

# An *Arabidopsis* Mutant Depleted in Glutathione Shows Unaltered Responses to Fungal and Bacterial Pathogens

Mike J. May<sup>1</sup>, Jane E. Parker<sup>2</sup>, Michael J. Daniels<sup>2</sup>, Christopher J. Leaver<sup>1</sup>, and Christopher S. Cobbett<sup>3</sup>

<sup>1</sup>Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK; <sup>2</sup>The Sainsbury Laboratory, Norwich Research Park, Colney, Norwich, NR4 7UH, UK; <sup>3</sup>Department of Genetics, The University of Melbourne, Parkville, Australia, 3052

Received 5 September 1995. Accepted 1 March 1996.

We have previously shown that the leaf glutathione (GSH;  $\gamma$ -glutamylcysteinyl glycine) content of a cadmium-sensitive mutant of *Arabidopsis thaliana*, *cad2-1*, was decreased to 30% of that in the wild-type parental accession Columbia (Col-0) (R. Howden et al., *Plant Physiol.* 107:1067–1073, 1995). Here we show that GSH deficiency in this mutant does not alter responses to fungal and bacterial pathogens. The timing and extent of asexual (sporangiophore) and sexual spore (oospore) development of a virulent strain of *Peronospora parasitica*, *Noco2*, in *cad2-1* were identical to those in Col-0. The resistance of *cad2-1* to a strain of *P. parasitica*, *Wela1*, which is avirulent in Col-0, was not altered. Furthermore, attempted infection by *Wela1* was associated with a 2.5-fold increase in the level of glutathione in *cad2-1* and a 1.3-fold increase in Col-0, and in accumulation of PR1 mRNA in both mutant and wild-type plants. However, while in Col-0 levels of glutathione dropped to the initial levels 3 days after inoculation, the increase was maintained in the mutant, *cad2-1*. A virulent strain of *Pseudomonas syringae* pv. *tomato* (DC3000) grew at the same rate and to the same titer in both *cad2-1* and Col-0 and both plant lines were equally resistant to the same strain carrying the avirulence gene *avrB* from the soybean pathogen *P. syringae* pv. *glycinea*. Both *cad2-1* and Col-0 acquired resistance to *Noco2* and *P. syringae* pv. *tomato* (DC3000) after treatment with 5  $\mu$ M or 10  $\mu$ M 2,6-dichloroisonicotinic acid (INA) and this was associated with accumulation of PR1 mRNA. A 1.6-fold increase in the level of glutathione in Col-0 and a 2.3-fold increase in *cad2-1* were also observed and were maintained in both lines during the course of the experiment. These results demonstrate that the depletion of glutathione levels by 70% in *Arabidopsis* does not alter its responses to the pathogens analyzed. The impact of these results on the proposed functions of GSH and the way in

which active oxygen species are generated and reduced in plant-pathogen interactions is discussed.

*Additional keywords:* disease resistance, pathogenesis.

Fungal, viral, and bacterial pathogens elicit a vast array of biochemical responses in plants (Keen 1990; Lamb 1994). When the host responds promptly to attempted infection with the mobilization of defense systems, the establishment and spread of the pathogen is prevented. Such resistance is often accompanied by a hypersensitive response (HR). In many genetically characterized plant-pathogen interactions, the HR-associated resistance response is triggered by recognition between single gene products in the host and the pathogen (Keen 1990; Bennetzen and Jones 1992). The HR has been shown to be associated with a transient oxidative burst, plant defense gene activation, accumulation of antimicrobial compounds (phytoalexins), and oxidative cross-linking of the cell wall, resulting in a zone of localized necrosis at the site of attempted infection that limits pathogen growth (Lamb 1994; Mehdy 1994). In addition, the HR is often accompanied by increased resistance to subsequent infection throughout the plant and distant from the site of infection. This is termed systemic acquired resistance (SAR) (Kuc 1982). The complexity of this process and the speed with which defenses are deployed have presented obstacles to characterizing how individual components of the HR contribute to plant defense. Furthermore, while effective defense is likely to be the result of a combination of these factors it remains unclear how biochemical events of such widely differing nature are regulated in a coordinate manner upon resistance (*R*)-gene activation. Since the physiological symptoms associated with HR and SAR are different, it may be that the molecular bases of the respective resistance mechanisms differ greatly. A commonly adopted approach to determining the relative contribution of factors in the establishment of a resistance response is the treatment of plants with molecules that have no intrinsic antimicrobial activity yet modulate the levels and activity of components of plant defense. Considerable attention has been paid to the potential role of the ubiquitous thiol tripeptide, glutathione, (GSH;  $\gamma$ -glutamylcysteinyl glycine) and a number of independent lines of evidence have implicated a central role

Corresponding author: Mike J. May. Present address: Laboratorium voor Genetika, Universiteit Gent, KL Ledeganckstraat 35, B-9000, Gent, Belgium; Tele.: (32) 9 264 5010; Fax: (32) 9 264 5349; E-mail: mimay@gengenp.rug.ac.be

The first two authors contributed equally to this work.

for GSH in a variety of processes during host defense activation. Depletion of GSH or increases in its oxidation state in plants treated with sulfhydryl reagents is correlated with the accumulation of phytoalexins (Stossel 1984; Gustine 1987) and H<sub>2</sub>O<sub>2</sub> may exacerbate this effect (Guo et al. 1993). Evidence has been presented that this may occur at the level of transcription (Wingate et al. 1988). Increasingly, evidence suggests an important role for an accumulation of activated oxygen species (AOS) production in plant-pathogen interactions (Apostol et al. 1989; Legendre et al. 1993; Levine et al. 1994). AOS may injure the pathogen (Kim et al. 1988) and limit colonization through callose formation and oxidative cross-linking of cell-wall proteins (Bradley et al. 1992) and the promotion of cell death at the site of attempted infection (Levine et al. 1994). A role for hydrogen peroxide as a second messenger that diffuses from the site of incompatible plant-pathogen interactions has been proposed (Levine et al. 1994). A variety of defense responses are elicited; among these is the induction of glutathione-S-transferase (GST) gene expression, as has been reported previously (Mauch and Dudler 1993), and expression of glutathione peroxidase (Gpx). Adequate GSH in cells surrounding the site of localized necrosis, as an essential component of antioxidant defenses (Alscher 1989) and as a substrate for the induced enzyme activities would be necessary to prevent inopportune oxidative damage. It is clear that GSH and GSH-dependent processes potentially play a pivotal role in determining the outcome of plant-pathogen interactions.

The interaction between *Arabidopsis thaliana* (L.) Heynh. and its fungal and bacterial pathogens is emerging as a valuable model system to investigate the role of components of plant defense in the establishment of effective resistance; the model permits facile genetic analysis of both host and pathogens (Dangl et al. 1993). The availability of *cad2-1*, a mutant of *Arabidopsis* with drastically reduced GSH levels (Howden et al. 1995), presented a genetic basis on which to address important questions concerning the role of GSH and GSH-related processes in plant-pathogen responses. In this report we describe the response of *cad2-1* to both virulent and avirulent fungal and bacterial pathogens. We also analyze the gene expression of a defense-related protein, PR1, both during pathogen attack and also in response to 2,6-dichloroisonicotinic acid (INA) treatment, an inducer of SAR (Mettraux et al. 1991). Severe depletion of GSH does not impair the capacity of *A. thaliana* to resist an avirulent pathogen and does not lead to greater susceptibility to a normally virulent pathogen.

## RESULTS

### Responses of *cad2-1* and Col-0 to *Peronospora parasitica* infection.

Genetically determined resistance in wild-type Col-0 plants to the *Wela1* isolate of *Peronospora parasitica* (Pers.:Fr.) Fr. is controlled by a single resistance locus, *RPP6* (Holub et al. 1994). In contrast, Col-0 plants are susceptible to *Peronospora parasitica* isolate *Noco2*, (Parker et al. 1993). Therefore, the reaction phenotypes of Col-0 and *cad2-1* plants to these two fungal isolates were assessed. Individual cotyledons of Col-0 and *cad2-1* seedlings were inoculated with conidia of either *Wela1* or *Noco2* and infections were evaluated daily for asexual sporulation on cotyledons and leaves. Material was also stained with lactophenol-trypan blue and examined microscopically to assess disease severity and the nature of host cell responses. Results in Table 1 show that there was no difference between *cad2-1* and Col-0 in their resistance or sensitivity profiles to *P. parasitica* isolates *Wela1* and *Noco2*, respectively. The response phenotypes were evaluated by scoring the numbers of sporangiophores and sexual spores (oospores) in each inoculated cotyledon and by assessing the extent of plant cell necrosis surrounding sites of fungal ingress. Also, systemic spread of the fungus was recorded and was similar in both mutant and wild-type plants. As a control, inoculation of *La-er* seedlings (Table 1) showed that *Wela1* was pathogenically competent on *La-er*, which is normally susceptible to this *P. parasitica* isolate, and that *Noco2* was avirulent due to recognition by the resistance locus *RPP5*, (Parker et al. 1993).

### INA-induced resistance is effective in *cad2-1*.

To compare the responsiveness of Col-0 and *cad2-1* to INA-induced resistance, seedlings were inoculated with *Noco2* conidia 3 days after a soil drench with 5 or 10 μM INA or with wettable powder alone and plants were monitored for fungal development. Treatment with 5 μM INA caused a reduction in sporulation of *Noco2* in both Col-0 and *cad2-1* after 4 days, compared with plants treated with wettable powder alone. Treatment with 10 μM INA resulted in almost complete protection in both lines. Table 2 shows the development of *Noco2* in seedlings treated with 10 μM INA and stained 5 days after inoculation. These data confirm the macroscopic assessment and show that *cad2-1* is as responsive as Col-0 to INA. Cytological examination of *cad2-1* and Col-0 pretreated with INA and inoculated with *Noco2* demonstrated that INA-triggered

**Table 1.** Growth and development of *Peronospora parasitica* *NoCo 2* and *Wela 1* in wild-type (Col-0), glutathione-depleted (*cad 2-1*) and control (*La-er*) hosts<sup>a</sup>

Plant host	<i>Wela 1</i>				<i>NoCo 2</i>			
	Sp. <sup>b</sup>	Oosp. <sup>c</sup>	Cell nec. <sup>d</sup>	Systemic spread <sup>e</sup>	Sp. <sup>b</sup>	Oosp. <sup>c</sup>	Cell nec. <sup>d</sup>	Systemic spread <sup>e</sup>
Col-0	–	–	+	–	+	+++	–	+
<i>cad 2-1</i>	–	–/(+)	(+)/+	–	+	+++	–	+
<i>La-er</i>	+	(+)	–/(+)	–/+	–	–	+	–

<sup>a</sup> Plants stained 6 days after inoculation. Results represent average scorings from at least 5 plants per treatment. All plants in a single treatment gave similar results.

<sup>b</sup> Asexual sporophores: (+), 1 to 5; +, 5 to 15; ++, 15 to 40; +++, >40 per cotyledon

<sup>c</sup> Sexual oospores, same scale as asexual sporophores.

<sup>d</sup> Plant cell necrosis: –, no necrosis; (+), 1 to 5 cells; +, >5 cells around sites of fungal ingress

<sup>e</sup> Mycelium and sporangiophores observed on leaves.

resistance is associated with localized necrosis around the site of attempted infection in both lines. There were no differences in the size of the lesion nor in the sharpness of the border (data not shown).

#### Responses of *cad2-1* and Col-0 to bacterial infection.

In order to assess any effect of severely depleted GSH in *cad2-1* on genetically determined resistance to a bacterial pathogen, leaves were inoculated with the virulent strain DC3000 of *Pseudomonas syringae* pv. *tomato* (Whalen et al. 1991) and the same strain harboring the avirulence gene *avrB* from the soybean pathogen *P. syringae* pv. *glycinea* (DC3000/

pVB01, referred to as DC3000/*avrB*), which is avirulent to Col-0 (Innes et al. 1993). Symptom development and bacterial growth were measured over 5 days. Results indicated that *cad2-1* and Col-0 supported growth of the virulent strain DC3000 (Fig. 1A) and plants showed similar symptom expression (Fig. 2A). They were equally resistant to DC3000/*avrB* (Figs. 1A and 2B). As a control DC3000/*avrB* grew and induced symptoms in *Arabidopsis* accession Bla-2, which does not interact incompatibly with *avrB* (Innes et al. 1993; data not shown). In addition, the response of *cad2-1* to INA-induced resistance when inoculated subsequently with strain DC3000 was equal to that of Col-0 (Figs. 1B and 2C). In both

**Table 2.** Growth and development of *Peronospora parasitica* NoCo 2 in Col-0 and *cad2-1* plants after INA pretreatment<sup>a</sup>

Plant host	Control (wp) <sup>b</sup>				INA (10 μM)			
	Sp. <sup>c</sup>	Oosp. <sup>d</sup>	Cell nec. <sup>e</sup>	Systemic spread <sup>f</sup>	Sp. <sup>c</sup>	Oosp. <sup>d</sup>	Cell nec. <sup>e</sup>	Systemic spread <sup>f</sup>
Col-0	++	+++	-/(+)	+	(+)	-	(+)	-
<i>cad2-1</i>	++	+++	-	+	(+)	-/+	(+)	-

<sup>a</sup> INA = 2,6-dichloroisonicotinic acid. Plants stained 5 days after inoculation with *NoCo 2*. Results represent the average scorings from at least 5 plants per treatment.

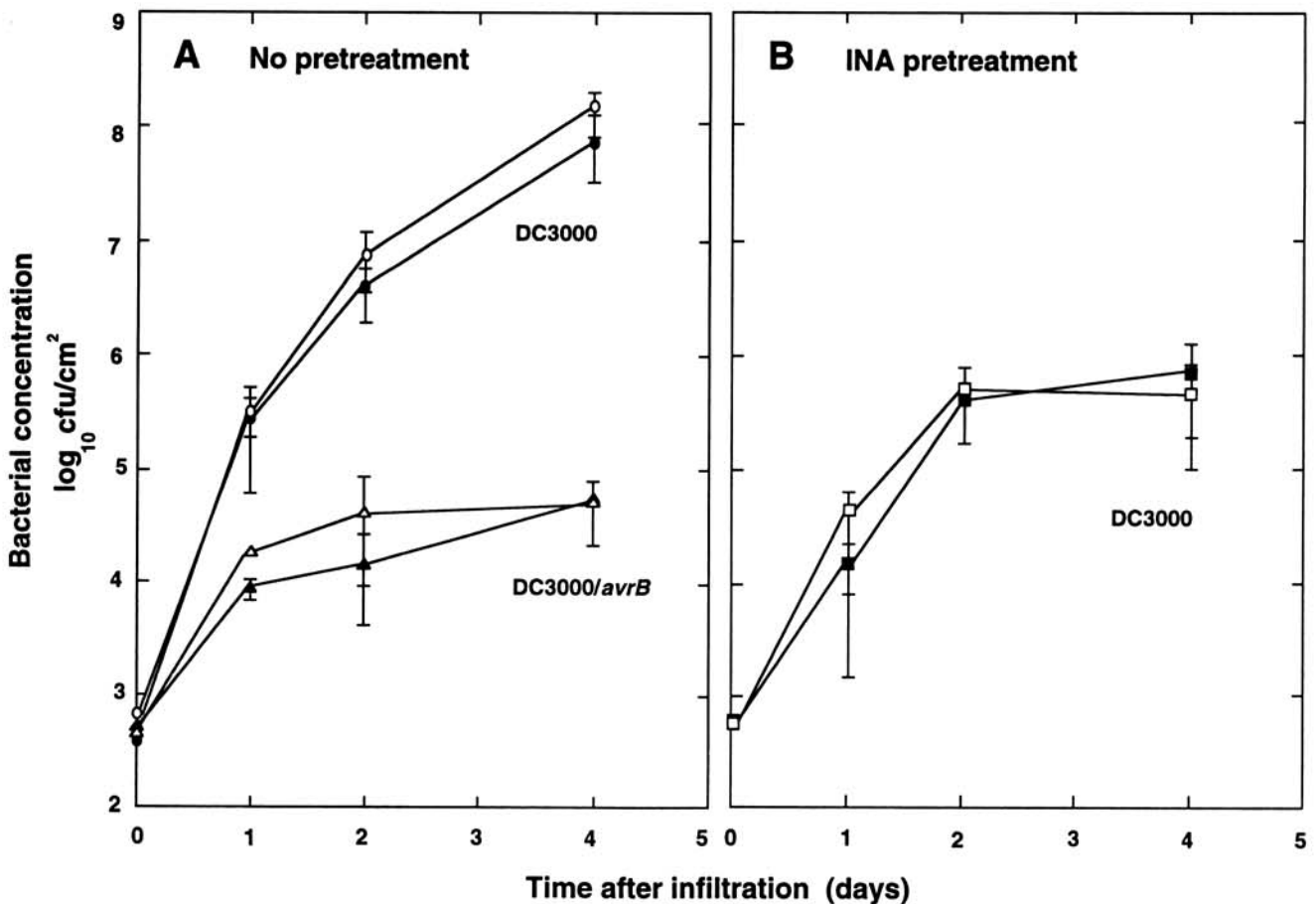
<sup>b</sup> Wettable powder formulation without active INA ingredient.

<sup>c</sup> Asexual sporophores: (+), 1 to 5; +, 5 to 15; ++, 15 to 40; +++, >40 per cotyledon

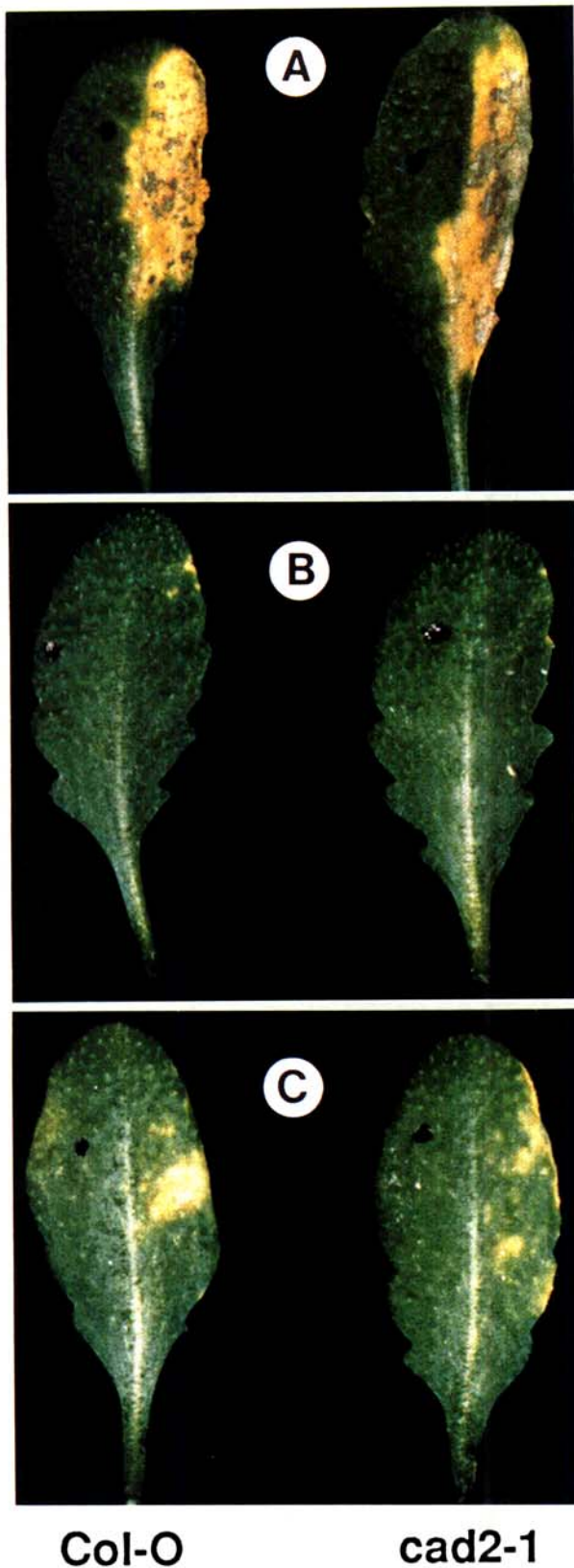
<sup>d</sup> Sexual oospores, same scale as asexual sporophores.

<sup>e</sup> Plant cell necrosis: -, no necrosis; (+), 1 to 5 cells; +, >5 cells around sites of fungal ingress

<sup>f</sup> Mycelium and sporangia observed on leaves.



**Fig. 1.** A, Growth of *Pseudomonas syringae* pv. *tomato* strain DC3000 in leaves of *Arabidopsis* Col-0 (●) and *cad2-1* (○) plants and of *P. syringae* tomato DC3000 expressing *avrB* (DC3000/*avrB*) in Col-0 (▲) and *cad2-1* (△). Leaves were infiltrated with a suspension of  $2 \times 10^5$  CFU/ml. B, Growth of *P. syringae* tomato strain DC3000 in leaves of *Arabidopsis* Col-0 (■) and *cad2-1* (□) plants 3 days after pretreatment with a soil drench of 10 μM 2,6-dichloroisonicotinic acid (INA). Leaves were infiltrated with a bacterial suspension of  $2 \times 10^5$  CFU/ml.



**Fig. 2.** Symptom expression in *Arabidopsis* Col-0 and *cad2-1* leaves 5 days after infiltration of untreated leaves with *Pseudomonas syringae* pv. *tomato* strain DC3000 (A) and DC3000/*avrB* (B), and of DC3000 in leaves pretreated with 10  $\mu$ M 2,6-dichloroisonicotinic acid (INA) (C). Leaves were infiltrated with a bacterial suspension of  $2 \times 10^5$  CFU/ml<sup>-1</sup>.

mutant and wild-type plants, INA pretreatment caused a 100-fold reduction in the recovery of viable bacteria from inoculated leaves.

#### Defense gene expression.

Marked changes in gene expression are frequently observed in response to attempted invasion by avirulent pathogens. Typical of this incompatible response is the expression of genes encoding "pathogenesis-related" (PR) proteins (Bol et al. 1990). Furthermore, their expression is strongly induced after INA treatment (Uknes et al. 1992). A 760-bp DNA fragment encoding *Arabidopsis* PR1 (Uknes et al. 1992) was used as a hybridization probe against Northern (RNA) blots isolated from *cad2-1* and Col-0 plants under two different inoculation conditions. In the first experiment, plants were spray inoculated with the avirulent *P. parasitica* isolate *Wela1* or with distilled water only. In a second experiment, plants were pretreated for 3 days with INA or with wettable powder only and then inoculated with the normally virulent *P. parasitica* isolate *Noco2*. The results shown in Figure 3 demonstrate that the timing and magnitude of PR1 gene expression in both *cad2-1* and Col-0 were identical, in response to *Wela1* inoculation. A weak induction of the PR-1 transcript was observed 24 h after inoculating Col-0 and *cad2-1* plants. A stronger PR-1 transcript induction was observed in both Col-0 and *cad2-1* in response to INA treatment, and raised levels of PR-1 mRNA were maintained during subsequent protection from *P. parasitica* *Noco2* (Fig. 3). The lack of PR-1 mRNA induction in the control plants (pretreated with wettable powder only) before and after *Noco2* inoculation indicated that growth of the normally virulent fungus in Col-0 and *cad2-1* plants did not induce PR-1 under these experimental conditions.

#### Glutathione determination.

It has previously been observed that the steady state levels of cellular thiols are increased after treatment of bean and alfalfa suspension cultures with fungal elicitors from *Colletotrichum lindemuthianum* (Edwards et al. 1991) and in cultured liverwort cells treated with elicitors (Nakagawara et al. 1993). We determined the concentration of glutathione in leaf extracts prepared from *cad2-1* and Col-0 at various time points after inoculation with *P. parasitica* isolates *Noco2* (virulent) and *Wela1* (avirulent) with or without pretreatment of 10  $\mu$ M INA. Significant increases in the extractable glutathione content were only measured during the incompatible reaction (1.3-fold, Col-0; 2.5-fold, *cad2-1*) compared with water-treated controls 1 day after inoculation. In Col-0, this dropped to control levels 3 days after inoculation (Table 3) although in *cad2-1* elevated glutathione levels were maintained throughout the period of analysis. Concurrently, significant increases in the level of oxidized glutathione (GSSG) occurred 1 day after inoculation, indicating a perturbation of the redox state during the establishment of the incompatible reaction. Changes in the level of GSSG during the compatible interaction could not be measured since they were below the detection level of the assay used. When seedlings were treated with 10  $\mu$ M INA prior to inoculation with *Noco2*, a 1.3-fold increase in the extractable glutathione was measured in extracts of Col-0 and a 1.4-fold increase in *cad2-1* 3 days after pretreatment. The levels rose to 1.6-fold in Col-0 and 2.3-fold in *cad2-1* 2 days after *Noco2* inoculation compared with the

wettable powder-treated controls (Table 3) and these levels were maintained in both plant lines.

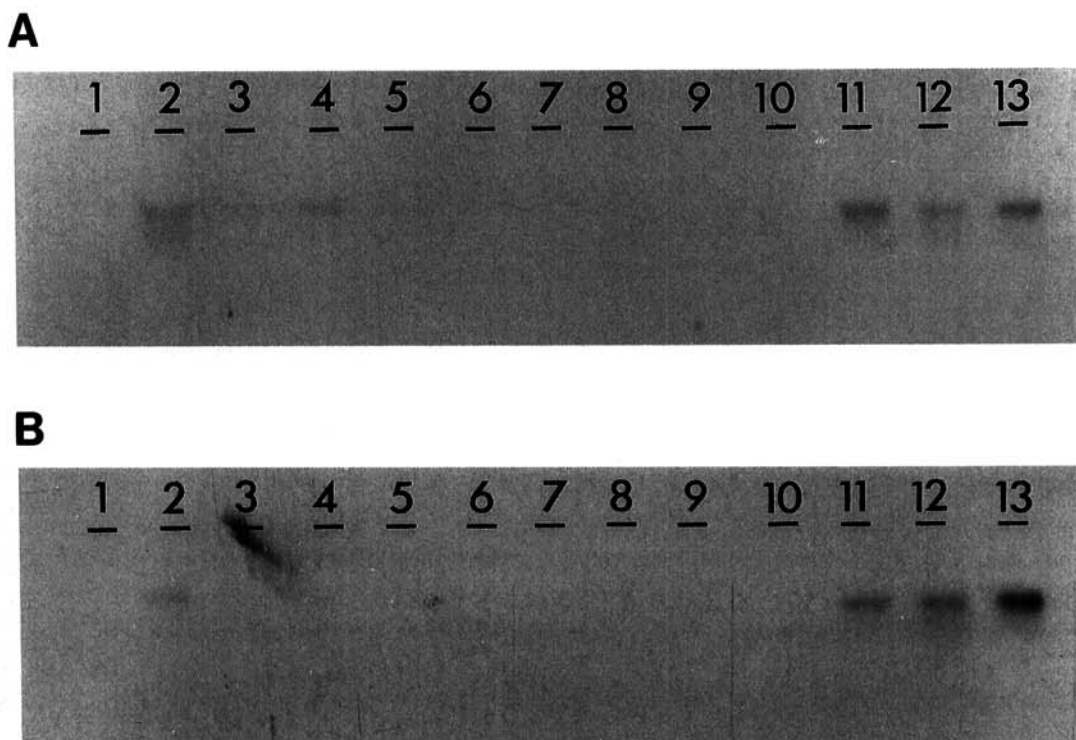
## DISCUSSION

The consequence of plant disease resistance gene action, commonly manifested as the HR, is highly complex. Mutants defective in specific components of the HR should establish more clearly the relative contribution of individual gene prod-

ucts to the establishment of effective resistance and clarify how such biochemically diverse processes are coordinated in a challenged plant.

We have exploited a mutant of *Arabidopsis*, *cad2-1*, in which GSH levels are 70% lower than in the wild-type parental ecotype, Col-0, to investigate the role of GSH in plant-pathogen interactions.

Molecular, microscopic, macroscopic, and biochemical analyses of the response of *cad2-1* to virulent and avirulent



**Fig. 3.** Northern (RNA) blot analysis of PR1 expression in *Arabidopsis* Col-0 (A) and *cad2-1* (B). Total RNA was extracted from leaf tissue after the following treatments: Lanes 1 to 3, *Peronospora parasitica* WeLa1, day 0, 1, and 3, respectively; and lanes 4 and 5, control water treatment for 1 and 3 days, respectively. Lanes 6 and 7, wettable powder treatment for 0 and 3 days; and lanes 8 and 9, 24 and 48 h postinfection with *Peronospora parasitica* Noco2 after 3 days wettable powder pretreatment. Lanes 10 and 11, 2,6-dichloroisonicotinic acid (INA) treatment for 0 and 3 days; and lanes 12 and 13, 24 and 48 h postinfection with *P. parasitica* Noco2 after 3 days INA pretreatment.

**Table 3.** Steady state levels of total (reduced [GSH] plus oxidized [GSSG]) glutathione and GSSG measured in extracts of wild-type (Col-0) and GSH-depleted (*cad 2-1*) *Arabidopsis* after fungal inoculation or chemical treatment<sup>a</sup>

Treatment	Time <sup>b</sup>	Col-0		<i>cad 2-1</i>	
		GSH/GSSG	GSSG	GSH/GSSG	GSSG
WeLa 1	0 d	206.6 +/- 14.7	14.5 +/- 1.1	34.95 +/- 5.8	ND <sup>c</sup>
WeLa 1	1 d	264.1 +/- 18.2	32.1 +/- 4.3	87.93 +/- 7.9	12.1 +/- 1.5
WeLa 1	3 d	201.8 +/- 26.3	15.5 +/- 6.1	78.52 +/- 8.3	11.5 +/- 3.1
H <sub>2</sub> O	1 d	206.3 +/- 15.2	15.2 +/- 3.4	37.68 +/- 2.1	ND
H <sub>2</sub> O	3 d	205.1 +/- 14.4	14.8 +/- 2.5	38.8 +/- 8.2	ND
WP <sup>d</sup>	0 d	202.9 +/- 17.1	13.3 +/- 3.2	33.2 +/- 1.5	ND
WP	3 d	211.4 +/- 14.1	15.4 +/- 4.4	32.12 +/- 4.0	ND
WP + Noco 2	24 hpi	185.6 +/- 18.4	16.1 +/- 2.1	24.21 +/- 3.5	ND
WP + Noco2	48 hpi	212.1 +/- 24.4	18.4 +/- 1.0	36.0 +/- 5.0	ND
INA	0 d	201.4 +/- 15.3	12.1 +/- 1.5	35.32 +/- 2.9	ND
INA	3 d	266.4 +/- 27.4	14.4 +/- 3.2	46.3 +/- 8.4	ND
INA + Noco2	24 hpi	239.2 +/- 18.1	17.0 +/- 2.6	84.34 +/- 12.6	17.2 +/- 3.2
INA + Noco2	48 hpi	329.3 +/- 21.4	25.5 +/- 6.8	80.1 +/- 6.2	15.3 +/- 2.5

<sup>a</sup> Tissue samples taken at the time points indicated and frozen in liquid nitrogen until use. Glutathione levels are expressed as nmol per g fresh weight.

<sup>b</sup> d, days; hpi, hours postinoculation.

<sup>c</sup> Not detected (below the minimum detection level of the assay).

<sup>d</sup> Wettable powder formulation without active 2,6-dichloroisonicotinic acid ingredient.

fungal and bacterial pathogens revealed no differences when compared with the wild-type parental strain Col-0. Both lines were equally resistant to an avirulent strain of *Peronospora parasitica*, *Wela1*, and no differences in the size and structure of necrotic lesions surrounding the site of attempted ingress were evident. Furthermore, the timing and magnitude of PR1 gene expression, hallmarks of the incompatible response, were identical in both lines in response to attempted infection by *Wela1*. Quantitatively and qualitatively there were no differences in the ability of mutant and wild-type plants to restrict the growth of virulent fungal (*Noco2*) and bacterial (*P. syringae* pv. *tomato*, DC3000) pathogens. Also, INA, a chemical inducer of SAR, induced resistance in both lines to the virulent *P. parasitica* isolate *Noco2* and the bacterial pathogen *P. syringae* pv. *tomato* DC3000.

The similarities in the localized reactions of *cad2-1* and Col-0 to avirulent pathogens and in the systemic responses to chemical treatment is initially perplexing. Adequate GSH, as an essential component of antioxidant defenses, would in theory be required for scavenging AOS to arise during both the HR (Apostol et al. 1989; Legendre et al. 1993; Levine et al. 1994) and SAR (Chen et al. 1993) for the prevention of oxidative damage in unchallenged tissues (Alscher 1989).

Furthermore, a potentially decisive role for GSH in plant pathogen interactions could be envisaged since it has been demonstrated that GSH selectively elicits defense gene activation (Wingate et al. 1988). GSH-responsive elements on the promoters of genes involved in the synthesis of phytoalexins have been identified (Dron et al. 1988) and net increases in the level of GSH have been measured in cell suspensions of bean and alfalfa treated with fungal elicitors (Edwards et al. 1991) and in elicitor-treated liverwort cells (Nakagawara et al. 1993). More recently it has been shown that depletion of GSH through inhibition of its synthesis triggers phytoalexin synthesis and that addition of H<sub>2</sub>O<sub>2</sub> can mimic this response (Guo et al. 1993), suggesting that cellular redox status plays an important role in this process. Similar redox-potential mediated accumulation of phytoalexins has been reported in soybean and Ladino clover treated with sulfhydryl reagents, (Stossel 1984; Gustine 1987). Redox mechanisms regulate plant transcription factors (Babiychuk et al. 1994), RNA binding proteins (Zhang and Mehdy 1994), and gene expression (Herouart et al. 1993; Levine et al. 1994) during plant responses to biotic and abiotic stresses. It has been shown that genes involved in the metabolism of GSH, (GST and GPx) are induced in response to an elicitor-stimulated oxidative burst (Levine et al. 1994). A likely role of increased activity of specific isoforms of GST would be the detoxification of the products of lipid peroxidation generated as a result of AOS accumulation (Vera-Estrella et al. 1992). For such detoxification to be effective, an adequate supply of GSH is essential. Thus, alterations in the level of glutathione or a shift in the ratio of GSSG/GSH could be predicted to act at multiple levels and have a profound impact upon the outcome of plant-pathogen interactions.

There are a number of possible explanations why differences were not observed in the interactions tested. First, the GSH present in *cad2-1* or the measured increase in its steady state levels are sufficient to ensure the correct intracellular environment for effective resistance mechanisms to function and for the restriction of AOS arising during the HR. Second,

in the absence of adequate GSH, other antioxidant molecules or enzymes can compensate, and thus restrict oxidative damage. This is possible, since in animal systems depleted of GSH, ascorbate levels rise significantly and addition of ascorbate to GSH-depleted cells confers resistance to oxidative stress. Importantly, the ascorbate levels of *cad2-1* are significantly increased and it is not significantly more sensitive to abiotic oxidative stress than Col-0 (M. J. May, in preparation). Increases in the abundance of other molecules essential for the maintenance of cellular redox equilibrium such as thioredoxin may also compensate effectively. Interestingly, *Escherichia coli* mutants doubly deficient in thioredoxin and glutaredoxin are not more sensitive to DNA-damaging agents due to the combined activities of other compensatory GSH-dependent mechanisms (Miranda-Vizuete et al. 1994). Thus, it is emerging that complex mechanisms have evolved to ensure the maintenance of antioxidant defense such that when one component is limiting, other processes are upregulated to compensate.

Third, although we have clearly demonstrated that in the *cad2-1* mutant a drastic depletion of the level of GSH to 30% of that of the wild type does not alter the response of *Arabidopsis* to the virulent and avirulent pathogens used in this study, this may not be the case in interactions of *Arabidopsis* with other pathogens and it may not reflect the situation in other plant species. For example, while an enhanced cellular oxidation state has been correlated with phytoalexin accumulation in some species (Guo et al. 1993; Stossel 1984; Gustine 1987), the observation that GSH accumulates in elicitor-treated cells concurrently with phytoalexin formation in other species (Edwards et al. 1991; Nakagawara et al. 1993) suggests that in different species the relative contribution of redox factors to phytoalexin formation may differ. Thus, in *Arabidopsis*, in which phytoalexin accumulation is not a necessary component of the HR (Glazebrook and Ausubel 1994) depletion of GSH may not alter responses to pathogens. It will be of interest to determine whether GSH depletion in plant species in which phytoalexin accumulation is a necessary component of plant defense alters their response to pathogens.

It is also possible that pathogens that elicit a very strong oxidative burst or that can invade before cellular antioxidant defenses can be mobilized may more efficiently infect this mutant as a result of the host's reduced capacity for AOS scavenging. In this respect, high levels of glutathione are induced and maintained in the *cad2-1/WeLa* interaction while they return to control levels in the Col-0/*Wela1* interaction 3 days after inoculation (Table 3). This is perhaps very significant since it indicates that, in Col-0, glutathione levels are saturating for these responses, while in *cad2-1* the available glutathione is not sufficient to fully quench AOS synthesis, resulting in a continuous oxidative stimulation of glutathione cycling. Similarly, differences between the responses of Col-0 and *cad2-1* to *Noco2* after INA treatment add further support to this concept; increases in GSH in *cad2-1* are twofold higher than those in Col-0. Thus, while the mutation has no effect on the outcome of the interactions tested it sets a lower limit to the threshold of glutathione required, and highlights important aspects of glutathione metabolism during these interactions. In the absence of mutants allelic to *cad2-1* or GSH null-mutants it is difficult to establish unequivocally a clear redundancy for GSH in the interactions between *Arabidopsis* and its fungal

and bacterial pathogens. The availability of cDNAs encoding GSH biosynthetic enzymes (May and Leaver 1994; Rawlins et al., in press) will permit the construction of transgenic plants that have an increased or decreased capacity for GSH synthesis. Analysis of plants or mutants with altered antioxidant mechanisms will be a key method to address questions concerning the coordination of antioxidant defenses and their role in plant pathogen interactions. Furthermore, the results presented here indicate that such plants would provide insights to the way in which AOS are generated and controlled during plant-pathogen interactions.

## MATERIALS AND METHODS

### Maintenance of bacterial strains and fungal isolates.

*Pseudomonas syringae* pv. *tomato* strain DC3000 containing the *avrB* gene in the broad host range vector pVSP61, DC3000/pVB01 (Innes et al. 1993), and containing pVSP61 without an insert were kindly provided by Brian Staskawicz (University of California, Berkeley). These were cultured as described previously (Innes et al. 1993). *Peronospora parasitica* isolate *Wela1* was kindly provided by Alan Slusarenko (Plant Biology Institute, Zurich). *Wela1* and the isolate *Noco2* (Parker et al. 1993) were cultured on *Landsberg-erecta* (*La-er*) and Columbia (Col-0) seedlings, respectively, as described (Parker et al. 1993).

### Growth of plants and INA treatment.

For bacterial inoculations, *A. thaliana* Col-0 and *cad2-1* (Howden et al. 1995) plants were grown in Levington's compost in a growth chamber at 23°C with an 8-h light period and a light intensity of 250  $\mu\text{E s}^{-1} \text{m}^{-2}$ . Plants were inoculated when they were 5 to 6 weeks old. For inoculations of *A. thaliana* cotyledons with *P. parasitica*, seed were sown individually into Levington's compost. Ten-day-old seedlings were used for inoculations. For *P. parasitica* spray inoculations, seed were sown at high density and grown under the same conditions as above except with a 16-h light period. Seedlings were inoculated after 3 weeks.

In experiments in which INA was applied, this was added as a soil drench to a final concentration of 5 or 10  $\mu\text{M}$  active ingredient (25% wt/vol wettable powder formulation) (Metraux et al. 1991) 3 days before inoculation of plants with bacteria or fungus. Preliminary experiments established that young seedlings did not show adverse toxicity symptoms at these concentrations.

### Pathogen inoculations.

*P. syringae tomato* DC3000/pVSP61 and DC3000/pVB01 were harvested from overnight cultures (Innes et al. 1993) and diluted in 10 mM  $\text{MgCl}_2$  to  $2 \times 10^5$  CFU  $\text{ml}^{-1}$ . These were then inoculated onto the underside of *A. thaliana* leaves by pressure infiltration as previously described (Parker et al. 1993). Five to six leaves per plant were infiltrated. Plants were then incubated in a high humidity chamber at 24 to 25°C. Bacterial growth in the leaves was monitored essentially as described by Innes et al. (1993).

To measure fungal growth in *A. thaliana* seedlings, freshly harvested *P. parasitica* conidia were prepared and inoculated onto individual cotyledons at a concentration of  $4 \times 10^4$   $\text{ml}^{-1}$  (Parker et al. 1993). Seedlings were then stained with lacto-

phenol-trypan blue and viewed under a light microscope as described previously (Parker et al. 1993). In experiments requiring larger amounts of plant tissue, conidia were sprayed onto the more densely grown older seedlings at a concentration of  $4 \times 10^5$   $\text{ml}^{-1}$ . Inoculated seedlings were incubated as described previously (Parker et al. 1993) and monitored daily for asexual sporulation.

### RNA analysis.

Tissue samples for RNA isolation were collected at the described time-points, frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until extraction. RNA was purified from the frozen samples by a guanidinium hydrochloride extraction procedure (Gurr and McPherson 1992). Samples of RNA (10  $\mu\text{g}$ ) were separated by electrophoresis through a formaldehyde agarose gel and blotted to nitrocellulose (Schleicher and Schuell, Dassel, Germany) as described previously (May and Leaver 1994). Ethidium bromide was included in the sample loading buffer at 20  $\mu\text{g ml}^{-1}$ , which allowed photography under UV light after electrophoresis to confirm equal sample loading. *A. thaliana* PR1 cDNA (Uknes et al. 1992) was a kind gift from Eric Ward (Ciba Geigy, Research Triangle Park, NC). Gel purified cDNA fragments were  $^{32}\text{P}$ -labeled by random priming (Feinberg and Vogelstein 1983). Hybridization and washing were as described previously (May and Leaver 1994).

### GSH determination.

Duplicate samples of approximately 0.1 to 0.2 g were harvested at various times during the treatments, frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. The day-0 samples were collected from nontreated plants. GSH was extracted and determined as described previously (May and Leaver 1993). Recovery experiments were performed in which a known concentration of GSH or GSSG was added prior to grinding. Recovery was found to be 87 to 92%.

## ACKNOWLEDGMENTS

We thank those who supplied bacterial and fungal strains and gratefully acknowledge the help of Louise Frost with technical support. This work was supported in part by the Agricultural and Food Research Council, The Ministry of Agriculture Fisheries and Food, a Violet and Samuel Glasstone Fellowship, and the Australian Research Council. The Sainsbury Laboratory is supported by the Gatsby Charitable Foundation.

## LITERATURE CITED

- Alscher, R. G. 1989. Biosynthesis and antioxidant function of glutathione in plants. *Physiol. Plant.* 77:457-464.
- Apostol, I., Heinstein, P. F., and Low, P. S. 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol.* 90:109-116.
- Babiychuk, E., Kushnir, S., Van Montagu, M., and Inze, D. 1994. The *Arabidopsis thaliana* apurinic endonuclease Arp reduces human transcription factors Fos and Jun. *Proc. Natl. Acad. Sci. USA* 91:3299-3303.
- Bennetzen, J. L., and Jones, J. D. G. 1992. Approaches and progress in the molecular cloning of plant disease resistance genes. Pages 99-124 in: *Genetic Engineering, Principles and Methods*, vol. 14. J. K. Setlow, ed. Plenum Press, London.
- Bol, J. F., Linthorst, H. J. M., and Cornelissen, B. J. C. 1990. Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* 28:113-138.
- Bradley, D. J., Kjeilborn, P., and Lamb, C. J. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell-wall pro-

- tein: A novel, rapid defense response. *Cell* 70:21-30.
- Chen, Z., Silva, H., and Klessig, D. F. 1993. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262:1883-1886.
- Dangl, J. L. 1993. The emergence of *Arabidopsis thaliana* as a model for plant-pathogen interactions. *Adv. Plant Pathol.* 10:127-156.
- Dron, M., Clouse, S. D., Dixon, R. A., Lawton, M. A., and Lamb, C. J. 1988. Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. *Proc. Natl. Acad. Sci. USA* 85:6738-6742.
- Edwards, R., Blount, J. W., and Dixon, R. A. 1991. Glutathione and elicitation of the phytoalexin response in legume cell cultures. *Planta* 184:403-409.
- Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Glazebrook, J., and Ausubel, F. M. 1994. Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proc. Natl. Acad. Sci. USA* 91:8955-8959.
- Guo, Z.-J., Nakagawara, S., Sumitani, K., and Ohta, Y. 1993. Effect of intracellular glutathione level on the production of 6-methoxymellein in cultured carrot (*Daucus carota*) cells. *Plant Physiol.* 102:45-51.
- Gurr, S. J., and McPherson, M. J. 1992. Pages 109-121 in: *Molecular Plant Pathology: A Practical Approach*. S. J. Gurr, M. J. McPherson, and D. J. Bowles, eds. IRL Press, Oxford, UK.
- Gustine, D. L. 1987. Induction of medicarpin biosynthesis in landino clover by p-chloromercuribenzoic acid is reversed by dithiothreitol. *Plant Physiol.* 84:3-6.
- Herouart, D., Inze, D., and Van Montagu, M. 1993. Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in *Nicotiana*. *Proc. Natl. Acad. Sci. USA* 90:3108-3112.
- Holub, E. B., Beynon, J. L., and Crute, I. R. 1994. Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* 7:223-239.
- Howden, R., Anderson, C. R., Goldsborough, P. B., and Cobbett, C. S. 1995. Cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. *Plant Physiol.* 107:1067-1073.
- Innes, R. W., Bisgrove, S. R., Smith, N. M., Bent, A. F., Staskawicz, B. J., and Liu, Y.-C. 1993. Identification of a disease resistance locus in *Arabidopsis* that is functionally homologous to the *RPG1* locus of soybean. *Plant J.* 4:813-820.
- Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24:447-463.
- Kim, K. K., Fravel, D. R., and Papavizas, G. C. 1988. Identification of a metabolite produced by *Talaromyces flavus* as glucose oxidase and its role in the biocontrol of *Verticillium dahliae*. *Phytopathology* 78:488-492.
- Kuc, J. 1982. Induced immunity to plant disease. *BioScience* 32:854-860.
- Lamb, C. J. 1994. Plant disease resistance genes in signal perception and transduction. *Cell* 76:419-422.
- Legendre, L., Rueter, S., Heinstein, P. F., and Low, P. S. 1993. Characterisation of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. *Plant Physiol.* 102:233-240.
- Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H<sub>2</sub>O<sub>2</sub> from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79:1-20.
- Mauch, F., and Dudler, R. 1993. Differential induction of distinct glutathione S-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiol.* 102:1193-1201.
- May, M. J., and Leaver, C. J. 1993. Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiol.* 103:621-627.
- May, M. J., and Leaver, C. J. 1994. *Arabidopsis thaliana*  $\gamma$ -glutamyl-cysteine synthetase is structurally unrelated to mammalian, yeast and *E. coli* homologs. *Proc. Natl. Acad. Sci. USA* 91:10059-10063.
- Mehdy, M. C. 1994. Active oxygen species in plant defence against pathogens. *Plant Physiol.* 105:467-472.
- Metraux, J.-P., Ahl Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. 1991. Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. Pages 432-439 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*, vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Miranda-Vizuete, A., Martinez-Glistero, E., Aslund, F., Lopez-Barea, J., Pueyo, C., and Holmgren, A. 1994. Null thioredoxin and glutaredoxin *Escherichia coli* K-12 mutants have no enhanced sensitivity to mutagens due to a new GSH-dependent hydrogen donor and high increases in ribonucleotide reductase activity. *J. Biol. Chem.* 269:16631-16637.
- Nakagawara, S., Nakamura, N., Guo, Z.-J., Sumitani, K., Katoh, K., and Ohta, Y. 1993. Enhanced formation of a constitutive sesquiterpenoid in cultured cells of a liverwort, *Calypogeia granulata* Inoue during elicitation; effects of vanadate. *Plant Cell Physiol.* 34:421-429.
- Parker, J. E., Szabo, V., Staskawicz, B. J., Lister, C., Dean, C., Daniels, M. J., and Jones, J. D. G. 1993. Phenotypic characterization and molecular mapping of the *Arabidopsis thaliana* locus *RPP5*, determining disease resistance to *Peronospora parasitica*. *Plant J.* 4:821-831.
- Rawlins, M. R., Leaver, C. J., and May, M. J. 1995. Characterisation of a cDNA encoding *Arabidopsis* glutathione synthetase. *FEBS Lett.* 376:81-86.
- Stossel, P. 1984. Regulation by sulfhydryl groups of glyceollin accumulation in soybean hypocotyls. *Planta* 160:314-319.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired resistance in *Arabidopsis*. *Plant Cell* 4: 645-656.
- Vera-Estrella, R., Blumwald, E., and Higgins, V. J. 1992. Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. Evidence for the involvement of active oxygen species. *Plant Physiol.* 99:1208-1215.
- Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3:49-59.
- Wingate, V. P. M., Lawton, M. A., and Lamb, C. J. 1988. Glutathione causes a massive and selective induction of plant defense genes. *Plant Physiol.* 87:206-210.
- Zhang, S., and Mehdy, M. C. 1994. Binding of a 50-kD protein to a U-rich sequence in an mRNA encoding a proline-rich protein that is destabilized by fungal elicitor. *Plant Cell* 6:135-145.