH1 was shown to undergo a redox-regulated reversible conformational change that modulates enzyme activity (Jez *et al.*, 2004). Developing strategies to make plants more stress- and disease-resistant by increasing their GSH pool would therefore be quite challenging.

GSH plays an important role in numerous plant–pathogen interactions

The accumulation of GSH in response to *P. brassicae* suggests an increased need by the host for GSH, presumably to counteract disturbances of redox status. GSH was found to accumulate in response to various pathogens (Fodor *et al.*, 1997; May *et al.*, 1996a,b; Mou *et al.*, 2003; Vanacker *et al.*, 2000). Interestingly, significant increases in GSH were measured in incompatible but not compatible interactions (May *et al.*, 1996b; Vanacker *et al.*, 2000). A significant inverse correlation between GSH content and disease incidence was also observed in chemically induced plants (Bolter *et al.*, 1993). GSH content was found to decrease in tomato leaves infected with *B. cinerea* (Kuzniak and Sklodowska, 1999). Considering the negative effect of GSH deficiency on Arabidopsis disease resistance against *B. cinerea* (Figure 7), one might speculate that GSH biosynthesis could be a virulence target for this pathogen.

The importance of GSH in abiotic stress resistance is much better documented than its role in biotic stress resistance (May *et al.*, 1998a; Mullineaux and Rausch, 2005; Noctor *et al.*, 1998a,b). In a test of its disease resistance phenotype, the *cad2-1* mutant did not show increased susceptibility to virulent and avirulent isolates of either the biotrophic oomycete *H. parasitica* or the bacterial pathogen *P. syringae* pv. *tomato* DC3000 (May *et al.*, 1996a). It was concluded that GSH was of minor importance for disease resistance of Arabidopsis. However, impaired pathogen defense against avirulent isolates of *P. syringae* pv. *tomato* was reported for *rax1-1* and *cad2-1* (Ball *et al.*, 2004), and virus-induced gene silencing of *GSH2* compromised GSH accumulation and the disease resistance of *Nicotiana megalosiphon* to bluemold (Borras-Hidalgo *et al.*, 2006). The reported resistance of *cad2-1* to some pathogens might be explained by its slightly higher GSH content than *pad2-1*. In support of this conclusion, disease resistance to *P. brassicae* was less affected in *cad2-1* than in *pad2-1* (Figure 5b). The *pad2-1* mutant was previously tested for altered disease resistance and found to be more susceptible to a number of pathogens (Table S1). Clearly, GSH deficiency in Arabidopsis interferes with disease resistance against many pathogens. The reason for the differential effect of GSH deficiency on disease resistance to different pathogens is not clear. A possible explanation is that GSH only becomes important when the first layers of defense are breached by the pathogen. This does not seem to be the case in the interaction of Arabidopsis with *E. orontii*, *L. maculans*, and some avirulent isolates of *H. parasitica* (Bohman *et al.*, 2004; Glazebrook *et al.*, 1997; Reuber *et al.*, 1998). Although Col-0 is resistant, *P. brassicae* managed to penetrate into the leaf and then triggered a hypersensitive response. This second phase of the interaction appears to be disturbed in *pad2-1* (Roetschi *et al.*, 2001), suggesting that GSH deficiency may interfere with pathogen-triggered oxidative signaling (Foyer and Noctor, 2005; Noctor *et al.*, 2002).

How GSH homeostasis might influence plant disease resistance

Why does GSH deficiency lead to increased disease susceptibility? An obvious hypothesis is that GSH deficiency leads to disturbances of cellular homeostasis that eventually result in compromised plant fitness. Although this possibility cannot be ruled out, there is increasing evidence for a role of GSH in plant defense beyond that of a mere redox buffer (Foyer and Noctor, 2005; Noctor, 2006). The level of *S*-nitrosothiol (*S*-nitrosoglutathione, GSNO) was recently shown to affect plant disease resistance (Feechan *et al.*, 2005). The GSH-deficient mutants *pad2-1*, *cad2-1* and *rax1-1* look phenotypically normal and are still capable of mounting a successful defense against some pathogens. Application of GSH can activate the expression of stress and defense genes in a number of plant species (Dron *et al.*, 1988; Gomez *et al.*, 2004; Loyall *et al.*, 2000; Wingate *et al.*, 1988; Zhang *et al.*, 1997). Our results suggest a positive role for GSH in pathogen-induced camalexin production. Increased camalexin synthesis in response to pathogens has been shown to be independent of SA-, JA- and ET-signaling but linked to oxidative stress (Thomma *et al.*, 1999; Zhao *et al.*, 1998). Perturbations in redox signaling could form the link between GSH deficiency and reduced camalexin accumulation. As an unexplored alternative possibility, GSH could be the cysteine-related intermediate Cys-R that was proposed to function as an *S*-donor in formation of the camalexin precursor *S*-dihydro-camalexic acid (Schuhegger *et al.*, 2006).

There is evidence for a connection between GSH and the accumulation of PR proteins. *PR1* expression was induced by *GSH1* overexpression and by GSH feeding, while inhibition of GSH synthesis suppressed *PR* gene expression in tobacco (Creissen *et al.*, 1999; Gomez *et al.*, 2004). Similarly, *PR1* transcript accumulation was suppressed in the GSH-deficient *cad2-1* mutant and was promoted in *GSH1*-overexpressing Arabidopsis (Senda and Ogawa, 2004). Finally, in contrast to Col-0, *pad2-1* showed no detectable accumulation of *PR1* transcripts in response to *P. brassicae* (Roetschi *et al.*, 2001). The mechanisms of how GSH levels influence *PR* gene expression are not known. The expression of *PR1* and other SA-regulated genes is under the control of the regulatory protein NPR1 which interacts with the TGA1 and TGA4 transcription factors to activate *PR* gene expression. The reduction of specific disulfides of NPR1 and TGA1/TGA4 is crucial for the induction process (Després *et al.*, 2003; Mou *et al.*, 2003). GSH deficiency could possibly interfere with the reduction of NPR1 and/or the TGA transcription factors, thus preventing the transcriptional activation of *PR* genes. Analysis of global gene expression supports a specific effect of GSH on the expression of defense-related genes (Ball *et al.*, 2004). The expression of defense-related genes, but surprisingly not genes encoding anti-oxidant proteins, was affected by GSH deficiency in *cad2-1* and *rax1-1*. Feeding of GSH had a positive effect on defense gene expression but transcript levels of anti-oxidative enzymes were not much affected (Gomez *et al.*, 2004).

Similar to the results with GSH-deficient mutants (Ball *et al.*, 2004), the most striking changes in transcript abundance in ascorbate-deficient mutants were not observed for genes encoding anti-oxidative enzymes but for genes involved in responses to biotic stress (Pastori *et al.*, 2003). GSH and ascorbate cooperate directly via the GSH–ascorbate cycle in anti-oxidant defense (Noctor and Foyer, 1998). It is therefore surprising that ascorbate and GSH deficiency have opposite effects on plant disease resistance: high ascorbate levels were found to suppress the expression of PR proteins, and ascorbate-deficient mutants showed increased SA content, constitutive accumulation of defense gene transcripts and increased resistance to virulent strains of *P. syringae* and *H. parasitica* (Barth *et al.*, 2004; Pastori *et al.*, 2003; Pavet *et al.*, 2005). The opposite effects of changes in the level of GSH and ascorbate are difficult to reconcile with their well-described cooperative function in anti-oxidant defense, and suggest additional more specific roles of these molecules in plant disease resistance.

Experimental procedures

Biological material and plant inoculation with pathogens

After stratification for 3 days at 4°C, *Arabidopsis thaliana* seeds of accession Col-0 and the various Arabidopsis mutants were grown in jiffy-7 peat pellets (42 mm, Samen Mauser AG, Winterthur, Switzerland) in a growth chamber with a 10/14 h day/night photoperiod at 19/17°C. Light intensity varied between 80 and 120 μE m^{-2} sec^{-1}. The following mutant lines (all in the Col background) were used: *pad2-1* and *pad2-2* (Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1996), *cad2-1* and *cad1-3* (Howden *et al.*, 1995), and *rax1-1* (Ball *et al.*, 2004).

P. brassicae isolate HH and *P. brassicae* isolate D (CBS179.89) were cultivated as described previously (Roetschi *et al.*, 2001). Plants were inoculated in two ways. Unless otherwise indicated, 6–7-week-old plants were inoculated by placing agar plugs with growing mycelia upside-down on the leaf surface (Roetschi *et al.*, 2001). Empty plugs were used for control treatments. In some experiments, 3-week-old plants were spray-inoculated at low pressure with zoospore suspensions at a concentration of 1–2×10^5 zoospores ml^{-1}. Zoospores were produced as described by Roetschi *et al.* (2001). The inoculated plants were incubated at 100% relative humidity at 17–19°C. Plants were inoculated shortly before the onset of the 14 h dark period. Plug-inoculated plants were scored for disease resistance on a scale of 0–4 based on symptom development. Zero corresponds to a completely susceptible leaf and 4 to a fully resistant leaf, while scores of 1, 2 or 3 represent leaves with lesions on about 75%, 50% or 25% of their surface, respectively.

P. syringae pv. *maculicola* strain ES4326 was cultured in King's B medium at ambient temperature (22–25°C). Plants used for *Psm* ES4326 infection were grown in a controlled-environment chamber at 22°C, 75% relative humidity and 100 μmol m^{-2} sec^{-1} fluorescent illumination on a 12 h light/12 h dark cycle. All plants were 4–5 weeks old at the time of infection. For assay of camalexin and bacterial growth, plants were infected at a concentration of 4×10^4 or 1×10^3 CFU cm^{-2} of leaf, respectively. Bacterial infection and determination of bacterial titer was performed as described previously (Glazebrook and Ausubel, 1994), except that each sample consisted of two leaf discs, and tissue was ground in deep 96-well plates using ball bearings and a paint shaker. *P. cucumerina* was grown in the dark at 18°C on potato dextrose agar (PDA) plates. *B. cinerea* was grown on PDA plates at room temperature. Plants were inoculated by applying 4 μl droplets of spore suspensions to the upper surface of the leaves. The spore concentration was 10^5 spores ml^{-1} for *P. cucumerina*. The spores of *B. cinerea* were applied in quarter-strength potato dextrose broth (PDB) at a concentration of 3×10^4 conidia ml^{-1}. The inoculated plants were incubated at 100% relative humidity at 20–24°C with a 12 h photoperiod. Symptom development was scored by measuring lesion diameter. Control inoculations were performed with sterile water or quarter-strength PDB, respectively.

Genetic complementation of pad2-1

The cDNA from *GSH1* of Arabidopsis (May and Leaver, 1994) was PCR-amplified using the primers F-GSH1-*Bam*HI/GW (5′-CACCGGATCCTATACCATGGCGCTCTTGTCTC-3′) and R-GSH1-*Sac*I (5′-GCGAGCTCCCGGAGACTCGAATTCTTCAG-3′). The resulting 1653 bp product was sequence-verified and mobilized into the Gateway pENTR donor vector by directional TOPO cloning to generate the pENTR:*GSH1* cDNA entry clone (Invitrogen, Basel, Switzerland). *GSH1* cDNA insertion was verified by PCR, and the cDNA of a positive pENTR:*GSH1* cDNA entry clone was mobilized into the Gateway destination vector pB2GW7 (Karimi *et al.*, 2002). In the resulting pB2GW7-*GSH1* plasmid, expression of the *GSH1* cDNA is under the control of the constitutive CaMV 35S promoter. *Agrobacterium tumefaciens* GV3101 was transformed by electroporation. Positive colonies were selected on LB plates containing spectinomycin (50 μg ml^{-1}), rifampicin (25 μg μl^{-1}) and gentamycin (25 μg μl^{-1}), and tested for the presence of the *GSH1* cDNA by PCR. One positive *Agrobacterium* colony was used for *in planta* transformation of Arabidopsis. Transformed Arabidopsis were selected based on their resistance to BASTA. Thirty-six primary transformants were tested for their GSH content, and three with a single T-DNA insertion were selected for further analysis.

Physiological complementation of pad2-1

For physiological complementation of camalexin deficiency, leaves were excised from 5-week-old plants and the petioles were placed in 1.5 ml microcentrifuge tubes containing various solutions. Leaves were infected with *Psm* ES4326 at a concentration of 4×10^4 CFU cm^{-2} of leaf and incubated in a controlled-environment chamber as described above. For physiological complementation of disease resistance against *P. brassicae*, excised leaves from 6-week-old Arabidopsis plants were incubated in test solutions 6 h prior to inoculation with a zoospore suspension of isolate D. The leaves were incubated under the conditions described above and analyzed at 7 dpi. The foliar thiol content was determined at the time of inoculation.

Biochemical analysis

GSH and cysteine were extracted and quantified by HPLC as described by Harms *et al.* (2000). The samples were analyzed on a reverse-phase HPLC column (C18, 250×4 mm, 5 μm particle size; Macherey-Nagel, Oensingen, Switzerland). A solvent system consisting of 10% v/v methanol, 0.25% v/v acetic acid, pH 3.9 (NaOH) and 90% v/v methanol with a flow rate of 1.5 ml min^{-1} was used. Chromatography was followed by fluorescence detection (excitation: 380 nm, emission: 480 nm, SFM 25 fluorescence detector; Kontron, Zürich, Switzerland). Chromatograms were quantified by integration of peak areas. An internal standard (homoglutathione, 50 μg g^{-1} plant fresh weight) was added to the plant material before extraction. The amounts of GSH and cysteine were calculated relative to homoglutathione using a calibration curve. The measurement of camalexin was performed as described previously (Glazebrook and Ausubel, 1994).

Analysis of gene expression

Total RNA was extracted from Arabidopsis leaves using the RNeasy Plant Mini Kit following the manufacturer's instructions, including a treatment with RNase-free DNase I (Qiagen, Hombrechtikon, Switzerland). Total RNA (2 μg) was reverse-transcribed into cDNA using the Omniscript RT Kit (Qiagen). Real-time PCR with a Rotor-Gene 2000 apparatus (Corbett Research, Sydney, Australia) was performed using SYBR-Green as fluorescent reporter dye (SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA, USA) with the following primers: F-GSH1 (5′-GTGTGCCCTGCCAGCTTTCT-3′), R-GSH1 (5′-CAGGACATCTTCAGCGACATGC-3′), F-GSH2 (5′-CTTGGTGCGAGAAGGCGTTTA-3′), R-GSH2 (5′-ACTCCAAAACCAGCTGCAACG-3′), F-actin (5′-GACCTTTAACTCTCCCGCTATG-3′) and R-actin (5′-CACCAGAATCCAGCACAATAC-3′).

The respective primer pairs amplified a 183 bp region of *GSH1* and 170 bp region of *GSH2* from the 3′ end of the target transcript. Each reaction was performed in triplicate. The specificity of the amplification was confirmed by melting curve analysis and agarose gel electrophoresis. The relative abundance of *GSH1* and *GSH2* transcripts was calculated and normalized to *At-Actin2* (At3g18780) transcript levels based on analysis with the Rotor-Gene 4.4 software package. Results represent the means and standard errors of two experiments. The real-time RT-PCR data of *GSH1* expression were qualitatively confirmed by RNA blot analysis using ^{32}P-radiolabeled *GSH1* cDNA.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Relative transcript levels of *GSH1* and *GSH2* in response to various abiotic and biotic stresses as shown by the 'Gene Correlator' tool of GENEVESTIGATOR.

Table S1 Overview of *pad2-1* disease resistance phenotypes reported in the literature

This material is available as part of the online article from http://www.blackwell-synergy.com.

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