

**Mechanisms and genes controlling the signalling network  
for biotic and abiotic stress defences  
in *Arabidopsis thaliana* (L.) Heyhn.**

**Functional cross-talk between photo-produced reactive oxygen species,  
photosynthesis and plant disease defence responses**



張寂禎

Christine Chi-Chen Chang  
Stockholm University, Department of Botany  
Doctoral thesis, 2005

To my father, Wen-Kui Chang 張文貴  
and my mother, Yen-Hua Chen 陳炎華

## PREFACE

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This thesis is based on the following published papers and manuscripts, which will be referred to in the text by their Roman numerals.

- I. **Chang, C.C.-C.**, Ball, L., Fryer, M.J., Baker, N.R., Karpinski, S., and Mullineaux, P.M. (2004). Induction of *ASCORBATE PEROXIDASE 2* expression in wounded *Arabidopsis* leaves does not involve known wound-signalling pathways but is associated with changes in photosynthesis. *Plant J.* 38, 499-511.
- II. Mateo, A., Muhlenbock, P., Rusterucci, C., **Chang, C.C.-C.**, Miszalski, Z., Karpinska, B., Parker, J.E., Mullineaux, P.M., and Karpinski, S. (2004). LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. *Plant Physiol.* 136, 2818-2830.
- III. **Chang, C.C.-C.**, Slesak, I., Sotnikow, A., Mullineaux, P. M., Karpinski, S., and Karpinska, B. (2005). Functional characterization of the chloroplastic glutathione peroxidases (cpGPXs) in *Arabidopsis thaliana*: its role in light acclimatory mechanisms. (manuscript).
- IV. **Chang, C.C.-C.**, Jorda, L., Slesak, I., Melzer, M., Moritz, T., Miszalski, Z., Mullineaux, P. M., Parker, J., Karpinska, B., Karpinski, S. (2005). Functional analysis of chloroplastic glutathione peroxidases (cpGPXs) in *Arabidopsis thaliana* provides a direct link between photo-oxidative stress and basal pathogen resistance mechanisms. (manuscript).

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My contributions to the papers were: (I) Planning, performing major part of the experiments and writing. (II) Analysis of salicylic acid effects. (III) Planning, performing major part of the experiments and writing. (IV) Planning, performing major part of the experiments and writing.

## ABSTRACT

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Excess excitation energy, mechanical injury and defence against pathogens, each trigger rapid production of reactive oxygen species (ROS) in *Arabidopsis thaliana* leaves. ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), are required for the induction of systemic acquired acclimation and may lead to redox changes in photosynthetic electron transport (PET). On one hand, enhanced ROS production during stress can destroy cells, and on the other, ROS can also act as signals for the activation of stress responsive and defensive pathways.

In this work, physiological and molecular analyses of *Arabidopsis* mutants and transgenic lines were applied to investigate the signalling network controlling biotic and abiotic stress responses. A key enzyme of the antioxidant network is encoded by *ASCORBATE PEROXIDASE 2* (*APX2*). Wounded leaves showed low induction of *APX2* expression and when exposed to excess light, *APX2* expression was increased synergistically. Signalling pathways dependent upon jasmonic acid, chitosan and abscisic acid were not involved in the wound-induced expression of *APX2*, but PET was required, and *APX2* induction was preceded by a depressed rate of CO<sub>2</sub> fixation.

Analysis of *lsd1* (*LESION SIMULATING DISEASE 1*) strongly suggests that light acclimatory processes and pathogen defences are genetically and functionally linked. It is important to know that *LSD1* type of mutants have mainly been studied with regard to pathogenesis. From this work, it reveals that association of *LSD1* with hypersensitive response may only be supplementary.

*GLUTATHIONE PEROXIDASES* (*GPXs*) are another major family of ROS scavenging enzymes. Analysis of the *Arabidopsis* genome database revealed a new open-reading frame, thus increasing the total number of *AtGPX* gene family to eight (*AtGPX1-AtGPX8*). *Arabidopsis thaliana* transgenic lines with reduced expression of both putative chloroplastic isoforms (*AtGPX1* and *AtGPX7*) and *AtGPX7* knock-out mutant (*ko-GPX7*) were more sensitive to photo-oxidative stress but had a reduced bacterial growth rate when inoculated with virulent strains *Pseudomonas syringae* pv. *tomato* DC3000 and *P.s.t. maculicola* strain ES4326, indicating increased resistance to pathogenesis. This, to our knowledge, is the first functional and genetic analysis of chloroplastic *GPXs* in plants, and confirms that light and chloroplastic ROS metabolism is important for basal resistance against virulent pathogens.

The above results confirm that light sensing, light acclimatory processes and photo-produced ROS also govern pathogen defence pathways. This has a great ecological relevance for Darwinian fitness of plants growing in the natural environment, where simultaneous pathogen attack and fluctuations in light, temperature and other environmental factors make rapid acclimation a constant necessity. Molecular, biochemical and physiological analysis of pathogen responses in mutants impaired in light sensing, EEE-dissipatory mechanisms, and similar analysis of light acclimatory processes in mutants impaired in pathogen defences may prove to be seminal.

Chang, C.C.-C. 2005. ISBN 91-7155-002-X, pp. 1-44.

Mechanisms and genes controlling the signalling network for biotic and abiotic stress defences in *Arabidopsis thaliana* (L.) Heyhn. : *Functional cross-talk between photo-produced reactive oxygen species, photosynthesis and plant disease defence responses*.

Department of Botany, Stockholm University, S-106 91 Stockholm, Sweden

[Christine.Chang@botan.su.se](mailto:Christine.Chang@botan.su.se)

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## ABBREVIATIONS

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ABA	Abscissic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	acyl coA oxidase gene
APX	Ascorbate peroxidase
Avr	pathogen avirulence proteins
BTH	benzothiadiazole
CAT	Catalase
<i>CHIB</i>	Basic chitinase
CRY	cryptochrome
DHAR	Dehydroascorbate reductase
DPI	Diphenylene iodonium
Eds	enhanced disease susceptibility
EEE	Excess excitation energy
EL	Excess light
ET	Ethylene
GPX	Glutathione peroxidase
GSH	Reduced glutathione
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HR	Hypersensitive response
INA	2,6-dichloroisonicotinic acid
ISR	induced systemic resistance
JA	Jasmonic acid
<i>Jr1</i>	jasmonate-responsive gene 1
<i>Jr2</i>	jasmonate-responsive gene 2
MDAR	Monodehydroascorbate reductase
MeJA	methyl jasmonate
NPQ	Non-photochemical quenching
OGA	Oligosaccharide
Pad	Phytoalexin deficient
PCD	programmed cell death
PET	Photosynthetic electron transport
PHOT	phototropin
PHY	phytochrome
PR1	Pathogenesis related protein 1
PSII	Photosystem II
qP	Photochemical quenching
R gene	resistance gene
rcd	Runaway cell death
ROS	Reactive oxygen species
SA	Salicylic acid
SAA	Systemic acquired acclimation
SAR	Systemic acquired resistance
SO* <sup>-</sup>	Superoxide
SOD	Superoxide dismutase
VSP	vegetative storage protein
WR	wound-responsive genes
<i>Wr3</i>	wound-responsive gene 3

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# 1. INTRODUCTION

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*“Leaves in the dark see the light”*

*(Foyer and Noctor, 1999)*

The sessile nature of plants implies that they must be able to adjust metabolic processes to constantly fluctuating light and other changing environmental factors. In full sunlight and optimal conditions, only a small fraction of absorbed light energy is used for CO<sub>2</sub> fixation (Foyer and Halliwell, 1976; Foyer et al., 1994; Asada, 1999). The amount of absorbed light energy in excess of that needed by plants for photosynthetic metabolism is termed as EEE or excess excitation energy (Karpinski et al., 1997; Karpinski et al., 1999; Niyogi, 2000; Fryer et al., 2003). During full sunlight, EEE is an ever-present problem for plants and light acclimation processes have several major compensatory functions: first, to optimise the energy status of the cell (NADPH/ATP ratio) for its metabolic needs; second, to optimise the use of absorbed light for photosynthesis and reactive oxygen species (ROS) formation; and third, to use changes in the light spectra and light energy as a source of information about diurnal or seasonal shifts (circadian or seasonal check points). All these objectives can be pivotal for the Darwinian fitness of plants and other photosynthetic organisms, which cannot escape from stressful conditions (Kulheim et al., 2002).

The above strategy of plants' acclimation processes necessitates accurate sensing and simultaneous processing of the incoming environmental signals such as changes in light intensity and quality, fluctuations in temperature and relative humidity, photoperiod length, water availability, nutritional status, wounding, pathogen attack and herbivores, etc. All these changes must be simultaneously conveyed to the plant cell in order to generate optimal acclimatory and defence responses, which will allow successful propagation.

The reaction of a plant cell to a stimulus is assumed to be composed of at least three elementary processes: signal perception, signal amplification and transduction, and primary and secondary responses. Signal perception includes physical and chemical interactions between the stimulus and a receptor. Subsequently, signal amplification and transduction of changes in the receptor generates specific effectors and downstream signalling cascades. The ultimate responses are specific changes in gene expression or enzyme activities and subsequent changes in the cellular metabolism. These interactions may be quite complex and are likely to involve both plant hormones such as jasmonic acid, ethylene, and abscisic acid (ABA), as well as other elicitors (Mullineaux and Karpinski, 2002).

## *1.1 Light perception*

The plant cell is equipped with sophisticated light-sensing mechanisms that are localized in the plasma-membrane, the cytosol, within chloroplasts and in the nucleus (Mullineaux et al., 2000). There are three families of photoreceptors that have been identified to date, phytochromes (PHY), cryptochromes (CRY), and phototropins (PHOT) (Clack et al., 1994; Briggs et al., 2001; Lin, 2002; Schäfer and Bowler, 2002). Only PHY are active in the long-wavelength region of the light spectrum: they absorb predominantly red-light and far-red-light, whereas CRY and PHOT monitor the blue-light spectra and the ultraviolet-A region

respectively (Ahmad et al., 1998). Presumably CRY functions by mediating a light-dependent redox reaction (Yang et al., 2001). PHY mediates the light control of all major phases of plant development from seed germination, through seedling establishment (de-etiolation, leaf expansion and chloroplast maturation) and determination of the architecture of the mature plant, to the induction of flowering and dormancy (Schäfer and Bowler, 2002). PHOT controls three distinct types of light-induced movement responses that are thought to optimize photosynthesis: phototropism, chloroplast relocation and stomatal opening. In *Arabidopsis thaliana*, there are two CRY genes (Lin, 2002), two PHOT genes (Lin, 2002) and five PHY photoreceptor genes (Schäfer and Bowler, 2002).

### *1.1.1 Photo-oxidative stress and excess excitation energy*

Diurnal and seasonal fluctuations in light intensity, as well as environmental stresses such as cold, drought, salinity, and nutrient deficiency limit CO<sub>2</sub> fixation and can result in the absorption of more light energy than can be utilized productively for photosynthetic metabolism (Karpinski et al., 1999; Mullineaux and Karpinski, 2002). Since acclimation responses that require alterations in gene expression are often too slow, plants utilize several “safety valves” to ensure that the harvested light energy does not inadvertently lead to ROS formation and cellular damage (Niyogi, 2000). Failure to dissipate EEE results in over-reduction of photosynthetic electron transport components and increased production of ROS in the chloroplast and peroxisomes. ROS include singlet oxygen(<sup>1</sup>O<sub>2</sub>), superoxide anion(O<sub>2</sub><sup>-</sup>), hydrogen peroxide(H<sub>2</sub>O<sub>2</sub>), and hydroxyl radical(OH<sup>•</sup>). Hyper-accumulation of ROS leads to photo-inhibition, photo-oxidative damage and eventually death of the cell, manifested as bleaching or chlorosis of the photosynthetic tissues (Karpinski et al., 1999; Kasahara et al., 2002).

Dissipation of EEE in plants is achieved by a combination of non-photochemical and photochemical quenching processes. Non-photochemical quenching processes include the transfer of excitation energy from antenna chlorophyll to zeaxanthin instead of the reaction centres. The extended NPQ is regulated by conformational changes in the PSII antenna and the reversible conversion of violaxanthin to zeaxanthin in the xanthophylls cycle. Both processes are dependent on the pH gradient across the thylakoid membrane. Additionally, carotenoids can scavenge <sup>1</sup>O<sub>2</sub> that emerges when excited chlorophyll converts to the triplet state and then induces a spin conversion in triplet O<sub>2</sub> (Havaux and Niyogi, 1999; Niyogi, 1999; Niyogi, 2000; Muller et al., 2001).

Photochemical quenching of EEE is the collective term for processes that increase the consumption of photosynthetic electrons by the deployment of additional metabolic sinks. These include the reduction of O<sub>2</sub> at PSII or PSI with subsequent return of the electrons to water by antioxidant enzymes (water-water cycle) and an increased photosynthetic and photo-respiratory metabolism. Photorespiration starts from the oxygenase reaction of Rubisco and results in the energy consuming release of CO<sub>2</sub> from fixed carbon (Kozaki and Takeba, 1996; Willekens et al., 1997; Wingler et al., 2000). Both the water-water cycle and photorespiration have a duplicated dissipation value, because they dissipate excess electrons and at the same time provide NADP<sup>+</sup> as electron acceptor.



### *1.1.2 Light acclimation responses*

Light acclimation processes are aimed to optimize light harvesting and CO<sub>2</sub> fixation. Plants will dissipate EEE or minimize its formation by diminishing the capacity of the leaf to capture light energy (Walters and Horton, 1994; Niyogi, 2000; Bailey et al., 2001).

Among the acclimatory pathways, a short-term chromatic adaptation known as state-1/ state-2 transition is required when the flux of electron excitation of PSII and PSI is unequal (Allen, 1995; Haldrup et al., 2001; Wollman, 2001; Allen, 2003). In oxygenic photosynthesis, two separate reactions centres named PSI and PSII, which have distinct light absorption properties, are coupled in sequence by a chain of electron carriers. PSI is enriched in chlorophyll *a*, absorbing in the far-red region (absorption maximum  $\lambda \approx 700$  nm) whereas PSII is enriched in chlorophyll *b*, with absorption maximum at shorter wavelengths in the orange/red region ( $\lambda \approx 650 - 680$  nm) (Wollman, 2001). Thus, spectral imbalances of the exciting light may result in an uneven excitation of the two photosystems leading to lower or higher ROS production (Karpinski et al., 1999; Karpinska et al., 2000; Wollman, 2001; Mullineaux and Karpinski, 2002; Fryer et al., 2003). During state transitions, a part of the PSII antenna is moved towards PS I or back to PS II to level out imbalanced excitation of the two photosystems.

Short-term processes are subsequently complemented by long-term light acclimation processes if unbalanced excitation pressure persists (Karpinski et al., 2003). Common long term acclimation responses include increasing the capacity for photosynthetic electron transport and CO<sub>2</sub> fixation, as well as decreasing the size of the light-harvesting antennae that are associated with photosystems (Noctor and Foyer, 1998; Pfannschmidt et al., 1999). This acclimation responses involve the regulation of gene expression (nuclear and chloroplastic) by redox potential, and/or proteolytic degradation of existing light harvesting complexes and other proteins (Allen, 1993; Durnford and Falkowski, 1997; Bailey et al., 2001; Pfannschmidt et al., 2001a; Pfannschmidt et al., 2001b; Pfannschmidt et al., 2003).

Although potentially damaging, ROS are also needed to trigger other protective responses, such as the down-regulation of PSII activity, as well as to induce cellular and systemic signals that can promote redox changes in the proximity of PSII in unstressed chloroplasts, thus inducing protective mechanisms in remote chloroplasts (Karpinski et al., 1999; Mullineaux et al., 2000; Kovalchuk et al., 2003).

### *1.1.3 ROS protection responses*

In addition to light-dependent regulatory systems used to avoid the formation of ROS, plants are equipped with enzymatic (mainly water soluble) and non-enzymatic (water and lipid soluble) ROS scavenging systems (Foyer and Halliwell, 1976; Foyer et al., 1994; Jimenez et al., 1997; Mittler, 2002; Mittler et al., 2004). Enzymatic antioxidant systems consist of superoxide dismutase (SOD, EC : 1.15.1.1) ascorbate peroxidase (APX, EC : 1.11.1.11), catalase (CAT, EC : 1.11.1.6), monodehydro- and dehydro-ascorbate reductase (MDAR, EC : 1.6.5.4 and DHAR, EC : 1.8.5.1) glutathione reductase (GR, EC : 1.6.4.2) and glutathione

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peroxidase (GPX, EC : 1.11.1.9). Detailed information on the isoforms encoded in the *Arabidopsis* genome is listed in Table 1.

**Table 1.** Summary of ROS scavenging enzymes and corresponding gene family in the *Arabidopsis* genome

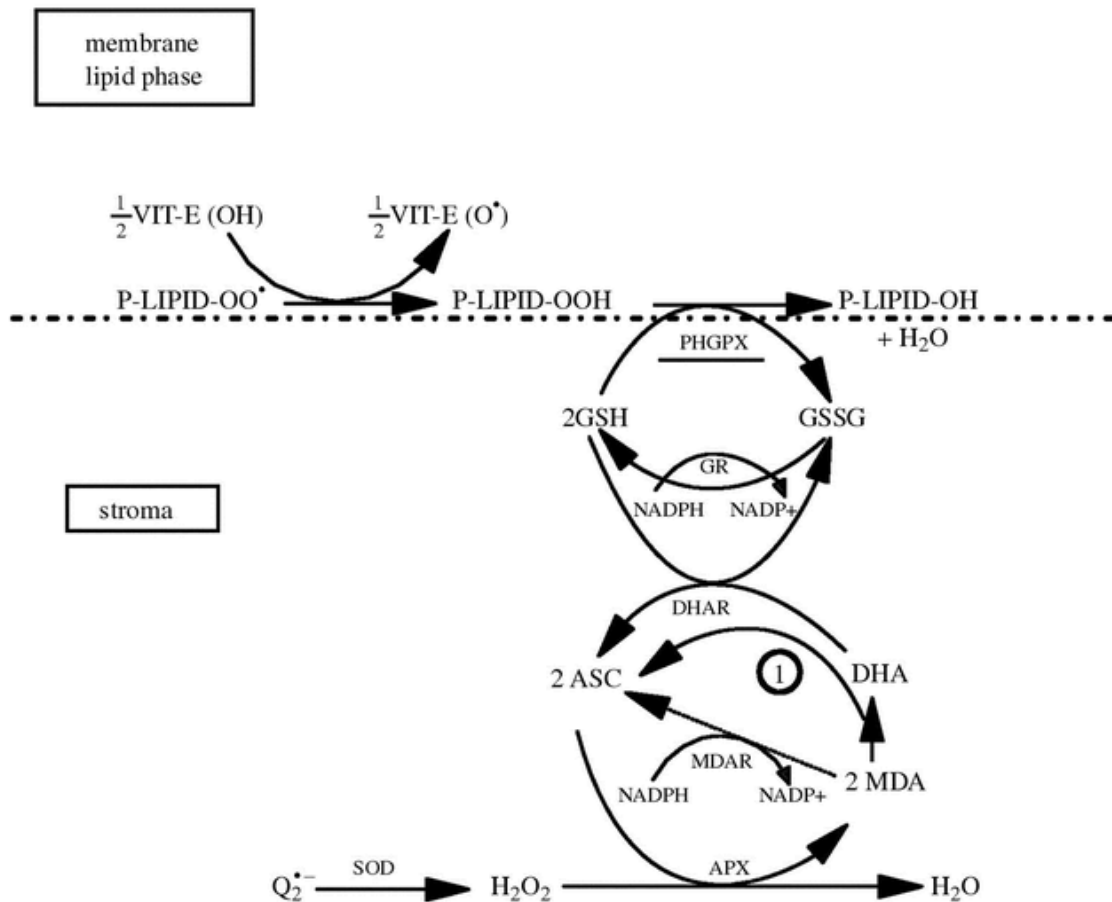
Name	Reaction	Gene family in Arabidopsis	Accession number	Localization	References
SOD	$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	FSD1	At4g25100	<b>chl</b>	(Camp et al., 1990)
		FSD2	At5g51100	<b>chl</b>	(Kliebenstein et al., 1998)
		FSD3	At5g23310	<b>chl</b>	(Kliebenstein et al., 1998)
		CSD1	At1g08830	<b>cyt</b>	(Hindges and Slusarenko, 1992)
		CSD2	At2g28190	<b>chl</b>	(Kliebenstein et al., 1998)
		CSD3	At5g18100	<b>per</b>	(Kliebenstein et al., 1998)
		MSD1	At3g10920	<b>mit</b>	(Kliebenstein et al., 1998)
		MSD-like	At3g56350	-	-
APX	$H_2O_2 + 2Asc \rightarrow 2H_2O + 2MDA$	APX1	At1g07890	<b>cyt</b>	(Kubo et al., 1993a)
		APX2	At3g09640	<b>cyt</b>	(Kubo et al., 1992)
		APX3	At4g35000	<b>per</b>	(Zhang et al., 1997)
		APX4	At4g09010	<b>chl</b>	(Schubert et al., 2002)
		APX5	At4g35970	<b>cyt</b>	-
		APX6	At4g32320	<b>chl</b>	-
		thylakoidAPX	At1g77490	<b>chl</b>	(Shigeoka et al., 2002)
		stromal APX	At4g08390	<b>chl</b>	(Shigeoka et al., 2002)
MDAR	$NADH + H^+ + 2 MDA \rightarrow NAD^+ + 2Asc$	MDAR	At1g63940	<b>chl/cyt</b>	-
		MDAR	At3g09940	<b>cyt</b>	-
		MDAR	At3g27820	<b>cyt/mit</b>	-
		MDAR	At3g52880	<b>cyt</b>	-
		MDAR	At5g03630	<b>cyt</b>	-
DHAR	$DHA + 2GSH \rightarrow Asc + GSSG$	DHAR	At5g16710	<b>chl/cyt</b>	-
		DHAR	At5g36270	<b>cyt</b>	-
		DHAR	At1g75270	<b>cyt</b>	-
		DHAR	At1g19550	<b>cyt</b>	-
		DHAR	At1g19570	<b>cyt</b>	-
GR	$2GSH + NADP^+ \rightarrow$ glutathione disulfide $+ NADPH + H^+$	GR	At3g24170	<b>cyt</b>	-
		GR	At3g54660	<b>chl</b>	(Kubo et al., 1993b)
CAT	$2H_2O_2 \rightarrow 2H_2O + O_2$	CAT 1	At1g20630	<b>per</b>	(Frugoli et al., 1997)
		CAT 2	At4g35090	<b>per</b>	(Chevalier et al., 1992)
		CAT 3	At1g20620	<b>per</b>	(Frugoli et al., 1997)

Localization is based on sequence analysis and computational predictions unless stated elsewhere in the cited reference. “-“ means there is no specific citation at the moment, however one can find the locus information in *Arabidopsis* complete genome sequence analysis from “The *Arabidopsis* Information Resource (TAIR)”, [www.arabidopsis.org/aboutarabidopsis.html](http://www.arabidopsis.org/aboutarabidopsis.html), on [www.arabidopsis.org](http://www.arabidopsis.org), November 23, 2004 (Rhee et al., 2003). SOD-superoxide dismutase, FSD-Fe SOD, CSD-Cu/Zn SOD, MSD-Mn-SOD, APX-ascorbate peroxidase, MDAR, monodehydroascorbate reductase, DHAR, dehydroascorbate reductase, GR, glutathione reductase, CAT-catalase, chl-chloroplast, cyt-cytosol, per-peroxisome, mit-mitochondria.

## Introduction

Non-enzymatic antioxidants, such as ascorbate (vitamin C), glutathione,  $\alpha$ -tocopherol (vitamin E), pigments and phenolic components, prevent or interrupt cascades of uncontrolled oxidation of cellular compounds (Asada, 1994; Noctor and Foyer, 1998; Bailly, 2004).

GPXs catalyse the reduction of  $H_2O_2$  or different kinds of lipid peroxides by using reduced glutathione (GSH) as an electron donor (Ursini et al., 1995; Eshdat et al., 1997). Similar to APXs, GPXs comprise of eight isozymes (seven were previously found and one was newly identified by our present analysis) that protect cells against oxidative damage



**Figure 1.** The scavenging of active oxygen species in the chloroplast in both the lipid membrane phase and the stroma, linked to redox cycles for ascorbate and glutathione and the oxidation of  $\alpha$ -tocopherol (Vitamin E). Abbreviations are as follows: P-LIPID -OO, phospholipid peroxy radical; P-LIPID-OOH, phospholipid peroxide; P-LIPID-OH, phospholipid alcohol; VIT-E (OH),  $\alpha$ -tocopherol (vitamin E); VIT-E ( $O^*$ ),  $\alpha$ -chromanoxyl radical; PHGPX, phospholipid hydroperoxide-dependent glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; DHAR, dehydroascorbate reductase; ASC, ascorbic acid; DHA, dehydroascorbate; MDA, monodehydroascorbate free radical; MDAR, monodehydroascorbate free radical reductase; APX, ascorbate reductase; SOD, superoxide dismutase;  $O_2^{\bullet-}$ , superoxide ion. Reaction 1 is the non-enzyme-catalyzed spontaneous dismutation of two MDA molecules to one ASC and one DHA, respectively. From (Mullineaux et al., 2000).

generated by ROS. Several groups have reported the presence of GPXs in plants, but the specific role of each GPX isoform in plants has not been well documented. The putative subcellular localizations of the encoded GPX proteins are the cytosol, chloroplast,

mitochondria, and endoplasmic reticulum (Milla et al., 2003). There are a plethora of antioxidants, all of which are worthy of individual study. However, it is likely that it is the network of reactions that is important *in vivo* and which provides comprehensive control of ROS in cells, rather than the action of any single reaction. The first antioxidant network to be proposed for plants was the ascorbate-glutathione cycle of the chloroplast stroma (Figure 1) (Foyer and Halliwell, 1976; Foyer and Mullineaux, 1994; Asada, 1999). While the individual enzymes described in the ascorbate-glutathione cycle still receive a considerable amount of attention (eg. SOD, APX), it is now apparent that many more complex schemes can be created that involve a wider range of enzymes and molecules. These networks now include the movement of antioxidant compounds by specific transporters between and within cells, as well as the use of membrane-spanning redox couples (Jimenez et al., 1997; Polle, 1997; Horemans et al., 2000; Foyer and Noctor, 2003). Such networks have been described for a range of tissues and organs, including photosynthetic tissues of the leaf, the developing embryo, roots, ripening fruit and the germinating seed (Jimenez et al., 2002; Mittler et al., 2004). In addition, beyond the ascorbate glutathione cycle, further compounds, proteins and enzymes have since been demonstrated to be important contributors to antioxidant defences. These include potent antioxidant phenolic compounds (Gray et al., 1997; Grace and Logan, 2000; Horemans et al., 2000), small protein molecules such as (2-cys) peroxiredoxins, glutaredoxins and thioredoxins (Meyer et al., 1999; Horling et al., 2003), enzymes which turn out to have potent ROS scavenging potential such as glutathione-S-transferases/peroxidases (Marrs, 1996; Cummins et al., 1999), and the repair enzymes of oxidatively damaged proteins such as peptide methionine sulfoxide reductases (Bechtold et al., 2004).

### *1.2 Signalling network and cross-talk*

Due to the complexity of the antioxidant network, it is easily concluded that the network coordinating the antioxidant defence with the basic metabolism and other defence responses has to be even more intricate. Mutant analysis has contributed greatly to our understanding of the signalling transduction network in plants. Table 2 summarizes the mutants discussed in this thesis, which are valuable for studies of the signals involved in cross-talk between biotic and abiotic stresses. Widespread analysis of different biotic and abiotic stress responses has led to the conclusion that considerable "horizontal" interactions exist between many components of different signalling pathways (Gomez et al., 2004).

So-called "cross-talk" is now a subject of intense scrutiny, especially in laboratories using *Arabidopsis* as their experimental species (Rojo et al., 1999; Schenk et al., 2000; Swidzinski et al., 2002; Mahalingam et al., 2003; De Paepe et al., 2004). The application of post-genomic techniques and the analysis of considerable amounts of microarray data and mutants give an impression of a complex network of responses from the sub-cellular to whole plant level. Such integrated "systems biology" is seen by many as the way forward in studying plant-environment interactions (Raikhel and Coruzzi, 2003). Current research has barely begun to scratch the surface of how a response to multiple stresses is organised and why such signalling networks have evolved. Thus the reality of how signalling pathways integrate their responses to environmental stimuli is akin to the complex behaviour of neural networks and attempts to describe how such networks might operate have just begun (Genoud and Métraux, 1999; Pastori and Foyer, 2002).

**Table 2.** Signalling and cell death mutants discussed in this work

Mutant	Accession number	Reference
<i>jar 1-1</i> ( <i>jasmonate resistant 1-1</i> )	At2g46370	(Staswick et al., 1992)
<i>ein 2</i> ( <i>ethylene insensitive 2</i> )	At5g03280	(Guzman and Ecker, 1990)
<i>lsd 1</i> ( <i>lesion simulating disease-resistance 1</i> )	At4g20380	(Jabs et al., 1996)
<i>sid 2</i> ( <i>salicylic acid induction deficient 2</i> )	At1g74710	(Wildermuth et al., 2001)
<i>coi 1</i> ( <i>coronotone insensitive 1</i> )	At2g39940	(Feys et al., 1994)
<i>eds 1</i> ( <i>enhanced disease susceptibility 1</i> )	At3g48090	(Parker et al., 1996)
<i>pad 4</i> ( <i>phytoalexin deficient 4</i> )	At3g52430	(Glazebrook et al., 1996)
<i>cao</i> ( <i>chlorophyll a/b binding protein organelle specific</i> )	At2g47450	(Amin et al., 1999)
<i>abi1-1</i> ( <i>abscisic acid insensitive 1-1</i> )	At4g26080	(Gilmour and Thomashow, 1991)
<i>abi2-1</i> ( <i>abscisic acid insensitive 1-2</i> )	At5g57050	(Gilmour and Thomashow, 1991)

Accession number and references can be found in *Arabidopsis* complete genome sequence analysis from “The *Arabidopsis* Information Resource (TAIR)”, [www.arabidopsis.org/aboutarabidopsis.html](http://www.arabidopsis.org/aboutarabidopsis.html), on [www.arabidopsis.org](http://www.arabidopsis.org), November 23, 2004 (Rhee et al., 2003).

### 1.2.1 Wounding responses

Besides abiotic stress factors, plants also encounter various kinds of biotic attacks. Plants try to limit damage from injuries caused by chewing-insects or larger herbivores with pre-existing physical barriers. Moreover, ubiquitous mechanical stimuli such as rain or wind are known to result in morphological changes in plants. A localized injury activates defence mechanisms throughout the plant both in the directly damaged tissues (local response) and in the non-wounded areas (systemic response).

In reaction to mechanical injury, the activation of a series of wounding-responsive (WR) genes, several components like oligosaccharides, peptide systemin, jasmonic acid (JA), abscisic acid, ethylene and electrical signals, have been shown to support a complex signalling network (Guan and Scandalios, 2000; Pei et al., 2000). WR genes can be separated into three major groups according to their expression patterns: the JA-dependent group of genes, such as *VSP* (vegetative storage protein), JA-independent group of genes, including *CHIB* (basic chitinase) and the last category which involve both JA-dependent and independent signalling, such as *ACO* (acyl coA oxidase). Although the involved wounding signals are conserved in higher plants, there are several discrepancies between *Arabidopsis* and tomato in the regulatory system for wound responses (Leon et al., 2001). All these data strongly support the idea that JA may not be the only general primary signal transducer of wound responses for all plant species (Nishiuchi et al., 1997; Titarenko et al., 1997; Leon et al., 1998; Ellis and Turner, 2002).

### 1.2.2 Mechanisms of the plant ‘immune’ response

Multiple signalling pathways were also found to be involved in the regulation of defence responses against microbial attacks. Disease occurs only when a pathogen is able to avoid early detection by the plant. The innate immune system is the first line of inducible defence

against disease. A key function of innate immunity is the detection of pathogen-associated molecular patterns produced by infectious agents but not by host cells (Asai et al., 2002). Strong disease resistance of plants often is conditioned by gene-for-gene interactions: when a pathogen has an avirulence (*avr*) gene and a plant has a corresponding resistance (*R*) gene, the plant can rapidly recognize the pathogen and effectively deploy defence responses (Dangl and Jones, 2001). According to this model, the specific recognition of an *avr* gene product by a plant resistance gene product leads to a defence response (so called hypersensitive response or HR) that is characterized by localized cell lesion of the infected tissue and induction of certain plant defence-related genes (Lamb et al., 1989). Recognition of an avirulent pathogen triggers the rapid production of ROS, and nitric oxide, which also participates as a signal in the immune and vascular systems in plant disease resistance (Delledonne et al., 1998).

When a plant survives the infection of a pathogen at one site, it often develops increased resistance to subsequent attack at sites throughout the plant and enjoys protection against a wide range of pathogen species. This phenomenon, called systemic acquired resistance (SAR), develops over a period of several days following initial infection (Chen et al., 1993; Dietrich et al., 1994). *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance and thus potentially enhances protection against diverse pathogen types (Petersen et al., 2000).

### 1.2.3 EEE and defence against pathogens

Several common features characterize the response of plants to both EEE and infection by incompatible pathogens. They are characterized by a rapid increase in the foliar concentrations of ROS, the depletion of antioxidant pools, the chlorosis and necrosis of leaves, local and systemic defence responses (systemic acquired acclimation and systemic acquired resistance) and induction of defence gene expression.

However, in cells infected by an avirulent pathogen, the sub-cellular origin of the ROS burst is thought to be NADPH oxidases at the plasma membrane, which is different from EEE that stimulates ROS production mainly in chloroplasts and peroxisomes. It has been proposed that ROS produced during the oxidative burst act as the central regulator of the HR (Levine et al., 1994). The manipulation of endogenous ROS alters the outcome of cell death signals and the induction of cell death requires the controlled attenuation of ROS detoxification (Lamb and Dixon, 1997). In situations where ROS are actively involved in the induction of cell death, there are constitutive and induced ROS detoxification systems that could inhibit cell death. Insight into the ROS dependent regulation of cell death came from analysis of the *lesion simulating disease 1* (*lsd1*) mutant, which was initially characterized for its O<sub>2</sub><sup>-</sup>-dependent spreading of chlorotic/necrotic lesions (a phenotype termed runaway cell death, *rcd*) that develop under long or continuous photoperiods or after infection with an avirulent pathogen (Dietrich et al., 1994; Jabs et al., 1996). LSD1 has been suggested to be a negative regulator of cell death by acting as a ROS rheostat. Short day photoperiods and low photosynthetically active photo flux density are permissive conditions for the growth of *lsd1*. These observations strongly imply the relationship between prevailing light conditions and the initiation of the runaway cell death in *lsd1*. Therefore, the *lsd1* mutant is a good candidate

## Aim of the present studies

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to study the role of photo-produced ROS in the regulation of cell death and the role of LSD1 in light acclimation processes in *Arabidopsis*.

## **2. AIM OF THE PRESENT STUDIES**

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The aim of the work presented in this thesis is to test the hypothesis that light acclimatory processes and resistance mechanisms of plants against pathogen are functionally or/and genetically linked.

In particular, the following important questions are addressed:

- What is the role of chloroplast-derived redox signalling and ROS in regulation of biotic stress responses?
- What is the role of light acclimatory processes in plant defence responses?
- What is the function of genes regulating plant defence responses in regulation of light acclimatory processes?
- What is the function of genes controlling excess light acclimatory processes in regulation of plant defence responses?

### 3. RESULTS AND DISCUSSION

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#### *3.1 The role of chloroplast-derived redox signalling and ROS in regulation of wounding responses*

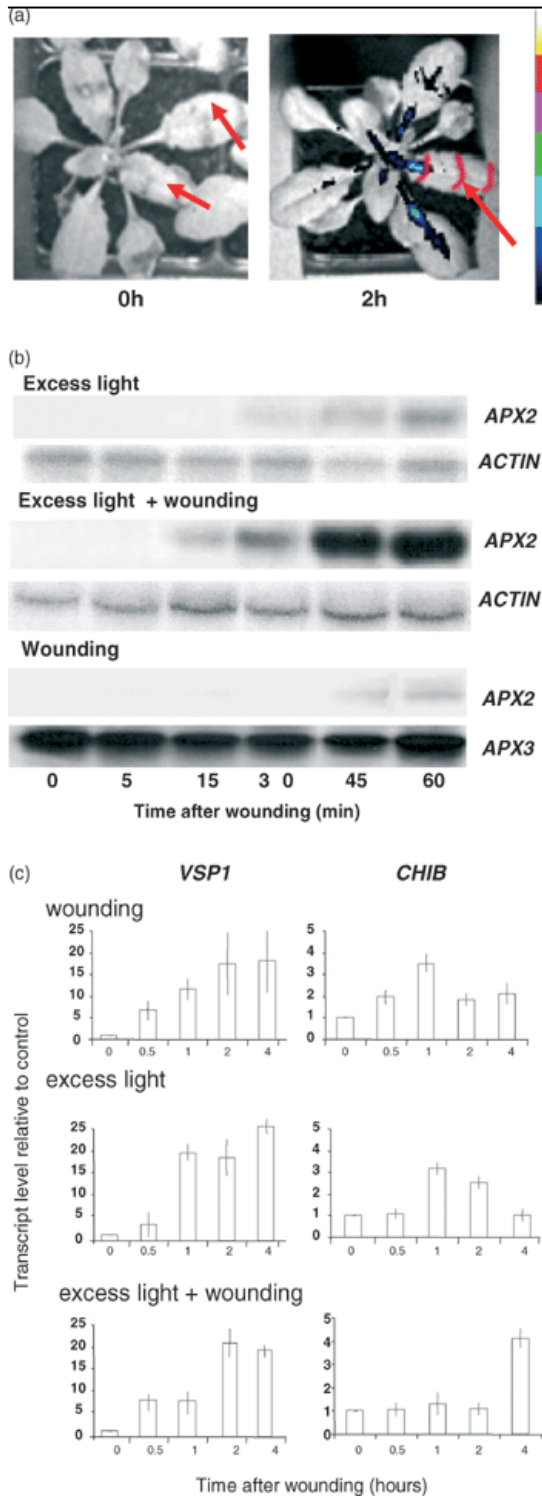
Previously published observations showed that the expression of the firefly luciferase gene (*APX2LUC*) fused to the *APX2* promoter in transgenic *Arabidopsis* (Karpinski et al., 1999) was enhanced in the veins closest to the wounded tissue of detached leaves partially exposed to excess light (Mullineaux et al., 2000). This raised the possibility that the signalling pathway controlling the expression of *APX2* could be integrated into one or more wound-signalling pathways or could share common components, or is responsive to the same effectors molecules. However, further investigations reported here led to a different and unexpected conclusion. Presented results suggest that in wounded leaves exposed to light, there is an increased diversion of photosynthetic electron flux to  $O_2$  in the vascular regions leading to redox changes in photosynthetic electron transport and an increased production of  $H_2O_2$  that, in turn, triggers induction of *APX2* expression. This has important implications for how “wound-specific” gene expression is viewed.

##### *3.1.1 Synergistic effect of wounding and excess light on APX2 expression*

The induction of *APX2* expression in wounded leaves could be detected by induction of expression of *APX2LUC* reporter system in transgenic *Arabidopsis* lines (Karpinski et al., 1999). Luciferase activity could be imaged in the central vein and apical region of both the wounded leaf and adjacent unwounded leaves (Figure 2a). *APX2* transcript could be detected as little as 45 minutes after wounding (Figure 2b). Under these conditions, the wounded plants expressed the JA-dependent wound-inducible *VEGETATIVE STORAGE PROTEIN 1* (*VSP1*) and JA-independent wound-inducible *BASIC CHITINASE* (*CHIB*) genes (Rojo et al., 1999; Ellis and Turner, 2001). *APX2* expression has been shown to be induced in leaves subjected to excess light treatments at times as little as 7 minutes after the onset of the challenge (Karpinski et al., 1997). To determine whether the regulation of *APX2* expression shared any similarities in wounded leaves compared with those subjected to excess light stress, the effect on expression of these combined stresses was compared with undamaged leaves subjected to a 5-fold excess light stress. Using RNA gel blots, *APX2* transcripts was detected 15 minutes earlier and they accumulated to a >10-fold higher level in wounded, excess light-stressed leaves than in their undamaged counterparts (Figure 2b). It should be noted that in wounded leaves, induction of *APX2* transcript could only be detected using a RT-PCR and was undetectable on RNA gel blots (data not shown and Figure 2b). Therefore, it was concluded that the combined effect of wounding and excess light had a synergistic effect on the level of induced *APX2* transcript, compared with either excess light or wounding stress alone. Both *VSP1* and *CHIB* expression were induced further by excess light treatment, but no synergistic effect could be discerned of the combined stresses on their transcript levels (Figure 2c).



## Results and Discussion



**Figure 2.** Induction of *APX2* expression in wounded leaves.

(a) Imaging of luciferase activity from an *APX2LUC* transgenic *Arabidopsis* line (Karpinski et al., 1999) 2 h after wounding (right panel). The wounding was carried out by crimping with forceps at the points shown by red bands in the right panel. Wounded tissue can also be seen in the left panel at the start of the experiment as slightly darkened bands (arrowed).

(b) Effect of combined excess light and wounding on levels of *APX2* transcript. Individual plants, undamaged or wounded as in (a), were subjected to a fivefold excess light treatment for one of the time points indicated, and their RNAs were extracted and analysed by blotting of RNA gels and probing with an *APX2*-specific cDNA. The *ACTIN* probe was used as a loading control. For early detection of *APX2* transcript after wounding, 3' RACE PCR was used to amplify cDNA followed by Southern blotting, gene-specific probes and detection using a phosphorimager. Here cDNA derived from *APX3* transcript is used as a loading control and to check for presence of cDNA in early time points.

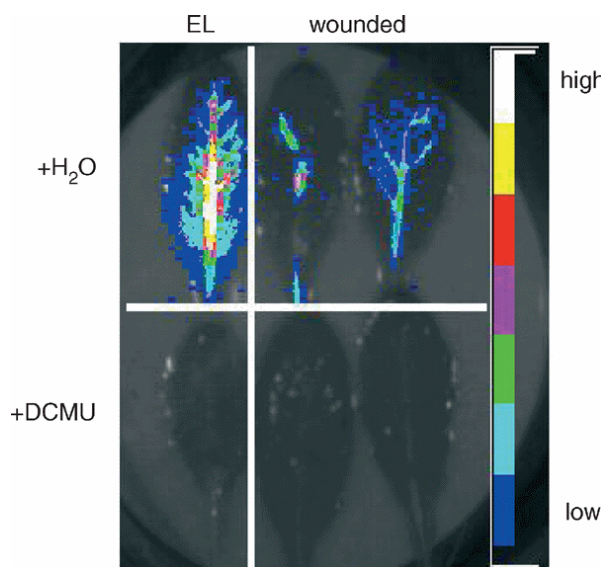
(c) Levels of *VSP1* and *CHIB* transcripts in wounded, excess light treated, and combined treatments. The levels of transcripts were assayed using a quantitative RT-PCR procedure, normalized for loading using 18S RNA standard and expressed relative to untreated controls.

The observation that wounded leaf tissue showed enhanced expression of *APX2* under ambient and excess light conditions (Figure 2) raised the possibility that wound signalling pathways in *Arabidopsis* might share common components. This data presented here indicates that neither the JA-dependent nor JA-independent wound signalling pathways known in *Arabidopsis* (McConn et al., 1997; Nishiuchi et al., 1997; Titarenko et al., 1997; Rojo et al., 1998; Rojo et al., 1999; Leon et al., 2001) are implicated in *APX2* expression in wounded leaves (paper I), despite both pathways being operative in our experimental conditions as evidenced by the induction of *VSP1* and *CHIB* expression, respectively (Figure

2b). While not examined in detail, it was interesting to note that high light – mediated induction of *VSP1* expression was not JA-dependent (Figure 2b), adding further weight to the argument that the JA signalling pathway plays no direct role in governing responses of gene expression to high light stress.

### 3.1.2. Photosynthetic electron transport is required for wound-induced *APX2* expression

The highest levels of *APX2* expression were always observed in leaves subjected to both wounding and excess light (Figure 2c). Wounded leaves treated with the photosynthetic inhibitor DCMU did not show any induction of *APX2* expression (Figure 3). This led us to hypothesize that the mechanism of *APX2* induction in wounded *Arabidopsis* leaves was qualitatively the same as that described previously for excess light-stressed leaves, depending on redox changes in photosynthetic electron transport that generate  $H_2O_2$  and augmented by



**Figure 3.** The inhibition of wound-induced *APX2LUC* expression by the photosynthesis inhibitor DCMU.

Leaves were vacuum infiltrated for 3 min with 10  $\mu$ M DCMU and then incubated in the same solution for 3 h. At the end of this period, the leaves were wounded, incubated on wetted blotting paper at growth PFD for 2 h and sprayed with luciferin, and the luciferase activity was imaged. The color bar code gives the range of luciferase activity from background to 1000 RLU (blue) to 1800 RLU (yellow).

ABA (Karpinski et al., 1999; Fryer et al., 2003). This hypothesis would mean that induction of *APX2* expression would differ between wounded and excess light-stressed leaves only in the degree of response. The synergistic effect of excess light and wounding on *APX2* expression and its inhibition by DCMU were consistent with this hypothesis.

### 3.1.3 Wound and excess light-induced *APX2* expression is not controlled by known wound signalling pathways in *Arabidopsis*

*Arabidopsis* has at least two wound signalling pathways, JA-dependent and JA-independent (Titarenko et al., 1997; Rojo et al., 1999; Ellis and Turner, 2002). Communication between these pathways is mediated by ethylene generated in damaged tissues (Rojo et al., 1999). The mutant *coronatine-insensitive 1-1 (coi 1-1)* (Feys et al., 1994) is blocked in the perception of JA and *COI 1-1* may play a role in cross-talk between the JA pathway and those directed by ethylene and ABA (Feys et al., 1994; Ellis and Turner, 2002). The *coi 1-1* mutant showed no difference from wild-type plants in the abundance of *APX2* transcript when wounded or subjected to excess light (paper I). *VSP1* transcript induction was blocked in wounded, but not in high light-stressed *coi 1-1* plants (paper I). Similarly, wounded or high light treated

mutants impaired in their perception of ethylene, *ethylene insensitive 2-1* (Guzman and Ecker, 1990), and *ethylene resistant 1-3* (Bleecker et al., 1988), or JA (Staswick et al., 1992) showed no inhibition of *APX2* expression (paper I).

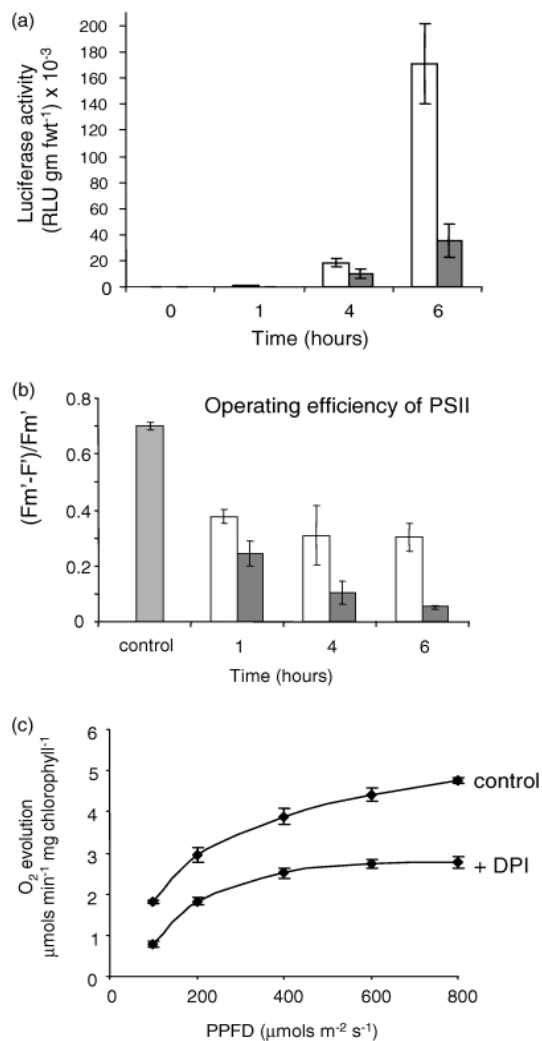
*APX2LUC Arabidopsis* rosettes sprayed with either JA or chitosan, an elicitor of the JA-independent pathway (Rojo et al., 1999), did not induce luciferase activity under ambient light conditions (paper I). The effects of JA and chitosan in the leaves were confirmed by *CHIB* and *VSP1* gene induction (paper I). Taken together these data indicate that the wound induction of *APX2* expression was not regulated by either of the known JA-dependent or JA-independent pathways in *Arabidopsis* and in contrast to wounding, *VSP1* induction of expression in excess light stressed leaves also was not JA-dependent.

### 3.1.4 Sources of $H_2O_2$ for induction of *APX2* expression

It has been proposed that NADPH oxidase(s) could be responsible for the generation of  $H_2O_2$  in the peri-veinal tissue of wounded leaves (Allan and Fluhr, 1997; Orozco-Cardenas et al., 2001; Bolwell et al., 2002). This suggestion was based on the observation that the wound-induced accumulation of  $H_2O_2$  can be inhibited by pre-treatment of leaves with diphenylene iodonium (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas et al., 2001). Therefore, the effect of DPI was tested on the wound-induced *APX2* expression and on photosynthetic electron transport.

DPI inhibited the wound-induced expression of *APX2LUC* in fully expanded leaves by >80 % after 6 hours (Figure 4a). This inhibition of *APX2LUC* expression by DPI was associated with a decline in the PSII operating efficiency indicative of decreases in the rate of photosynthetic electron transport (Figure 4b). These data suggest that DPI is a potent inhibitor of photosynthetic electron transport in wounded leaves. This conclusion was further supported by the inhibition of the rate of photosynthetic  $O_2$  evolution over a range of PPFDs (photosynthetically active photon flux density) in DPI-treated leaves (Figure 4c). Furthermore, the rate of  $O_2$  evolution in DPI-treated leaves saturated above a PPFD of 400  $\mu\text{mol m}^{-2}\text{s}^{-1}$  in DPI-treated leaves, whereas control leaves required in excess of 800  $\mu\text{mol m}^{-2}\text{s}^{-1}$  for saturation.

This observation has led to the proposal that wound-induced  $H_2O_2$  accumulation may be catalysed by a bundle sheath located NADPH oxidase (Orozco-Cardenas et al., 2001). Similar conclusions about the origins of  $H_2O_2$  have been made from DPI treatments of stomatal guard cells, wounded fruit mesocarp tissue and elicitor-treated suspension culture cells (Levine et al., 1994; Tenhaken et al., 1995; Watanabe and Sakai, 1998; Murata et al., 2001; Zhang et al., 2001). There is considerable biochemical and molecular genetic evidence for the existence of at least the gp96<sup>phox</sup> subunit of plant NADPH oxidases, homologous to that in mammalian phagocytes (Tenhaken et al., 1995; Keller et al., 1998; Torres et al., 1998). However, DPI has been shown to inhibit other haem-containing enzymes such as xanthine oxidase, nitric oxide synthase (Delledonne et al., 1998; Moulton et al., 2000) and cell wall peroxidases (Bolwell et al., 1999). Some of these enzymes have been implicated as sources of ROS at least for the HR (Allan and Fluhr, 1997; Bolwell et al., 1999; Bolwell et al., 2002).



**Figure 4.** Effect of DPI on the induction of *APX2LUC* expression (a), operating efficiency of PSII in wounded leaves (b) and photosynthetic O<sub>2</sub> evolution (c).

(a, b) DPI (50 μm) or water was injected into the mid-vein of leaves while still attached to the plant 1 h before wounding (as in Fig.3a) at time zero. Three plants per time point were used with a single wounded leaf taken from each rosette. The data shown are means (±SE) combined from two experiments. Duplicate assays for luciferase activity were performed from cell-free extracts prepared from the wounded leaves from which the operating efficiency of PSII, estimated from  $(F_m' - F_v')/F_m'$ , had been calculated from chlorophyll *a* fluorescence measurements using a modulating fluorimeter in which the detector was placed over wounded tissue at the times indicated

(c) The rate of photosynthetic O<sub>2</sub> evolution in leaf discs (Karpinski et al., 1997) prepared from DPI-treated leaves (after 4 h incubation) of *APX2LUC* plants was compared with that in control water-treated leaves at a range of PPFDs. The data are the means (±SE) of three independent experiments, each conducted with a single fully expanded leaf from four plants for each treatment (*n* = 12). The differences observed between DPI- and water-treated leaves are statistically significant (*P* < 0.001).

The data presented here indicate that the effects of DPI may include inhibition of photosynthesis, complicating the interpretation of data when using this compound on leaves. DPI inhibited wound induction of *APX2LUC* expression by >80 % (Figure 4a) but this was accompanied by an inhibition of photosynthetic electron transport as well (Figures 4b,c). There is considerable evidence for the requirement of photosynthetic electron transport for expression of *APX2* (Karpinski et al., 1997; Karpinski et al., 1999; Fryer et al., 2003). Therefore, we favour the inhibition of photosynthetic electron transport as the explanation for the action of DPI on *APX2* expression rather than implying a direct role for an NADPH oxidase.

The signal transduction pathway, which includes H<sub>2</sub>O<sub>2</sub> from the chloroplast and achieves regulation of *APX2* expression in bundle sheath cells of both high light stressed and wounded *Arabidopsis* leaves remains to be determined (Mullineaux and Karpinski, 2002). However, it is clear that the signalling role of ROS derived from changes in photosynthetic electron transport may be a common feature in controlling the expression of genes in response to environmental stimuli, such as in wounded leaves described here.

### 3.2 LESION SIMULATING DISEASE 1 controls acclimation to excess light

The *lesion simulating disease 1* (*lsd1*) mutant was first characterized for its O<sub>2</sub><sup>-</sup>-dependent spreading chlorotic/necrotic phenotype that develops under long (>16h) or continuous photoperiods or after infection with an avirulent pathogen (Dietrich et al., 1994; Jabs et al., 1996). Short day photoperiods (8h) and low PPFD (typically circa 100 ± 50 μmol m<sup>-2</sup>s<sup>-1</sup>) are permissive conditions for the growth of *lsd1* (Dietrich et al., 1994; Jabs et al., 1996). These observations suggest a strong relationship between prevailing light conditions and the propagation of the *lsd1* phenotype.

#### 3.2.1 Role of stomata and photorespiration in development of the *lsd1* phenotype

Examination of the chlorophyll *a* fluorescence parameters did not reveal any effect of LSD1 on thermal dissipation or other non-photochemical quenching processes (paper II). Therefore attention was focused on the EEE dissipatory process that consumes electrons, such as photorespiration. Closure of stomata in *Arabidopsis* leaves occurs rapidly in response to even small (as low as 3-fold) increases in PPFD and leads to increased photorespiration (Wingler et al., 2000; Noctor et al., 2002; Fryer et al., 2003). Stomatal conductance in both Ws-0 and *lsd1* grown under short day low light conditions was measured (paper II). Relative stomatal conductance values in *lsd1* plants in these permissive conditions were 50 % lower than in Ws-0 (0.146 ± 0.0109 and 0.315 ± 0.0246 cm<sup>-2</sup>s<sup>-1</sup>, respectively), indicating that stomata were more closed in the mutant than in wild-type plants. Mutations in *PAD4* (*PHYTOALEXIN DEFICIENT4*) and *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*) block *lsd1*-conditioned runaway cell death phenotype triggered by long photoperiods, pathogen inoculation, ROS provision, or supply of the phenolic signalling molecule, salicylic acid (Rusterucci et al., 2001). Significantly lower stomatal conductance observed in *lsd1* was not observed in the *pad4-5/lsd1* or *eds1-1/lsd1* double mutants (paper II).

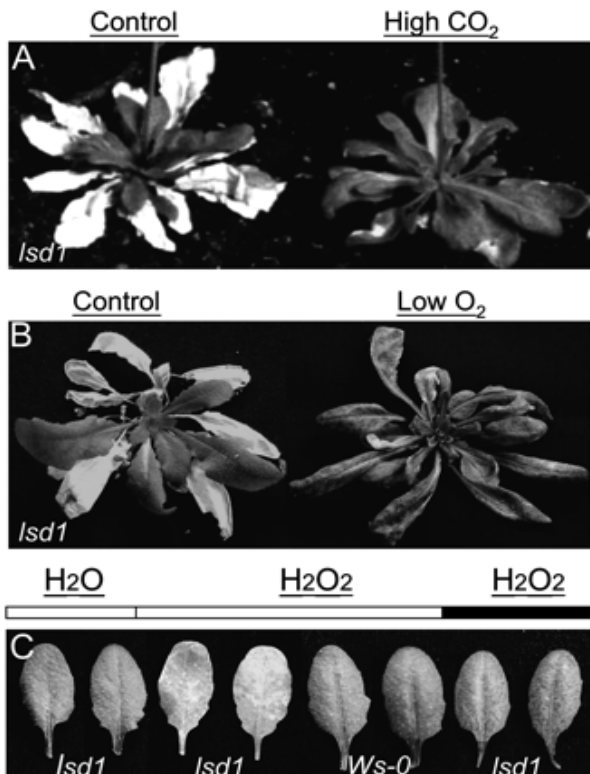
If impairment in stomatal conductance could be an important contributory factor in the runaway cell death phenotype in *lsd1*, then artificial blocking of stomatal pores and gas exchange by smearing lanolin on the lower surface of *lsd1* leaves would promote lesion formation under otherwise permissive light conditions. After 24h, a readily detectable increase in foliar H<sub>2</sub>O<sub>2</sub> content was observed in lanolin treated leaves of Ws-0 and *lsd1* by diaminofluorescein (DAF) staining (Figure 3 in paper II). The increase in foliar H<sub>2</sub>O<sub>2</sub> and runaway cell death phenotype as a consequence of limiting gas exchange were not observed in the *pad4-5/lsd1* and *eds1-1/lsd1* double mutants (Figure 3 in paper II). Our data suggest that localised increases in photorespiration caused by blocking gas exchange (during EEE stress or artificially) can induce runaway cell death phenotype in *lsd1*.

#### 3.2.2 Attenuation of *lsd1* phenotype in non-photorespiratory conditions

If photorespiration is a major source of H<sub>2</sub>O<sub>2</sub> promoting runaway cell death phenotype in *lsd1*, we reasoned that preventing the oxygenase reaction of Rubisco by incubating plants under high CO<sub>2</sub> or low O<sub>2</sub> tensions should diminish the lesion phenotype in *lsd1*. Results in Figures 5A and B show that placing *lsd1* plants either in an atmosphere of 0.12 % (v/v) CO<sub>2</sub> (3-fold above ambient concentration) or 2 % (v/v) O<sub>2</sub> (10 % ambient concentration)

## Results and Discussion

substantially attenuated the lesion phenotype under non-permissive long day conditions. Treatment of *lsd1* leaves with 20 mM H<sub>2</sub>O<sub>2</sub> under permissive light conditions also caused spreading lesions after 48 h (Figure 5C). These were not observed on H<sub>2</sub>O<sub>2</sub> treated Ws-0 leaves or in *lsd1* leaves kept in the dark (Figure 5C).



**Figure 5.** Reactive oxygen species (ROS) originating from photorespiration are critical for runaway cell death (red) in *lsd1*.

(A) Four-week-old *lsd1* plants cultivated in LD and which had initiated lesion formation were fumigated either with a 0.12 % CO<sub>2</sub> and 21 % O<sub>2</sub> (high CO<sub>2</sub>) atmosphere LD (16 h) for a period of 2 weeks or (B) with a 2 % O<sub>2</sub> and 0.04 % CO<sub>2</sub> (low O<sub>2</sub>) atmosphere in continuous light (CL) for a period of 1 week.

(C) Pictures of representative leaves of Ws-0 and *lsd1* after 2 d incubation either in light or dark in a solution of 20 mM H<sub>2</sub>O<sub>2</sub>. Control leaves were incubated in water.

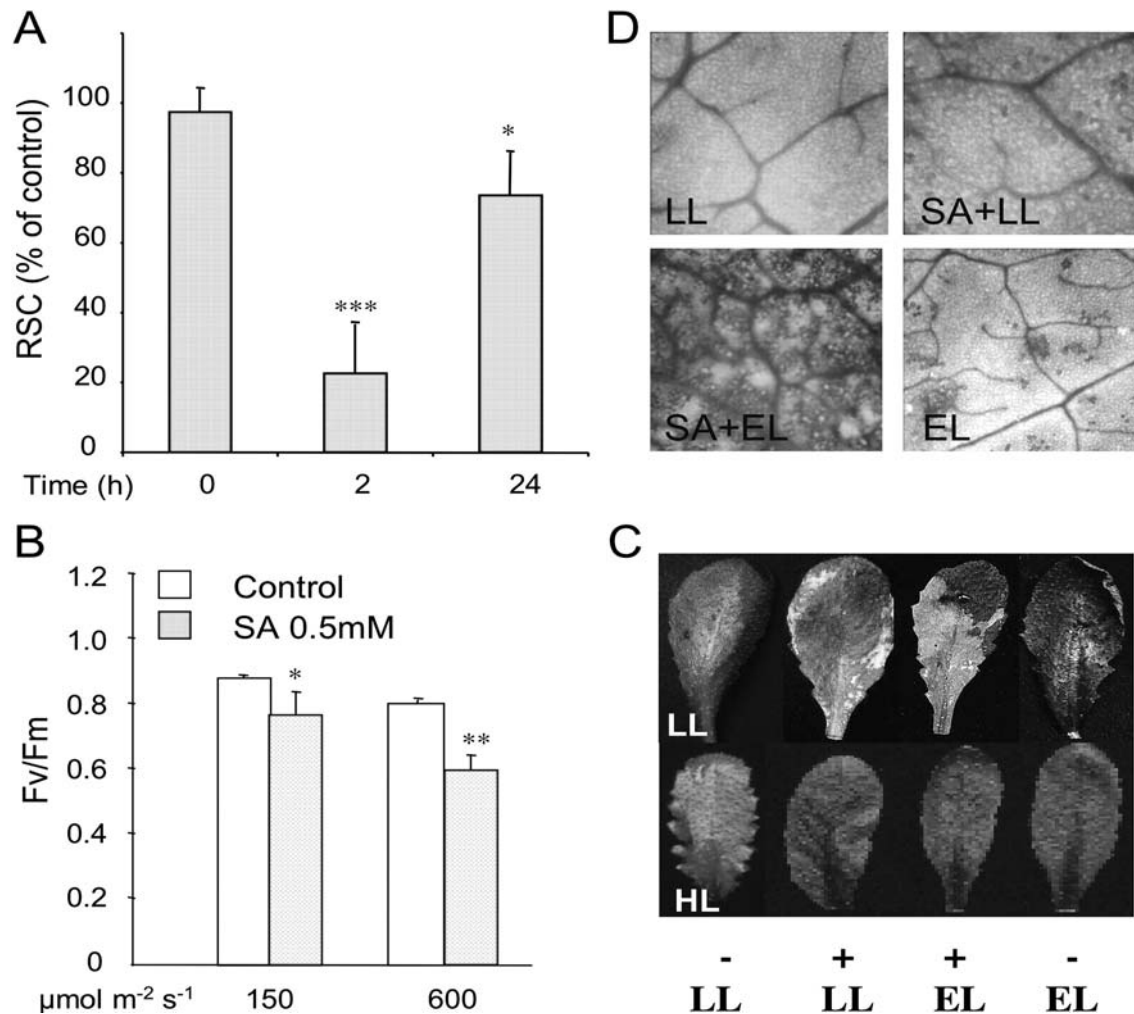
Acclimation to EEE in higher plants is signalled by significant increases in H<sub>2</sub>O<sub>2</sub> levels (Karpinski et al., 1997; Karpinski et al., 1999; Karpinska et al., 2000; Fryer et al., 2003). It can be concluded from the above experiments that LSD1 promotes the effectiveness of photorespiration by reducing the damaging effects of H<sub>2</sub>O<sub>2</sub> accumulation during light acclimation.

### 3.2.3 Salicylic acid, photo-oxidative stress and stomatal conductance

Salicylic acid treatment induces rcd in *lsd1* under otherwise permissive conditions (Jabs et al., 1996). Interestingly, rapid closure of stomata and an increase in foliar H<sub>2</sub>O<sub>2</sub> have been shown to occur upon SA treatment of leaves (Manthe et al., 1992; Chen et al., 1993; Rao et al., 1997; Shirasu et al., 1997; Mori et al., 2001). This may be associated with SA-dependent inhibition of catalase (CAT) activity (Sanchez-Casas and Klessig, 1994; Conrath et al., 1995; Durner and Klessig, 1996; Chen et al., 1997). If peroxisomal CAT levels prior to a change in the light environment are crucial to successful acclimation or recovery, it can be anticipated that wild-type leaves treated with SA would generally behave in a manner similar to *lsd1*.

## Results and Discussion

Treatment of wild-type *Arabidopsis* leaves with SA under short day conditions induced rapid stomatal closure within 2 h (Figure 6A). Furthermore such leaves showed enhanced inhibition of photosynthetic electron transport under low light conditions (Figure 6B) and enhanced photo-bleaching (Figure 6C). Photobleaching in wild type leaves exposed to EL



**Figure 6.** SA impairs acclimation to EEE in low light-acclimated plants.

- (A) Relative stomata conductance (RSC) in wild-type leaves of rosette grown in SD treated with SA (0.4 mM) in comparison to control leaves treated with water.
- (B) Photosynthetic parameter  $F_v/F_m$  in LL-acclimated leaves treated with 0.5 mM SA and then exposed to LL ( $100 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or HL ( $450 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 2 h.
- (C) LL- and HL-acclimated leaves treated with 0.4 mM SA (+) for several hours and exposed to EL ( $2,000 \pm 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 90 min exposure).
- (D) TB staining of representative leaves. Pictures were taken 2 h after treatments and represents of  $n = 15$  from three independent experiments ( $P \leq 0.05^*$ ,  $P \leq 0.01^{**}$ , and  $P \leq 0.001^{***}$ ).

was characterised by the appearance of a delimited area of cell death revealed by lactophenol-trypan blue (TB) staining (Figure 6D). These effects were strongly accelerated by a combined effect of SA-treatment and EL exposure (Figures 6B,C,D). It is important to note here that stomatal gas exchange in SA-treated wild-type leaves before EL exposure was reduced approximately by a factor of 4 (Figure 6A), while in combined treatment of wild type leaves by a factor of 12 ( $0.027 \pm 0.0185 \text{ cm}^{-2}\text{s}^{-1}$ ). SA treatment of *lsd1* leaves in low light caused reduction of stomatal gas exchange by a factor of 14 ( $0.024 \pm 0.0161 \text{ cm}^{-2}\text{s}^{-1}$ ) in comparison to wild-type control values. The data suggest that SA induces an increase in EEE under low light intensities such that failure to tolerate EL treatment ensued. This notion was reinforced by the observation that high light acclimated leaves, already dissipating or limiting EEE, were tolerant to SA treatment (Figure 6D) and have higher foliar SA levels (Karpinski et al., 2003).

It can be concluded that the effectiveness of photorespiration in dissipating EEE may be limited by the efficiency of  $\text{H}_2\text{O}_2$  scavenging. The above results show that the *Arabidopsis* mutant *lsd1* failed to acclimate to light conditions that promote photo-oxidative stress, and that LSD1 function was required for the expression of *CATALASE1*, encoding a subunit of the peroxisomal catalase (paper II). Through this regulation LSD1 can control the effectiveness of photorespiration in dissipating EEE absorbed by photosystem II. It can also be concluded that *lsd1* had reduced stomatal conductance in permissive conditions and induced  $\text{H}_2\text{O}_2$  accumulation together with runaway cell death when stomatal gas exchange was impeded. Previously  $\text{H}_2\text{O}_2$  had been excluded as a molecular trigger of *lsd1* runaway cell death phenotype (Jabs et al., 1996). These light sensitive *lsd1* phenotype depended on the defence regulators *EDS1* and *PAD4* and PSII light harvesting antenna size. Salicylic acid, which induces stomatal closure, inhibited catalase activity and triggered the runaway cell death phenotype of *lsd1*, also impaired acclimation of wild type plants to conditions that promote EEE. It can be proposed that the roles of LSD1 in light acclimation and regulation of stomatal conductance, as well as in restricting pathogen-induced cell death are functionally linked (paper II).

### 3.3 Functional characterization of the chloroplastic glutathione peroxidases (*cpGPXs*) in *Arabidopsis thaliana*

Glutathione peroxidases (GPXs) are commonly considered to be important ROS scavengers because their broader substrate specificities and stronger affinity for  $\text{H}_2\text{O}_2$  than catalases (Brigelius-Flohe and Flohe, 2003). Several plant ROS scavenging proteins are encoded by families of closely related genes that typically display specific structural and regulatory features (Ferrari et al., 2003). Sub-cellular compartmentation of ROS scavenging systems is crucial for efficient removal/control of ROS at their sites of generation (Greene, 2002). DNA and derived amino acid sequence analysis suggests that *Arabidopsis* GPXs also belongs to this category of enzymes with putative localization in the cytosol, chloroplast, mitochondria and endoplasmic reticulum (ER). Analysis of the *Arabidopsis* genome sequence revealed one additional open-reading frame encoding protein closely related to seven previously described GPXs (Milla et al., 2003), thus increasing the total number of genes to eight (*AtGPX1-AtGPX8*; Figure 7A). Phylogenetic analysis of *Arabidopsis* GPXs suggests three pair-group - *AtGPX1* and *AtGPX7* (chloroplastic group); cytosolic *AtGPX4* and ER-*AtGPX5*; cytosolic

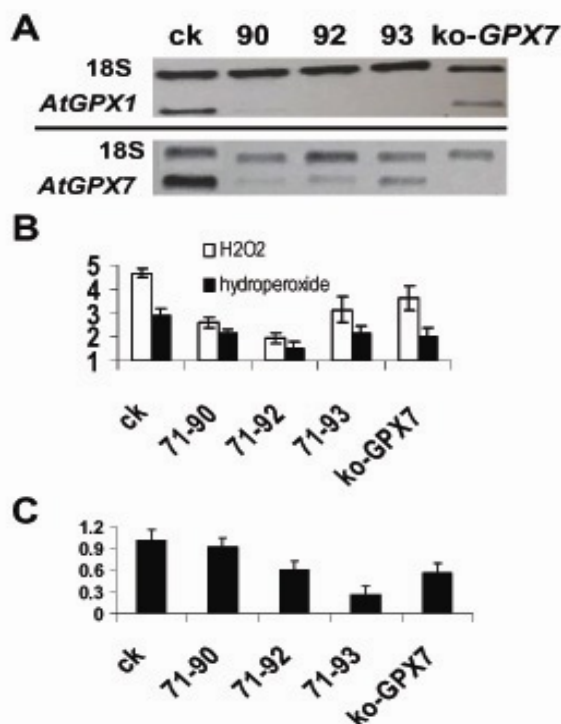




### 3.3.1 *Arabidopsis* plants with reduced expression of chloroplastic GPXs

Most plant GPXs have been analysed only partially and the biological function of these enzymes have not been sufficiently elucidated. In order to analyse the role of two chloroplastic isoforms of GPXs, we have generated *Arabidopsis thaliana* transgenic lines with reduced cpGPX activity. Results presented here suggest that cpGPXs play important protective and regulatory roles during acclimation to photo-oxidative stress conditions.

*Arabidopsis* plants were transformed with a construct consisting of the cDNA encoding GPX1 in antisense orientation under the control of the cauliflower mosaic virus (CaMV) 35S. Twelve independent transformed of AS-cpGPX lines were thereby produced. For analyses, homozygous T<sub>4</sub> plants from lines carrying single insertions were used. As revealed by RT-PCR and protein level analysis, AS-cpGPX lines (71-90, 71-92 and 71-93) showed significantly lower expression of both *AtGPX1* and *AtGPX7* whereas knockout (ko)-*GPX7* suppress *AtGPX7* only (Figure 8A). Furthermore, GPX activity measured in intact chloroplasts was significantly lower in AS-cpGPX lines than in control plants, while ko-*GPX7* mutant was only slightly reduced (Figure 8B). We also employed enzyme-linked immunosorbent assay (ELISA) to quantify the content of cpGPX and 71-93 line showed the most reduced among AS-cpGPX lines compared to control plants (Figure 8C). The different cpGPX suppression levels in AS-cpGPX lines could be due to the positional effects that depend on the insertion place of the transgene into the *Arabidopsis* genome.



**Figure 8.** Selected transgenic AS-cpGPX and ko-GPX7 of the chloroplastic AtGPXs transgenic plants have reduced cpGPX activity

(A) Transcript abundance analysis for *AtGPX1* and *AtGPX7* in selected transgenic *Arabidopsis* lines (90, 92, 93 and ko-GPX7) by using RT-PCR quantitative assay and 18S RNA as an internal standard as described in *Material and Methods*.

(B) GPX activity measured in intact chloroplast extracts. *t*-butyl peroxide and hydrogen peroxide were used as substrates and enzyme unit expressed as  $\mu\text{M}/\text{min}/\text{mg}^{-1}\text{FW}$

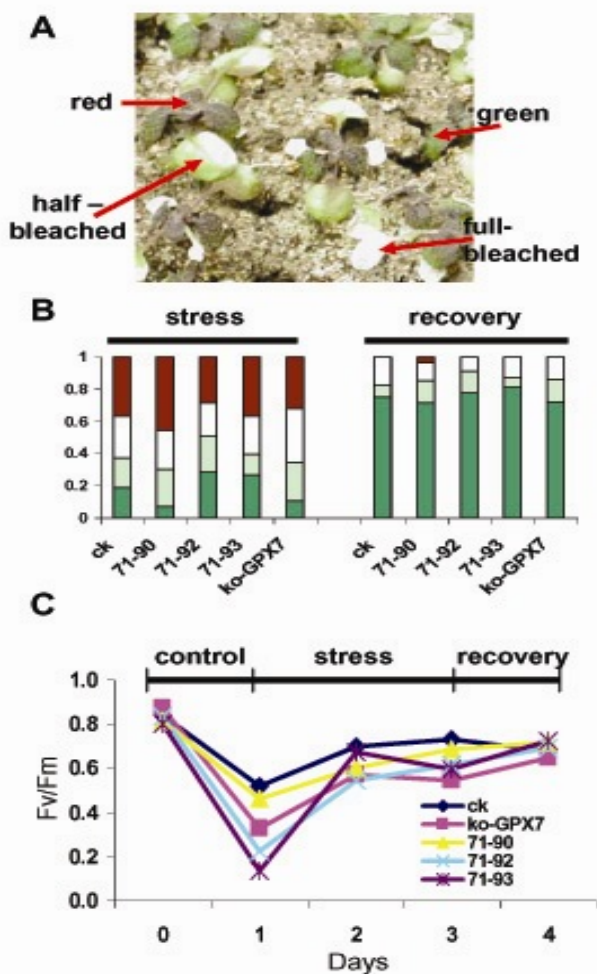
(C) cpGPX protein level analysis by enzyme-linked immunosorbent assay (ELISA) and expressed as ratio compared to control plants (ck).

### 3.3.2 Enhanced photo-inhibition in chloroplastic glutathione peroxidases deficient lines

When two-week-old AS-cpGPX lines were challenged with continuous high light combined with chilling stress (continuous high light  $650 \pm 50 \mu\text{mol s}^{-1}\text{m}^{-2}$  at  $4^\circ\text{C}$ ), seedlings did not

## Results and Discussion

show more visible photo-bleaching or photo-damage but did reveal different levels of photo-inhibition of photosynthetic electron transport (Figure 9 and paper III). Characteristic picture of young *Arabidopsis* plants exposed to such stress can be divided into four major categories; green, red, half-bleached and fully bleached leaves (Figure 9A). Photo-bleached leaves can be used as an indicator of the level of photo-oxidative stress. Percentages of the previous four categories are represented in Figure 9B. AS-cpGPX lines demonstrated enhanced inhibition of photosynthetic electron transport (Figure 9C). However, maximum photochemical efficiency (Fv/Fm) recovered equally as in control plants after four days of such a stress (Figures 9B,C). It is important to know that under this excessive stress condition, utilisation of light energy through photosynthetic carbon assimilation is decreased more than the rate of electron transport (Foyer et al., 1994). Therefore this could result in considerable increase in the sensitivity of PSII to photo-inhibition as observed in AS-cpGPX lines (Figure 9C).



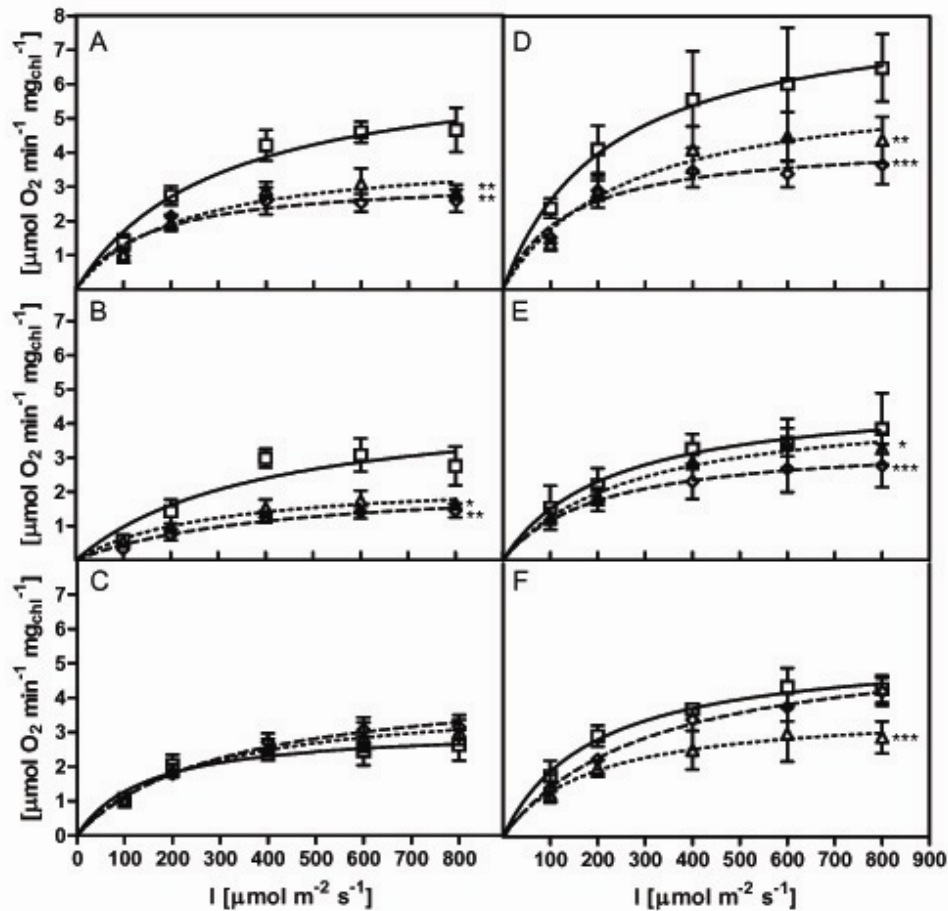
**Figure 9.** Antisense chloroplastic AtGPXs transgenic lines were more sensitive to photo-oxidative stress.

(A) Image of two-week-old *Arabidopsis* seedlings from low-light cultivation conditions followed by continuous high-light ( $700 \mu\text{mol s}^{-1}\text{m}^{-2}$ ) combined with chilling temperature (at  $4^\circ\text{C}$ ) for four days.

(B) Relative numbers (in percentage) of leaves of four different categories (green, red, half-bleached and fully bleached) which were shown in (A). Leaves number of twenty rosettes for each line were counted after four days of photo-oxidative stress and three days after recovery from such a stress.

(C) Photosynthetic maximal electron transport efficiency parameter (Fv/Fm) during photooxidative stress and recovery period ( $n > 20 + \text{SD}$ ).

Significant differences between low light (LL) and high light (HL) acclimated AS-cpGPX plants were observed for light response curves for oxygen evolution (Figure 10). Increases in photosynthesis-irradiance (PI) curves observed in Figure 10 reflect an increased capacity for electron transport, which in turn requires the provision of increased electron sink capacity, principally through the  $\text{CO}_2$  fixation via Calvin cycle, water-water cycle or photorespiration (Kozaki and Takeba, 1996; Asada, 1999; Mullineaux and Karpinski, 2002).



**Figure 10.** Analysis of P680 activity (oxygen evolution) in AS-cpGPX and ko-GPX7 lines cultivated in low-light (LL) and high-light (HL) conditions.

Light response curves for oxygen evolution in *A. thaliana* leaves grown under different light conditions (LL,  $100 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$  and HL,  $450 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Plants adapted to LL (A) and to HL (D). Plants exposed to excess light (EL,  $2,000 \pm 100 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 1 hour, for LL-adapted plants (B) and HL-adapted plants (E). Recovery (1h, R) from EL stress for LL-adapted plants (C), and HL-adapted plants (F). control plants ( $\square$ ), 71-92 ( $\blacktriangle$ ), mutant ( $\diamond$ ) were shown respectively. Values represent means  $\pm$  SE of pooled sample of three or four leaves from three independent experiments ( $n > 9$  -  $< 12$ ,  $N=3$ ). Photosynthetic  $\text{O}_2$ -evolution was measured in saturating  $\text{CO}_2$  atmosphere. One-way ANOVA and Turkey's multiple comparison post-test were used for statistical analysis of data. The asterisks indicate the significance of differences from control plants as revealed by Turkey's multiple comparisons post-test:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*)

Therefore reduced oxygen evolution (reduced photosynthetic electron production) observed in AS-cpGPX and ko-GPX7 plants could be explained as adaptive down regulation of P<sub>680</sub> activity due to reduced water-water cycle ROS scavenging capacity. Therefore, it can be suggested that cpGPXs activity is an important metabolic regulatory sink and link controlling photosynthetic electron transport stoichiometry.

### 3.3.3 Photo-produced reactive oxygen species and resistance against pathogen infection

GPXs have been shown to be induced during protection against various abiotic stresses including high light, salt, heat, cold, drought, mechanical wounding, heavy metals and

osmotic stress (Sugimoto and Sakamoto, 1997; Depege et al., 1998; Avsian-Kretchmer et al., 1999; Roxas, 2000; Swidzinski et al., 2002; Yoshimura et al., 2004). Involvement of GPXs in biotic stress responses has also been demonstrated (Kuniak and Skodowska, 2001) and GPX has become a robust molecular marker for biotic stress responses (Rao and Davis, 1999). However, when plant *GPX* was indexed for the first time as molecular marker for biotic stress responses, Levine and colleagues (1994) did not recognize that cDNA employed in their experiments encoded the putative chloroplastic isoform of selenium-independent GPX (Mullineaux et al., 1998).

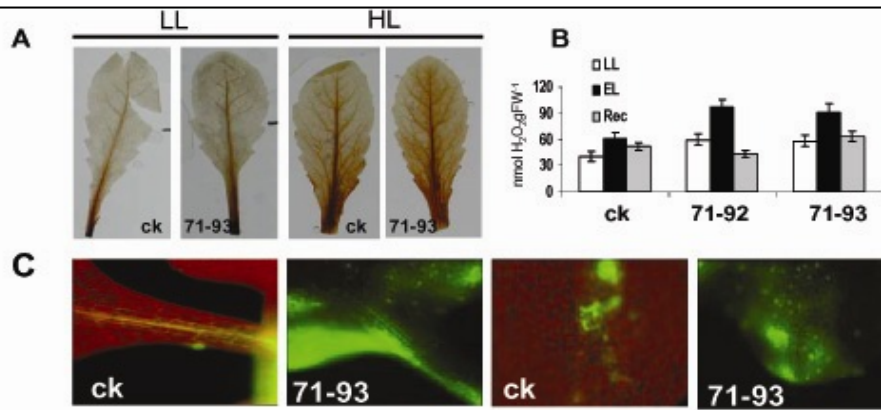
Both ROS and lipid peroxide play dual functions in cellular metabolism. On one hand, they are highly toxic and must be kept under tight control (Noctor and Foyer, 1998). On the other hand, ROS and lipid peroxides serve as substrates in metabolism and as signals for regulation of environmental stress responses (Foyer and Noctor, 2000). The chloroplast envelope is suggested as a main source of phospholipid peroxides which serve as substrates for oxylipin and jasmonates biosynthesis (La Camera et al., 2004). These in turn regulate many plant defence responses including infection with pathogens. Thus, ROS produced in chloroplast, especially hydrogen peroxide that can cross the membranes together with phospholipid peroxides may contribute to the defence network under pathogen infection. ROS- and lipoxygenase-mediated production of fatty acid hydroperoxides in chloroplasts are specific features of the hypersensitive response in plants and show the major role of this organelle in signalling during incompatible plant-pathogen interaction (Hammond-Kosack and Parker, 2003).

Our current results with five-week-old AS-cpGPX lines acclimated to both low light and high light conditions showed higher levels of hydrogen peroxide visualised by 3-3 diaminobenzidine (DAB) precipitate after excess light stress treatment (Figure 11A). Furthermore, quantification of foliar hydrogen peroxide levels was consistent with the above observation (Figure 11B). A readily detectable increase in foliar H<sub>2</sub>O<sub>2</sub> was observed in 71-93 from low light conditions both in leaf and petiole without any pre-treatment when we used more sensitive dichlorofluorescein staining, another H<sub>2</sub>O<sub>2</sub> accumulation index (Figure 11C). Since AS-cpGPX lines (71-92 and 71-93) showed more H<sub>2</sub>O<sub>2</sub> accumulation in both untreated and adapted to high light or exposed to excess light leaves, the above results confirm that reduction of cpGPX activity could be associated with higher foliar H<sub>2</sub>O<sub>2</sub> accumulation.

Furthermore, higher levels of ROS (Figure 11) in AS-cpGPX lines might lead to the possibility of cell death, and increase the plants' susceptibility to infection by avirulent strains. A visible HR can be demonstrated by lactophenol-trypan blue (TB) staining (Figure 12A). AS-cpGPX lines showed more extensive HR regions than the control plants after 72 hrs of avirulent pathogen strain (avrRPM1) infection. Ion leakage was used as a quantitative indicator of cell death (O'Donnell et al., 2001).

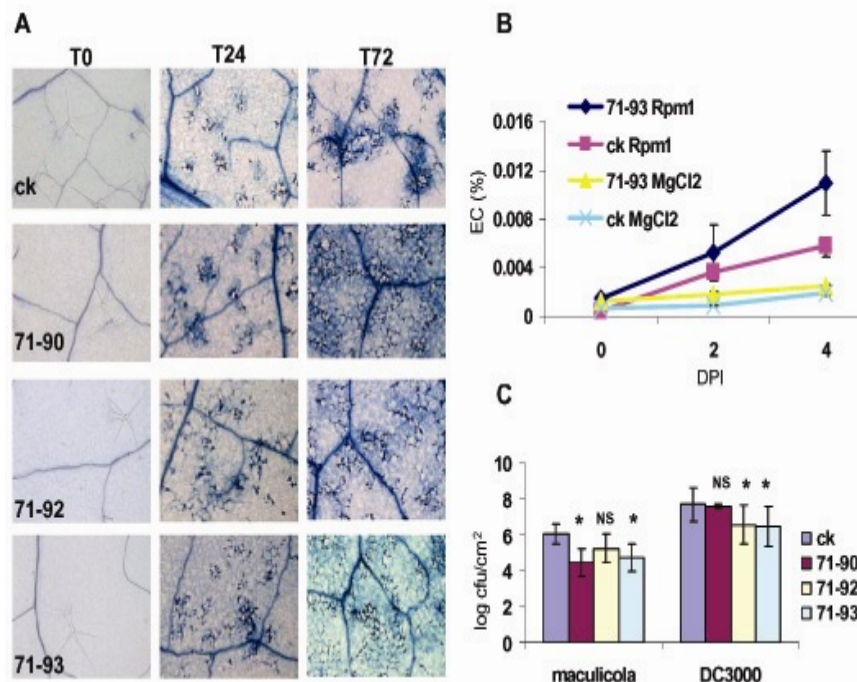
Consequently, ion leakage increased more in AS-cpGPX lines than in control plants in four days after the inoculation with avirulent strain (avrRPM1) (Figure 12B). However, in later times (after four days), the pathogen growth was not significantly different in transgenic and control plants (data not shown). These results suggest that the observed changes in HR were not able to restrict the pathogen growth and influence the plant resistance to avirulent

## Results and Discussion



**Figure 11.** Hydrogen peroxide accumulates to higher levels in AS-cpGPX transgenic lines.

(A) Five-week-old *Arabidopsis* control (ck) and 71-93 line leaves were taken from low light-adapted plants (LL;  $100 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and high light-adapted plants (HL;  $450 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), exposed to excess light (EL;  $2,000 \pm 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for one and half hour respectively and followed by 3,3-diaminobenzidine (DAB) staining. (B) Quantification of foliar hydrogen peroxide concentration from LL growth plants after one hour EL exposure, and after one hr recovery from such stress. Concentration is expressed in nmol of H<sub>2</sub>O<sub>2</sub> per gram fresh weight (gFW<sup>-1</sup>). (C) 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) staining of three-week-old seedlings. Data are representative for three independent experiments and several repetitions in one single experiment (N = 3 ± SD).



**Figure 12.** Transgenic lines with reduced cpGPX activity displayed enhanced basal resistance towards virulent pathogen infection.

(A) Leaves of 4-week-old plants were inoculated with a bacterial suspension containing  $10^5$  CFU (colony forming units) mL<sup>-1</sup>. (A) Trypan blue (TB) staining pictures were obtained after 0 (T0), 24 (T24) and 72 (T72) hrs of rosette leaves infiltration with avirulent *P. syringae* pv *tomato* DC3000 carrying *AvrRpm1* avirulence gene. (B) Ion leakage was measured after 0, 2 and 4 days followed by infiltration of leaves with avirulent *P. syringae* strain (avrRpm1), or with 10 mM MgCl<sub>2</sub> used as a control infiltration-induced wounding at 0, 2 and 4 days. (C) Bacterial growth at four days post inoculation of virulent strains *P. syringae* pv *maculicola* ES4326 and *P. syringae* pv *tomato* DC3000. Mean values ± SD of three or four different plants are depicted. Similar results were obtained in three independent experiments. The asterisks indicate the significance of differences from the control plants at the level at least  $P < 0.05$ .

pathogen attack. On the other hand, higher levels of ROS could possibly help to develop the basal resistance and defence against virulent pathogen attack (Figure 12C). To test this possibility, the bacterial growth of virulent pathogen strains *P. syringae* pv *maculicola* ES4326 and *P. syringae* pv *tomato* DC3000 was further monitored in AS-cpGPX lines and control plants. The initial rate of bacterial growth was similar in all tested lines (data not shown). However, at four days post inoculation, the bacterial titre was approximately 10-fold (significantly) lower in AS-cpGPX lines as compared to control plants, which indicates enhanced resistance against both virulent pathogens (Figure 12C). These results are in agreement with recent observations made in the analysis of vitamin C deficient mutants (*vtc1*, *vtc2*), virulent bacterial growth (*P. syringae* pv *maculicola* ES4326) was reduced in those mutants due to higher foliar ROS level (Barth et al., 2004). Both examples illustrate that photo-produced ROS play an important role in basal defence mechanisms. In addition, the above results also suggest that *AtGPX1* but not *AtGPX7* might be involved in defence against virulent pathogen infection. This hypothesis is supported by the observation that virulent pathogen growth was significantly reduced in AS-cpGPX lines, with the inhibition of both *AtGPX1* and *AtGPX7* (Figure 12C), while virulent pathogen growth in infected ko-*GPX7* line was not significantly reduced (data not shown).

#### 4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

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In this work, physiological and molecular analyses of *Arabidopsis* mutants and transgenic lines were applied to investigate the signalling network controlling biotic and abiotic stress responses. The signalling role of photo-produced ROS resulting from redox changes in the photosynthetic electron transport may be a common feature in controlling the expression of genes in response to environmental stimuli, such as wounding. *ASCORBATE PEROXIDASE 2 (APX2)* encodes a key enzyme of the antioxidant network. In excess light-stressed *Arabidopsis* leaves, H<sub>2</sub>O<sub>2</sub> and abscisic acid regulate *APX2* expression. Wounded leaves showed low induction of *APX2* expression and when exposed to excess light, *APX2* expression was increased synergistically. Signalling pathways dependent upon jasmonic acid, chitosan and abscisic acid were not involved in the wound-induced expression of *APX2*, but was shown to require PET and was preceded by a depressed rate of CO<sub>2</sub> fixation.

Analysis of *lsd1* in different mutant backgrounds puts forward a convincing argument for a loss of photorespiratory control in the LSD1 type of mutant and strongly suggests that light acclimatory processes and pathogen defences are genetically and functionally linked. It is important to know that LSD1 type of mutants have mainly been studied with regard to pathogenesis. From this work, it reveals that association of LSD1 with hypersensitive response may only be supplementary.

*GLUTATHIONE PEROXIDASES (GPXs)* are considered important ROS scavenging enzymes. Analysis of the *Arabidopsis* genome database revealed one additional open-reading frame encoding cytosolic protein closely related to seven previously described GPXs, thus increasing the total number of *AtGPX* genes to eight (*AtGPX1-AtGPX8*). Two putative chloroplastic isoforms showed differential expression patterns in various *Arabidopsis* organs in flower and in breaking of the ABA-dependent dormancy during seed germination. *Arabidopsis thaliana* transgenic lines with reduced expression of putative chloroplastic isoforms (*AtGPX1* and *AtGPX7*) and *AtGPX7* knock-out mutant (ko-*GPX7*) were more sensitive to photo-oxidative stress but manifested reduced bacterial growth rate when virulent strains *Pseudomonas syringae* pv. *tomato* DC3000 and *P.s.t. maculicola* strain ES4326) were inoculated. Functional analysis of cpGPXs confirms that light and chloroplastic ROS metabolism is important for basal resistance against virulent pathogens. Accordingly, to our knowledge, this is the first genetic analysis of chloroplastic GPXs in plants.

The presentation and discussion of the above results confirm that light sensing, light acclimatory processes and photo-produced ROS also govern pathogen defence pathways. This has a great ecological relevance for Darwinian fitness of plants growing in the natural environment, where simultaneous pathogen attack and fluctuations in light, temperature and other environmental factors make rapid acclimation a constant necessity. Molecular, biochemical and physiological analysis of pathogen responses in mutants impaired in light sensing, EEE-dissipatory mechanisms, and similar analysis of light acclimatory processes in mutants impaired in pathogen defences may prove to be seminal.

The following experiments are planned in the near future. Transgenic plants with cpGPXs overexpression and antisense cytosolic GPX lines which were also generated will be



used to further confirm already obtained data and to analyse more functional aspects of GPXs' roles in biotic and abiotic cross-talk. Furthermore, other *AtGPX* family mutants were obtained and purified from NASC. Investigation of these mutants will allow us to assess a more complete picture of the functional role of the *AtGPX* family. Generation of plants carrying GFP fusions with cDNAs encoding two isoforms of cpGPXs will help us to confirm the sub-cellular localization of these enzymes. In addition, massive metabolite analysis in transgenic plants and mutants will be another tool to give us the clue of metabolic connections and functions.

## 5. MATERIALS AND METHODS

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### *Plant Material*

*Arabidopsis thaliana* (L.) Heyhn. plants were germinated and grown in conventional soil (Topstar-Economa Garden AB, Sweden) for 4-5 weeks under controlled environmental conditions (PPFD of low light, LL,  $100 \pm 50 \mu\text{molm}^{-2}\text{s}^{-1}$  during a 8-9 hr (as indicated in the paper respectively) photoperiod at 22 °C in a relative humidity of 50 - 60 %). In all experiments, unless otherwise stated, the Col-0 ecotype of *Arabidopsis* was used. All mutants used in this study were obtained from the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, U.K.).

Continuous high light combined with cold stress treatments ( $650 \pm 50 \mu\text{molm}^{-2}\text{s}^{-1}$  at 4 °C) was used to test photo-oxidative stress tolerance in LL acclimated two-week-old seedlings. For recovery from such stress, after four days plants were returned to the initial growing conditions and analyzed for an additional three days.

During excess light (EL) experiments, 4 to 5 week old plants grown in LL or high light (HL;  $450 \pm 50 \mu\text{molm}^{-2}\text{s}^{-1}$ ) were exposed to EL ( $2,000 \pm 100 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) with an extra light source (HMI 1200 W/GS photo optic lamp; OSRAM, Germany) for 30-60 min. After EL-treatment, plants were returned to their previous growth conditions for recovery over a period of 60 min. For the HL experiment, 2-week-old plants grown in standard LL conditions were afterward exposed to HL with a supplementary light source (OSRAM Powerstar HQI-E 250W; Germany) until the end of the experiment. Temperature, photoperiod and humidity for HL experiments were similar as in LL conditions.

### *Antisense chloroplastic glutathione peroxidases constructs and selection of transgenic plants*

The cDNA clone (181O9T7) encoding chloroplast glutathione peroxidase (*AtGPXI*) in pBluescriptSKII+ (Alting-Mees and Short, 1989) was ordered from EMBL (<http://www.ebi.ac.uk/embl/>). cDNA clone was used to amplify the 500 bp PCR fragments carrying HindIII and EcoRI restriction sites from the 3' non-conserved part of the genes (forward: 5'-CGTACGGATCCTTCTCTACAGTCCG-3' and reverse: 5'-GGTCCATTTCACGTCAACCTTATCA-3'). The fragment was cloned in antisense orientation in the vector pJIT60 (Guerineau and Mullineaux, 1993) between the double CAMV 35S promoter and the CAMV 35S terminator. The whole 35S cassettes were

subsequently cut out using SstI and XhoI and inserted between SstI and SalI restriction sites of pBIN19 binary vector (Bevan, 1984). *Agrobacterium* strain GV3101. The integrity of the construct was verified by sequencing after isolation from *Agrobacteria* and amplification in *E. coli*.

Twelve primary transformant lines were isolated and self-pollinated. About 200 plants from T<sub>2</sub> generation for each line were analysed for kanamycin resistance. Lines segregated with a ratio 3:1 were selected and the T<sub>4</sub> generation was obtained for identification of homozygous lines. Selected lines were backcrossed to control plants and homozygous transgenic lines were selected to create comparable genetic backgrounds. Finally, three transgenic AS-cpGPX lines (71-90, 71-92 and 71-93) and one transgenic knockout of *AtGPX7* (ko-*GPX7*) from Sainsbury Laboratory *Arabidopsis thaliana* Transposants (SLAT) population which later compiled into *Arabidopsis* database (Tissier et al., 1999) were chosen for further experiments.

### *cpGPX Antibodies production and ELISA analysis*

In order to produce antibodies against cpGPX, relatively short 58 amino acid region between serine at position 175 and the C-terminal alanine of *AtGPX7* of which is the most different from cytosolic isoform of GPXs was chosen. This sequence has been expressed in fusion with thioredoxin protein and His-Tag using the pET32a vector (Novagen, CA, USA) in *E. coli* strain BL21DE3 strain. For purification of the fusion protein, we used the Chelating Sepharose Fast Flow (Amersham Biosciences, Sweden) charged with Ni ions. Purified protein has been loaded on the preparative polyacrylamide gel, visualized by Pierce Gel Code (Pierce, USA) staining then cut out of gel and used to immunize two rabbits. Immunization of rabbits was conducted by Agrisera (Umeå, Sweden) on commercial base. Obtained sera have been purified and tested in Western blot experiments on total and chloroplastic protein extracts from control plants and transgenic *Arabidopsis* lines. Both sera gave specific signal of expectable molecular weight of approximately 22 kDa in the crude protein extract.

For further quantification of cpGPX protein levels in *Arabidopsis*, enzyme-linked immunosorbent assay (ELISA) with modification (Mett et al., 2000) was used. The samples with proper dilution series were transferred to 96-well polystyrene microtiter plates (Nunc-Immuno-Plate, Maxisorb, Nunc, Denmark) at room temperature for one hour with shaking. Followed by adding blocking solution (0.5 % in PBS + 0.1 % Tween), primary antibodies, secondary antibody and color substrate OPD (Sigma-Aldrich, Sweden), detection of reaction was stopped after 15 minutes of incubation by the addition of 50 µl of 5M sulfur acid. The O.D. was determined at 450 nm on a Microplate reader (Bio-Tek Instruments, Vermont, USA).

### *GPX activity*

GPX activity was assayed spectrophotometrically according to (Drotar et al., 1985) with H<sub>2</sub>O<sub>2</sub> or *t*-butyl hydroperoxide as substrates. The rate of peroxide removal was measured with respect to the rate of NADPH oxidation at 348 nm.

For further details please refer to *Material and Methods* parts in the attached papers.

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## ACKNOWLEDGEMENTS

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I would like to express my sincere thanks to everyone that in anyway has supported and helped me during my thesis project. In particular:

### *Private and public foundations*

Financial support from the Botany Studentship, Stockholm University and contributions from Formas (Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning), NorFa (Nordic Science Policy Council and Nordic Academy for Advanced Study), STINT (Swedish Foundation for International Cooperation in Research and Higher Education), VR (Swedish Research Council), Wallenberg scholarship, and C. F. Liljevalch j:ors travel scholarship.

### *Supervisors*

#### **Stanislaw Karpinski, main supervisor**

Thank you for endless enthusiasm, empathy and big ideas.

#### **Barbara Karpinska, co-supervisor**

For heaps of kind help, both practical and theoretical.

#### **Philip M. Mullineaux, co-supervisor**

For good discussions and a critical eye.

### *Past and current group members, in no particular order*

#### **Dietmar Funck**

For being patient, providing numerous help and constructive criticism, especially those emergency cases!

#### **Matt Geisler**

For enthusiasm, excellent input and a particularly long-distance assistance!

#### **Mercedes Tiemblo**

A terrific roommate whenever we go to conferences or workshops together!

#### **Alexy Sotnikov**

For nice cooperation and good knowledge for proteins!

#### **Regina Choo**

For taking care of things when I am writing!

#### **Per Mühlenbock**

For being a great officemate, fantastic sense of humor and wonderful hospitality to help me acclimatize to Swedish culture!

#### **Alfonso Mateo**

For good discussion and being a kind officemate!

### *Others:*

Botanists with nice interactions together at “fika” and frequent innerbandy group players including Martin, Mattias, Clara, Johanna, Ingvild, YaoDong, and Gustaf who brought me the strength and an engaging atmosphere!

Thank you, Dad, Mom, I-Fan and Wayne, for always being there with endless support for me.