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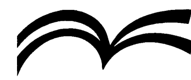

Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis

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Abstract

Sudden exposure of plants to high light (HL) leads to metabolic and physiological disruption of the photosynthetic cells. Changes in ROS content, adjustment of photosynthetic processes and the antioxidant pools and, ultimately, gene induction are essential components for a successful acclimation to the new light conditions. The influence of salicylic acid (SA) on plant growth, short-term acclimation to HL, and on the redox homeostasis of *Arabidopsis thaliana* leaves was assessed here. The dwarf phenotype displayed by mutants with high SA content (*cpr1-1*, *cpr5-1*, *cpr6-1*, and *dnd1-1*) was less pronounced when these plants were grown in HL, suggesting that the inhibitory effect of SA on growth was partly overcome at higher light intensities. Moreover, higher SA content affected energy conversion processes in low light, but did not impair short-term acclimation to HL. On the other hand, mutants with low foliar SA content (NahG and *sid2-2*) were impaired in acclimation to transient exposure to HL and thus predisposed to oxidative stress. Low and high SA levels were strictly correlated to a lower and higher foliar H_2O_2 content, respectively. Furthermore high SA was also associated with higher GSH contents, suggesting a tight correlation between SA, H_2O_2 and GSH contents in plants. These observations implied an essential role of SA in the acclimation processes and in regulating the redox homeostasis of the cell. Implications for the role of SA in pathogen defence signalling are also discussed.

Key words: *Arabidopsis*, cross tolerance, defence reactions, glutathione, hydrogen peroxide, light acclimation, photooxidative stress, photosynthesis, redox signalling, salicylic acid.

Introduction

In the natural environment plants are confronted by a multitude of biotic and abiotic stress factors. These environmental challenges must be perceived, integrated, and signalled in order to achieve a successful acclimation that will secure survival and reproduction. Light energy capture and carbon fixation by photosynthesis are the key determinants for plant life. However, light is among the most strongly fluctuating of the environmental parameters. Thus plants have evolved a highly plastic and dynamic regulatory system to maintain optimal rates of CO_2 fixation over a large range of light intensities.

Even under optimal environmental conditions, a portion of the light energy captured by the photosynthetic apparatus cannot be used to fix CO_2 or be dissipated safely as heat, but is diverted to the formation of reactive oxygen species (ROS; Asada, 1999; Ivanov and Khorobrykh, 2003). Besides fluctuations in light intensity, other environmental

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Abbreviations: APX, ascorbate peroxidase; CAT, catalase; DW, dry weight; EL, excess light; FW, fresh weight; GPX, glutathione peroxidase; GR, glutathione reductase; HL, high light; LL, low light; lN_2, liquid nitrogen; ROS, reactive oxygen species; SD, standard deviation; SA, salicylic acid; sHL, short-term HL treatment; SOD, superoxide dismutase; wt, wild type.

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factors such as changes in temperature, CO_2 availability, and water status can impair CO_2 fixation and increase the rate of ROS formation (van Rensen and Govindjee, 1999; Paul and Foyer, 2001; Tsonev *et al*., 2003). In many of these stress situations, adjusting stomatal conductance is a critical factor for the successful acclimatory response of the plant (Fryer *et al*., 2003; Mateo *et al*., 2004). Closing the stomata results in a rapid decline of the internal CO_2/O_2 ratio, thus enhancing photorespiration and concomitant H_2O_2 production (Wingler *et al*., 2000).

ROS are used as signalling molecules in light acclimation processes (Karpinski *et al*., 1999). However, excess ROS can induce oxidative damage to a multitude of cellular components (Apel and Hirt, 2004). Besides photosynthesis and photorespiration, additional sources of ROS in plants are oxidative mitochondrial respiration, peroxisomes, plasma membrane-localized NADPH oxidases, and cell wall peroxidases (Del Rio *et al*., 2002; Apel and Hirt, 2004). To counteract the oxidative pressure imposed by ROS formation, plants possess a multi-level antioxidant system, consisting of low-molecular-weight antioxidants like ascorbate and glutathione, as well as a multitude of ROS scavenging enzymes. Reduced glutathione (GSH) can react directly with ROS to detoxify them or can act as the substrate for ROS-scavenging glutathione peroxidases and glutathione-*S*-transferases (GPXs and GSTs; Ghezzi *et al*., 2005). Moreover GSH is used to regenerate oxidized ascorbate and oxidized thiol groups of proteins (Noctor *et al*., 2002). Oxidized glutathione (GSSG) is regenerated by glutathione reductase (GR) in an NADPH consuming reaction activated by the substrate (Wingsle and Karpinski, 1996). Glutathione is present in plant cells in millimolar concentrations and it is therefore regarded as a key determinant of the cellular redox status (Mullineaux and Rausch, 2005). Glutathione biosynthesis, concentration, and redox status were recently identified as the link between SA accumulation and Npr1-dependent induction of pathogen defence genes and were found to be involved in the regulation of genes coding for components of the antioxidant defence systems (Mou *et al*., 2003; Ball *et al*., 2004; Gomez *et al*., 2004; Senda and Ogawa, 2004). The overall extent of redox regulation of stress perception, signalling, and defence is just emerging (Foyer and Noctor, 2005).

Due to their agronomic relevance, pathogen defence mechanisms are among the most intensely studied environmental responses in plants. However, before this knowledge can be used in agronomy, the interdependence of pathogen defence and acclimation to abiotic stress needs to be determined. Most characterized mutants displaying constitutive pathogen resistance accumulate salicylic acid (SA; Jirage *et al*., 2001). *Constitutive expresser of pathogenesis related proteins* (*cpr*) mutants were identified in a screen for constitutive activation of the pathogen responsive BGL2/PR2 promoter (*cpr1-1*, *cpr5-1*, *cpr6-1*; Bowling *et al*., 1994, 1997; Clarke *et al*., 1998). *Cpr1* was mapped to an R-gene cluster on chromosome 4, thus representing the initial step of pathogen recognition (Stokes and Richards, 2002). Cpr5 is a membrane protein of unknown function and mutations in *Cpr5* have pleiotropic effects on the regulation of cell death, cell elongation, and trichome development (Kirik *et al*., 2001; Yoshida *et al*., 2002). *Cpr6* has not yet been identified molecularly, but has been genetically defined as a negative regulator of several defence pathways (Clarke *et al*., 2000). The *dnd1-1* (*defence no death*) mutation affects a cyclic nucleotide gated cation channel (AtCNGC2) and induces constitutive SA-dependent pathogen resistance in the absence of hypersensitive cell death (Yu *et al*., 1998; Clough *et al*., 2000). On the other hand, a mutant defective in SA synthesis (*sid2-2*) and transgenic NahG plants expressing a bacterial salicylate hydroxylase both display impaired pathogen defence (Bowling *et al*., 1994; Nawrath and Métraux, 1999; Wildermuth *et al*., 2001).

The effects of SA deficiency or constitutive SA accumulation on pathogen response and on cold or heat tolerance, flowering, and reproductive fitness have been reported recently (Clarke *et al*., 2004; Heidel *et al*., 2004; Martinez *et al*., 2004; Scott *et al*., 2004; Larkindale *et al*., 2005). However, little is known on the effects of SA levels on light acclimation and photo-oxidative stress tolerance. Moreover, all mutants with constitutive SA accumulation are retarded in growth, but so far it is unclear if this is a consequence of resource-consuming defence mechanisms or deteriorated energy capture and photosynthesis. In this study an analysis is presented of the influence of constitutively high or low levels of SA on photosynthesis and short-term acclimation of plants to high light as well as on redox homeostasis and the antioxidant system.

Materials and methods

Plant growth conditions

Arabidopsis thaliana (L.) Heynh., ecotype Col-0 (referred to as wild type, wt) or Landsberg *erecta* (L*er*) and derived mutants were cultivated in short day (9 h light period, 22/18 °C day/night temperature) on potting soil (Topstar-Economa Garden AB, Sweden) complemented with a thin layer of autoclaved clay. For the first 2 weeks, plants were kept in a growing room with mixed fluorescence tubes (L30W/77-fluora and L30W41-827 lumilux, OSRAM, Berlin, Germany), at a light intensity of 100 ± 20 µmol m^{-2} s^{-1} (LL). Two-week-old plants were repotted into 9 cm diameter pots and transferred to climate chambers (Conviron, Manitoba, Canada) with mercury bulbs and light intensities of either 100 ± 20 µmol m^{-2} s^{-1} (LL) or 450 ± 50 µmol m^{-2} s^{-1} (HL), 22/18 °C and a relative humidity of 50%. Fresh weight and hypocotyl diameters were determined directly on excised rosettes, for dry weight determination, the excised rosettes were dried for 3 d at 80 °C.

Light experiments and chlorophyll *a* fluorescence measurements

During short-term HL stress (sHL) experiments, 4–5-week-old Col-0 and mutant plants grown in LL were fully exposed to

750 ± 50 μmol m^{-2} s^{-1} with an extra light source in a light modulated cabinet for 3 h. Temperature and humidity for high light experiments were the same as in short day conditions. Chlorophyll *a* fluorescence was analysed immediately before and after the high light treatment. Plants were kept for 15 min in darkness to determine F_0 and F_m and then exposed for 10–15 min to actinic light (30 μmol m^{-2} s^{-1}) to determine F'_s and F'_m. The procedure and the derived photosynthetic parameters were recorded as described by Barbagallo *et al*. (2003) using a FluorImager and its associated software (Technologica Ltd, Colchester, UK). Calculations for maximum quantum efficiency of PSII ($(F_m–F_0)/F_m$), operating quantum efficiency of PSII $\Phi PSII = (F'_m - F'_s)/F'_m$, and non-photochemical quenching ($NPQ = (F_m - F'_m)/F'_m$ were carried out as described by Baker *et al.* (2001). Chlorophyll *a* fluorescence terminology is explained in detail elsewhere (Krause and Weis, 1991; Maxwell and Johnson, 2000).

Gas exchange and starch content

Gas exchange rates were derived from light response curves measured with a portable gas exchange system (Li-Cor 6400; Li-Cor, Lincoln; NE) equipped with an *Arabidopsis* chamber. Daytime respiration and maximal CO_2 assimilation were calculated using the software supplied with the instrument. Analysis of light response curves showed that the release of CO_2 measured at <0.5 μmol m^{-2} s^{-1} following light exposure was a reliable estimate of daytime respiration. Values were taken when a steady rate was recorded, which was 1–5 min after the end of illumination, depending on the genotype of the plant and the intensity of the preceding illumination (250 or 300 μmol m^{-2} s^{-1}). For all lines, saturation (A_{max}) was reached at 1200 μmol m^{-2} s^{-1} and no significant degree of photoinhibition was observed during the measurements. Stomatal conductance was determined at different light intensities in the linear range of light response curves. Data show representative responses at 250 μmol m^{-2} s^{-1} (LL). Leaves were illuminated with an external white light source ranging from 0–1200 μmol m^{-2} s^{-1} (halogen bulb HLX, 50 W, Osram, Germany). CO_2 concentration and temperature were maintained at 380 ppm and 25 °C, respectively. Leaf vapour deficits were maintained between 0.5 and approximately 1.6 kPa. For visualization of starch content, excised leaves were cleared in 80% ethanol at 60 °C, stained in aqueous 0.2% (w/V) KI_3 solution and de-stained in water until brown staining by iodine adsorbed to cell walls had nearly disappeared.

Quantification of H_2O_2, GSH and SA

Total foliar hydrogen peroxide was determined based on Guilbault *et al*. (1967) and as described by Jimenez *et al*. (2002), except that 100 mg of *Arabidopsis* leaf tissue per 1 ml of extraction medium was used per assay. A total of 100 mg fresh plant material was extracted immediately in 1 ml of ice-cold buffer containing 50 mM HEPES, pH 7.5, 50 mM $MgCl_2$ and 1 mM EDTA using a mortar and pestle. The extract was centrifuged at 14 000 *g* and the supernatant was used directly for analysis of H_2O_2. Glutathione was extracted from leaf material snap-frozen in liquid nitrogen (lN_2) and stored at −80 °C. The material was ground with a mortar and pestle in lN_2 and glutathione was extracted with 10 ml g^{-1} of 0.1 M HCl and the extract was incubated on ice for 30 min. After centrifugation at 14 000 *g* for 10 min, the supernatant was used to measure the content of glutathione (as GSH) by HPLC using the monobromobimane derivatization method (Newton *et al*., 1981) as described by Creissen *et al*. (1999). GSSG content was calculated from the difference between total glutathione from DTT-treated samples and GSH from non-DTT-treated samples. Determination of SA was performed as described by Meuwly and Métraux (1993) from leaves snap-frozen in lN_2.

Application of SA and GSH

Feeding of GSH or SA at concentrations of 5 mM or 0.5 mM, respectively, was performed by injecting 100 μl of buffered GSH or SA with a 1 ml disposable syringe pressed firmly to the lower epidermis into the leaf apoplast of attached mature rosette leaves of 5–6-week-old LL-cultivated plants. After the specified times, leaves were pooled from several plants and snap-frozen in lN_2. As a control, buffer alone or water was injected.

Enzyme activities

For the determination of enzyme activities, plant material was snap-frozen in lN_2 and stored at −80 °C until grinding and extraction. The activity of catalase was measured spectrophotometrically according to Aebi (1984) by monitoring H_2O_2 disappearance at 240 nm in 50 mM phosphate buffer pH 7.0 containing initially 13 mM H_2O_2. SOD activities were estimated by in-gel activity staining according to Beauchamp and Fridovich (1971). Ground leaf material was resuspended in 10 ml g^{-1} extraction buffer (100 mM potassium phosphate pH 7.5, 1 mM DTT, 3 mM EDTA, 0.4% (w/v) Triton X-100). Per lane, 20 μg of total protein was separated on native polyacrylamide gels. After staining, digital images of the gels were processed with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA). GR activity was determined as described in Connell and Mullet (1986). Activity of the enzyme is assessed in an NADPH containing HEPES buffer by the decrease in absorbance at 340 nm as NADPH is oxidized. Total protein content of the extracts was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA)

Statistical analysis

The Student's *t* test was used to analyse differences between the mutants or treatments. Significant differences were indicated for $P \leq 0.05$.

Results

Growth inhibition by high SA levels in LL is partially reverted in high light

Mutants with constitutively high levels of SA are severely retarded in growth in low light (LL; Fig. 1; Jirage *et al.*, 2001). The growth parameters for the low SA line *sid2-2* and for the SA accumulating mutants *dnd1-1*, *cpr1-1*, *cpr5-1* and *cpr6-1* were assessed and compared with the wild type (wt) Col-0 (Fig. 1). To determine if retarded growth was due to energy limitation or a consequence of altered developmental programmes, the growth parameters of these plants grown under either LL or high light (HL) for several weeks were compared. In LL, it took wt plants about 7 weeks to reach the same fresh weight (FW) as in 6 weeks in HL (Fig. 1A, B). At the same time points, dry weight (DW) and lateral growth of the hypocotyl for wt plants were 2 times higher in HL (Fig. 1C). The inability to synthesize SA through the isochorismate pathway in *sid2-2* or constant SA breakdown in NahG (data not shown) did not cause any major changes in growth parameters in LL or HL. However, all lines with constitutive high levels of SA showed significantly reduced biomass accumulation (Fig. 1B) in LL. Differences in biomass production between mutants and wt plants became less pronounced at higher

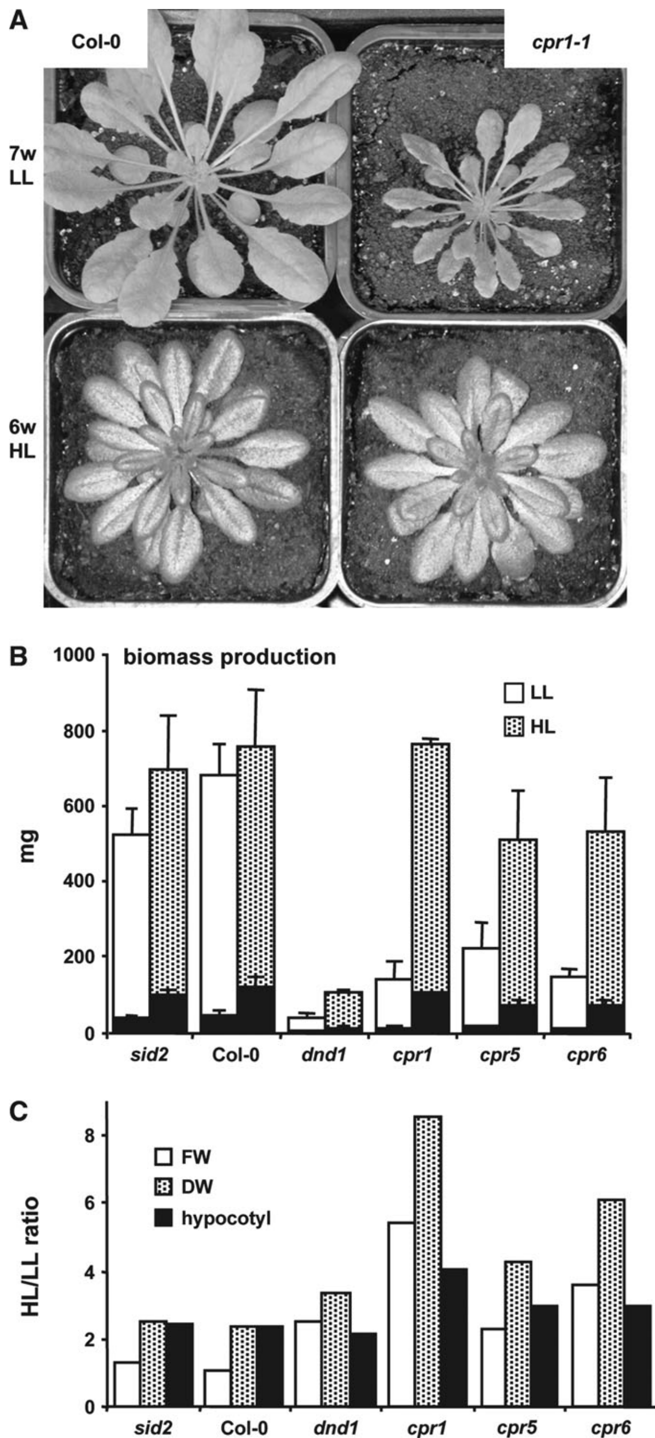

Fig. 1. The inhibitory effect of SA on growth is partially overcome at higher light intensities. Wt Col-0, SA-deficient *sid2-2* and SA-accumulating mutants were grown for 7 weeks in LL (100 μmol m^{-2} s^{-1}) or 6 weeks in HL (450 μmol m^{-2} s^{-1}) in short day. (A) Representative pictures of Col-0 and *cpr1-1* grown in LL and HL. (B) Biomass accumulation in mutants with low or constitutively high levels of SA. Open bars: FW after 7 weeks in LL, shaded bars: FW after 6 weeks in HL, solid bars: corresponding dry weight. Values presented are mean ±SD from five rosettes. (C) HL/LL ratios of fresh weight, dry weight and hypocotyl diameter. Very similar values for growth parameters and HL/LL ratios were obtained from two further batches of independently grown plants.

light intensities as indicated by higher HL/LL biomass ratios in SA-accumulating mutants (Fig. 1C). Interestingly, even the significantly lower FW/DW ratios in *dnd1-1* and *cpr5-1* in LL were increased to the wt levels in HL (data not shown). The strongest response to increased light intensity was observed in *cpr1-1*, which frequently was severely dwarfed and developed curled leaves in LL but reverted to almost normal growth in HL (Fig. 1A). We concluded that the inhibitory effect of SA on growth was partially overcome at higher light intensities.

Altered foliar levels of SA impair photosynthetic electron transport and energy dissipation mechanisms of photosynthesis

To investigate whether reduced growth and biomass accumulation of mutants with altered SA content in LL was caused by disturbances in light energy capture or photosynthetic processes, performance of the photosynthetic machinery was monitored by chlorophyll *a* fluorescence in LL and after short-time HL stress (sHL; 750 μmol m^{-2} s^{-1} for 3 h). Imaging of the maximum photochemical efficiency (F_v/F_m) as an indicator of damage to the PSII reaction centres revealed that, in LL conditions, *cpr5-1* and *dnd1-1* already had a lower photosynthetic capacity (Fig. 2A). The decrease in F_v/F_m in *cpr5-1* and *dnd1-1* was accompanied by increased heat dissipation of absorbed light energy (*NPQ*; Fig. 2B). All the mutant lines except *cpr6-1* displayed higher *NPQ* than wt. This suggested that altered SA levels impaired photochemical energy conversion and induced increased thermal dissipation. Reduced SA levels, either by constant breakdown or disturbed biosynthesis, as well as increased SA caused higher photoinhibition during sHL exposure, with the exception of *cpr6-1* where the reduction in F_v/F_m was not significant (Fig. 2A). Similarly, all analysed lines showed a lower operating efficiency of PSII (ΦPSII) than wt in LL conditions and after sHL (Fig. 2C). This was also reflected in the smaller proportion of open PSII reaction centres in all lines (q_P, data not shown). After HL treatment, ΦPSII was increasing significantly in the high SA content lines and wt, but not in the low SA content lines, suggesting an impairment of light acclimatory processes in the latter. The amount of anthocyanins after sHL treatment was also higher in NahG and *sid2-2*, suggesting higher oxidative stress in these lines (data not shown).

Among the SA-accumulating lines, *cpr6-1* showed the weakest differences to wt in LL or after sHL exposure (Fig. 2), thus we decided to test the excess light (EL) tolerance of this line. After 1 h exposure to EL (2000±200 μmol m^{-2} s^{-1}) mesophyll areas of *cpr6-1* showed a similar extent of photoinhibition compared with wt (Fig. 3A). In the vascular tissue, however, *cpr6-1* was much more sensitive to EL than wt. Even in wt, the vascular tissue was more susceptible to photoinhibition than the mesophyll area. After 1 h recovery, F_v/F_m in *cpr6-1* vascular tissue

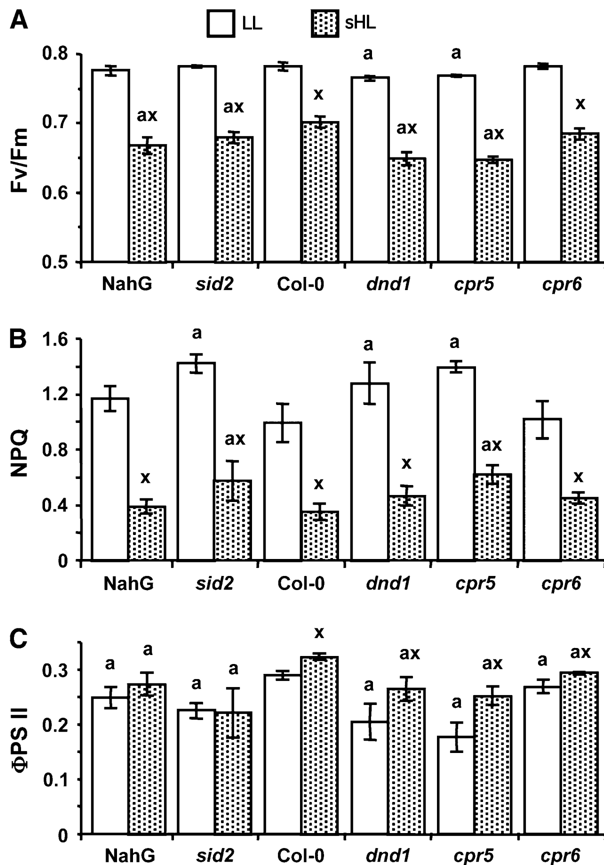

Fig. 2. Photosynthesis and short-term acclimation to HL are impaired in mutants with altered SA levels. Chlorophyll *a* fluorescence imaging was used to determine photosynthetic performance of wt and SA-deficient or -accumulating lines in LL and after 3 h exposure to HL stress (sHL, 750 μmol m^{-2} s^{-1}). (A) Maximum photochemical efficiency (F_v/F_m) as a measure of the proportion of functional PSII reaction centres. (B) Non-photochemical quenching (*NPQ*) as a measure of the proportion of absorbed energy that is dissipated as heat. (C) Operating efficiency (ΦPSII) as a measure of the quantum efficiency of photosynthetic electron transport in the light acclimated state. Photosynthetic parameters were averaged over the whole rosette area; values given are mean ±S.D. from at least four rosettes. a and x indicate significant difference to wt and LL, respectively.

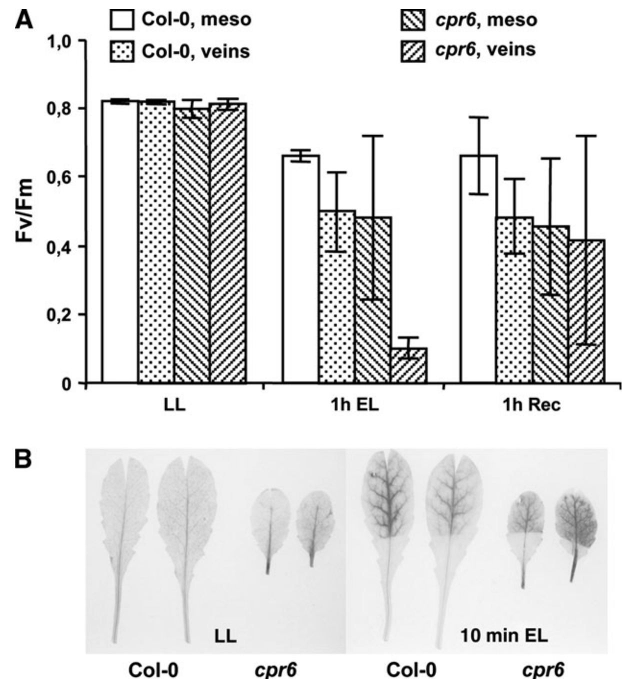

Fig. 3. Vascular tissue of *cpr6-1* was more susceptible to light stress than mesophyll area. (A) Rosettes of 6-week-old Col-0 and *cpr6-1* plants were exposed for 1 h to excess light stress. After 10 min dark adaptation, F_0 and F_m were determined separately for mesophyll area and vascular tissue. Calculation of F_v/F_m revealed the the strongest photoinhibition in the vascular tissue in *cpr6-1*. Error bars indicate SD, *n*=3. (B) Mature leaves of Col-0 and *cpr6-1* were infiltrated in very low light (approximately 30 μmol m^{-2} s^{-1}) with 3,3′-diaminobenzidine. After infiltration was complete, the distal parts of the leaf blades were exposed to EL for 10 min. Note that the vascular tissue of *cpr6-1* displayed strong staining for H_2O_2 even without EL exposure.

nearly recovered to wt levels (Fig. 3A). Staining with 3,3′-diaminobenzidine revealed higher levels of H_2O_2 in the vascular tissue of *cpr6-1* already in LL. Exposure to EL caused an increase of H_2O_2 in both wt and *cpr6-1* within 10 min, most prominently in the vascular tissue (Fig. 3B).

From the chlorophyll *a* fluorescence analysis it was concluded that both low and high levels of SA affect photosynthetic electron transport and dissipation mechanisms. SA-deficiency impaired the capability to acclimate during sHL, whereas plants with high SA levels acclimated similarly to wt plants.

Gas exchange but not starch content is correlated to biomass accumulation

Reduced efficiency of photosynthetic energy capturing was observed in all mutants with altered SA levels, but only SA-accumulating plants displayed reduced growth. Thus gas exchange capacity and CO_2 fixation were analysed as other parameters potentially limiting for growth (Fig. 4; see also supplementary Fig. 1 at JXB online). Stomatal conductance was determined at 250 μmol m^{-2} s^{-1}, in the linear range of light response curves. Stomatal conductance was generally lower in SA-accumulating lines whereas no significant differences were observed in lines with low SA levels (Fig. 4A). Similar results were obtained at other light intensities (data not shown). The lower stomatal conductance in *dnd1-1*, *cpr5-1*, and *cpr6-1* was paralleled by a lower maximal CO_2 assimilation (Fig. 4B) but also a higher daytime respiration in *dnd1-1* and *cpr5-1* (Fig. 4C). CO_2 assimilation at light intensities similar to the growing conditions showed similar trends, although the variation between individual plants of the mutant lines was higher (see supplementary Fig. 1 at JXB online). To detect whether impaired stomatal conductance and decreased maximal CO_2 assimilation were reflected by lower accumulation of photoassimilates, the starch content of the mutants with altered SA levels was analysed. Consistent with the results of gas exchange analysis, mature rosette leaves of *dnd1-1* had a very low starch content at the end

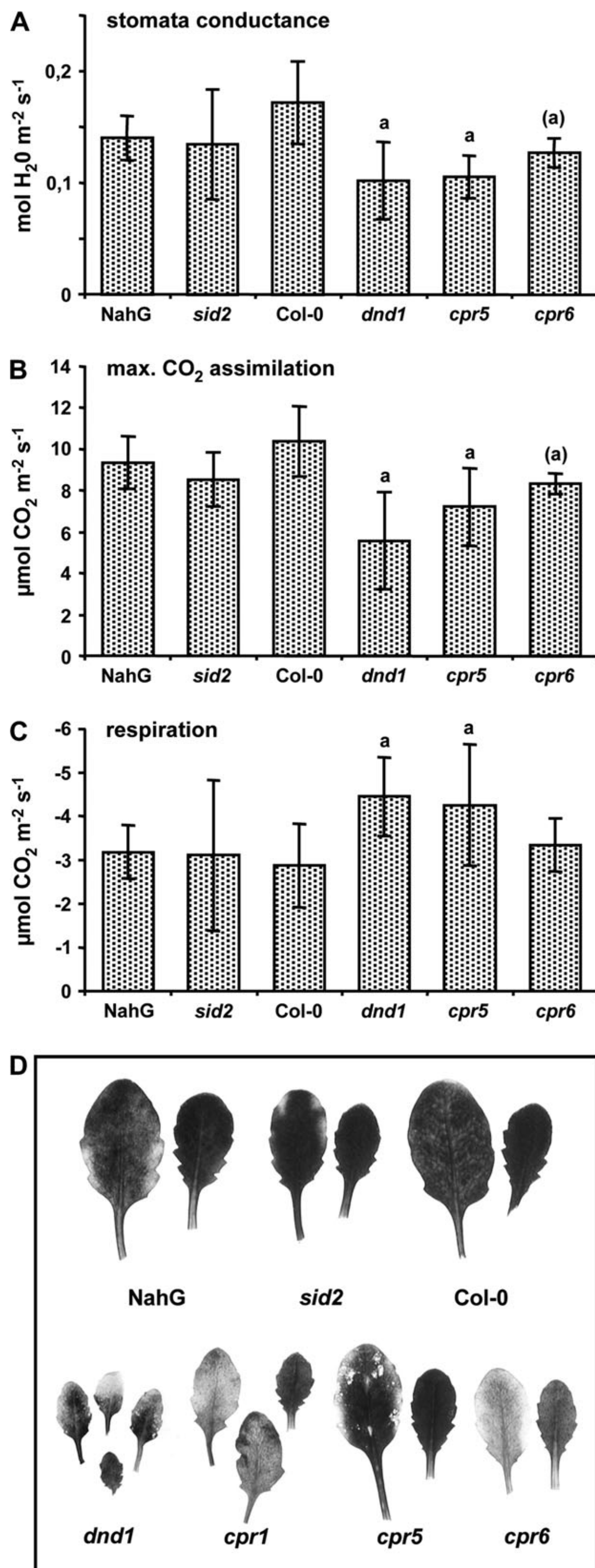

Fig. 4. Gas exchange and starch accumulation. (A) Stomatal conductance at 250 μmol m^{-2} s^{-1}. (B) Maximum CO_2 assimilation rates determined at 1200 μmol m^{-2} s^{-1}. (C) Daytime respiration calculated from CO_2 release at <0.5 μmol m^{-2} s^{-1}. Values in (A–C) are the mean ±SD of ⩾3 individual plants from two independent batches. a: indicates significant differences to wt. (a) Indicates that differences for *cpr6-1* were significant, but only three plants from a single batch were analysed. (D) Iodine staining for visualization of starch content. Pictures of representative mature rosette leaves of non-bolting plants are shown. All results were confirmed by replicates with independent batches of plants.

of the light period. A similar phenotype was observed in *cpr1-1* and *cpr6-1*, whereas plants with low SA levels and surprisingly also *cpr5-1* had starch contents similar to wt (Fig. 4D). Thus growth retardation by high SA levels in LL cannot solely be explained by impaired energy conversion and reduced gas exchange capacity.

SA induces ROS and glutathione accumulation

Limited gas exchange and impaired photochemistry are factors promoting oxidative stress (van Rensen and Govindjee, 1999; Paul and Foyer; 2001; Fryer *et al*., 2003). Therefore, the influence of SA levels on the content of H_2O_2 and the antioxidant glutathione was determined. All analysed *Arabidopsis* mutants with constitutive accumulation of SA had strongly increased H_2O_2 levels in LL (Fig. 5A). In lines with lower SA levels, H_2O_2 was decreased, indicating a strong correlation between SA levels and H_2O_2 content in the cell. SA-accumulating lines also had an increased content of GSH in LL, providing additional evidence for increased oxidative stress. However, the mutants were still able to maintain the same glutathione redox state as wt (Fig. 5B, C). Interestingly, deficiency of SA and lower H_2O_2 levels did not lead to significantly decreased GSH levels in LL, but in NahG, which constitutively degrades SA, the glutathione pool was more oxidized in LL conditions. After sHL treatment, the increase in GSH levels was lower in SA-deficient lines compared with wt or SA-accumulating lines, indicating a positive influence of SA on glutathione biosynthesis (Fig. 5). Exposure to sHL led to an oxidation of the glutathione pool in all analysed lines. However, *dnd1-1* and *cpr6-1* were able to maintain a significantly higher redox level (Fig. 5C). These findings demonstrate that constitutive pathogen resistance is not solely correlated with higher SA levels, but at the same time to higher GSH and H_2O_2 contents. Deficiency in SA however, results in lower H_2O_2 levels and an inability of the plant to increase the GSH level efficiently when it is exposed to sHL.

High SA content enhances catalase, Cu/Zn-superoxide dismutase, and glutathione reductase activities

Altered levels of ROS can result either from increased production or decreased scavenging. To distinguish between these two possibilities, the status of the antioxidant defence system in the SA-accumulating mutants was analysed. RT-PCR analysis of genes for key antioxidative genes did not reveal a strong and consistent correlation of SA, ROS, or GSH levels with gene expression (see supplementary Fig. 2 at JXB online). Since antioxidative

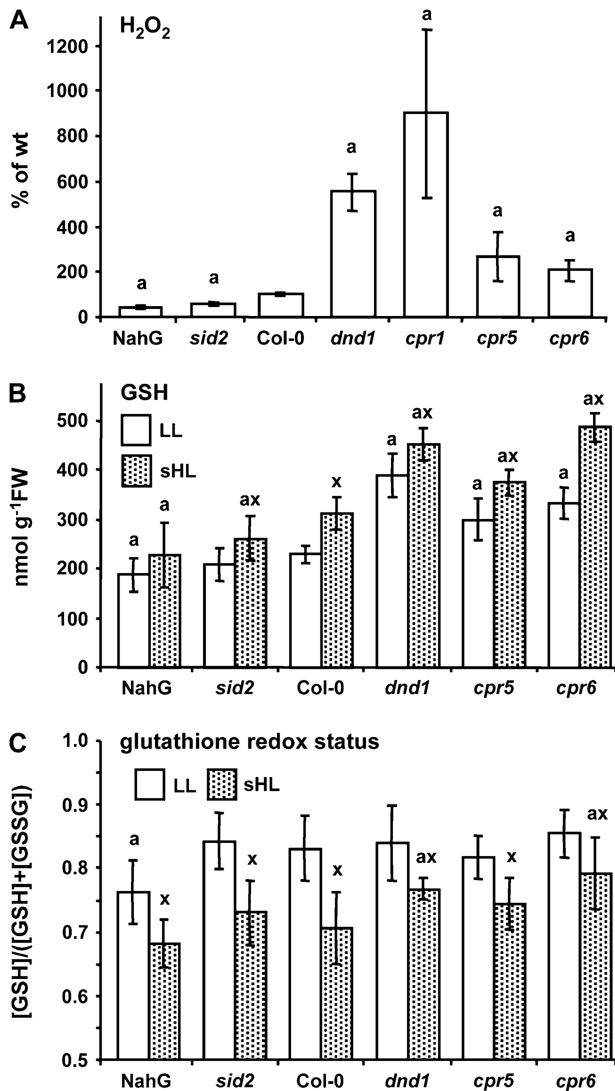

Fig. 5. Foliar accumulation of H_2O_2 and GSH in plants with constitutively high levels of SA. (A) Mutants with constitutive accumulation of SA had strongly increased H_2O_2 levels and in SA-deficient lines H_2O_2 was decreased, indicating a strong correlation between SA levels and H_2O_2 content in the cell (n=12 from three independent batches of plants). (B) SA-accumulating lines also had an increased GSH content in LL. Low SA and H_2O_2 levels in NahG were accompanied by decreased GSH levels in LL. After 3 h exposure to sHL, GSH levels were much lower in SA-deficient lines compared to wt or SA accumulating lines. (C) Redox status of the glutathione pool before and after 3 h sHL treatment. Glutathione redox potential was significantly lower in NahG in LL and significantly higher in *dnd1-1* and *cpr6-1* after sHL treatment (n=6 from two independent batches; bars represent mean ±SD; a and x: significantly different from wt and LL, respectively).

enzymes are often regulated post-transcriptionally, the activity of the major ROS-scavenging enzymes, catalases (CAT) and superoxide dismutases (SOD) was also measured. Inhibition of catalase activity by SA has been described before in the literature (Chen *et al.*, 1993). Surprisingly, under LL conditions, CAT activity was higher in SA-accumulating lines compared with wt, although the differences were significant only in *dnd1-1* and *cpr6-1* (Fig. 6A). It can be concluded that the accumulation of H_2O_2 observed in lines with high SA levels did not result from the inhibition of catalase activity. No differences to LL were observed after sHL treatment (data not shown), but after 1 h EL a 30–40% decrease in CAT activity was observed in all analysed lines independent of SA or H_2O_2 levels.

SODs convert highly reactive superoxide anions ($\cdot O_2^-$) to the more stable H_2O_2. The *Arabidopsis* genome contains eight genes for SODs belonging to three classes, induced with different kinetics during sustained stress conditions (Alscher *et al.*, 2002). MnSOD and FeSOD activities were the same in all analysed lines in LL or after sHL (data not shown). At noon, CuZnSOD activity was higher than in wt in NahG, *dnd1-1*, and *cpr6-1*, whereas *sid2-2* had slightly lower activity (Table 1). No difference was observed in *cpr5-1* plants. By the end of the light period, when SOD activities are highest, these differences were no longer observed (data not shown). Comparison with Fig. 5A indicates that H_2O_2 levels are correlated to SA and CAT activity, but not to SOD activity, thus conversion of $\cdot O_2^-$ by SODs does not seem to be the reason for altered H_2O_2 levels.

Since major changes in glutathione redox-state were not detected despite the high H_2O_2 levels in SA-accumulating lines, the activity of glutathione reductase (GR), which regenerates reduced GSH from oxidized GSSG was also analysed. In LL, GR activity was generally higher in the lines accumulating SA, indicating an increased oxidative pressure on the glutathione pool (Fig. 6B). Thus, maintenance of a normal cellular redox status in the presence of high SA and H_2O_2 levels seems to be at the expense of the NADPH-consuming GR-catalysed reaction. Lower SA content in NahG was accompanied by a lower GR activity. Low SA and H_2O_2 levels in NahG might prevent the activation of GR despite the lowered redox status of the glutathione pool (Figs 5, 6B).

Levels of SA and glutathione are physiologically coupled

From the observations of mutant lines that have adapted to their defect during development it was not possible to distinguish whether high glutathione content was a consequence of high SA levels or *vice versa*. Thus we decided to feed SA and glutathione directly into wt leaves under non-stressed conditions. Surprisingly, we found that SA and glutathione are inducing each other (Fig. 7A, B). Injection of physiological levels (0.5 mM) of SA into *Arabidopsis* leaves in LL caused an increase of both GSH and GSSG levels within 2 h. Glutathione levels were maximal at 4 h after SA application and started declining thereafter. A transient drop in the redox status of the glutathione pool after 2 h indicated the induction of oxidative stress by SA application. In the inverse experiment, SA levels were also responding to application of GSH in a very similar way. Free SA was significantly increased 2 h after

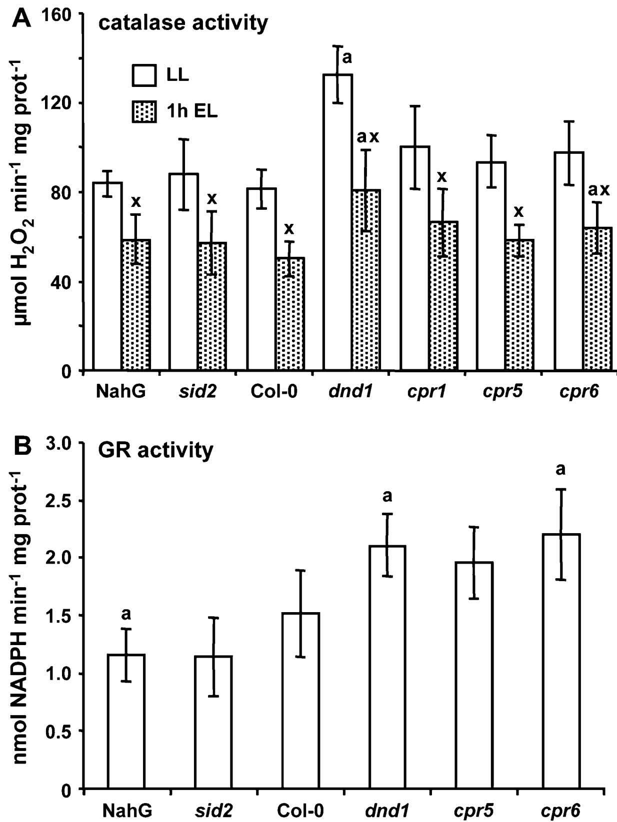

Fig. 6. Activity of key antioxidative enzymes. Enzyme activities were detected in total soluble protein extracts from mature leaves of 5–7-week-old plants. (A) Catalase activity prior to (LL) and after 1 h exposure to EL stress (2000±200 μmol m^{-2} s^{-1}). Values shown are the mean ±SD; *n*=6 samples from two independent batches of plants. (B) NADPH-dependent GR activity. Values are the mean ±SD; *n*=8 samples from two independent batches. (a and x: significantly different from wt and LL, respectively).

Table 1. *CuZnSOD activities*

SOD activity in extracts from LL-grown plants harvested between 13.00 h and 14.00 h was detected and quantified as described in the Materials and methods. The table gives the combined CuZnSOD activities relative to Col-0. Values are mean ±SD of seven samples from three independent batches of plants, 'a' indicates significant difference to Col-0.

Total CuZnSOD activity (relative to wt)	
Col-0	1.00
NahG	1.41±0.19 a
sid2-2	0.70±0.21 a
dnd1-1	2.53±0.79 a
cpr5-1	1.63±0.68
cpr6-1	2.64±1.05 a

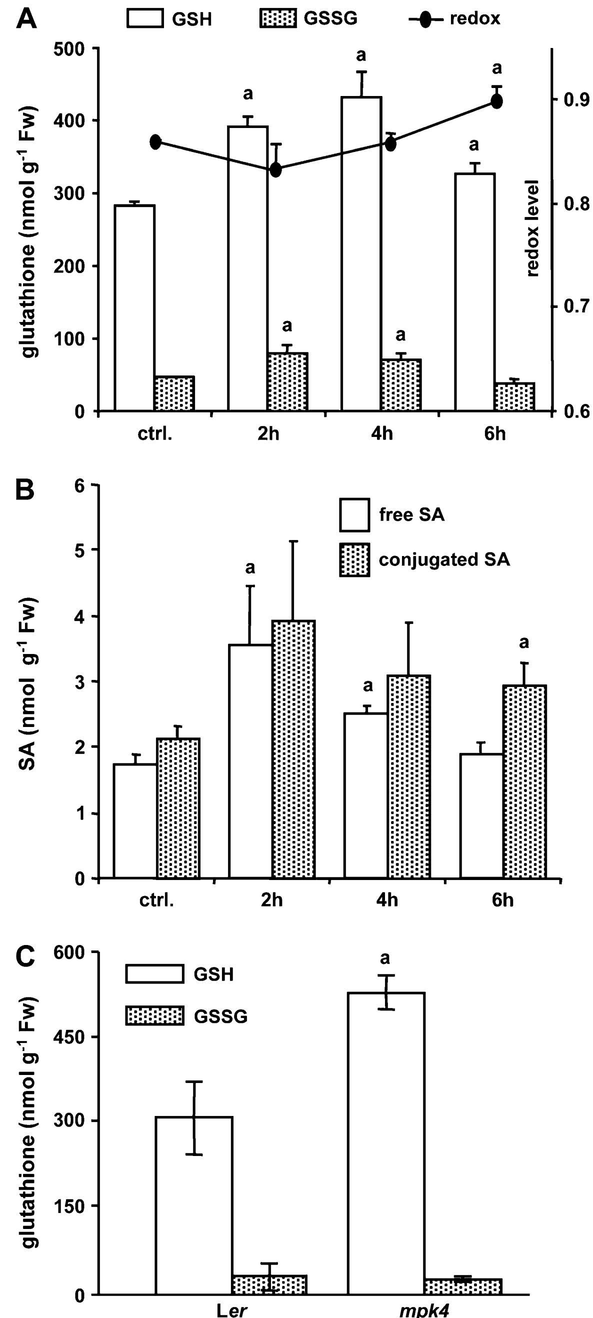

Fig. 7. SA and glutathione are interdependent. Injection of physiological levels of SA or GSH into mature rosette leaves of 6-week-old LL-grown Col-0 plants induced rapid changes in foliar GSH and SA content, respectively. (A) Levels of reduced GSH and oxidized GSSG after injection of 0.5 mM SA into mature rosette leaves. (B) Levels of free and conjugated SA after injection of 5 mM GSH. Values in (A) and (B) are the mean ±SD of four pooled samples of four leaves each. (C) Levels of GSH and GSSG in whole rosettes of 4-week-old LL-grown L*er* and *mpk4* plants. Values are the mean ±SD of three independent samples. (a: significantly different from untreated leaves or wt).

injection of 5 mM GSH into *Arabidopsis* rosette leaves (Fig. 7B). After 6 h, free SA levels were still increased, but had almost returned to the initial values. Conjugated SA also increased after 2 h but declined more slowly compared with free SA, possibly indicating removal of free SA by conjugation. No changes in SA or glutathione levels were observed after injection of water or buffer alone (data not shown).

To determine if high glutathione levels are a general symptom of SAR and SA-mediated resistance, the influence

of Mpk4, which was found to be a negative regulator of SA biosynthesis, was analysed. The *mpk4* mutant has constitutively high SA levels and subsequently enhanced pathogen resistance (Petersen *et al.*, 2000). Consistent with a close link between SA and glutathione, the *mpk4* mutant also had increased glutathione contents (Fig. 7C).

Discussion

SA, photosynthesis and plant growth in LL

Accumulation of SA is broadly accepted as a key signalling event in local and systemic acquired resistance against pathogens (Ryals *et al.*, 1995; Draper, 1997; McDowell and Dangl, 2000). Much less is known about the downstream events of perception and signal transduction. It is demonstrated in this paper that high levels of SA are tightly linked to further metabolic alterations, affecting photosynthetic performance and redox homeostasis in plants. Growth retardation in plants with constitutively elevated levels of SA had been observed previously, but the cause remained unclear (Bowling *et al.*, 1994; Mauch *et al.*, 2001). A set of mutants and transgenic plants was chosen (NahG, *sid2-2*, *dnd1-1*, *cpr1-1*, *cpr5-1*, *cpr6-1*), of which clear alterations of SA levels are well documented (Nawrath and Métraux, 1999; Jirage *et al.*, 2001). Plants with constitutively high SA levels showed decreased maximum efficiency of PSII (F_v/F_m; *dnd1-1*, *cpr5-1*), reduced quantum yield of PSII (ΦPSII; *dnd1-1*, *cpr5-1*, and *cpr6-1*), increased thermal dissipation of absorbed light energy (*NPQ*) and reduced stomatal conductance in LL (Figs 2, 4). The values for F_v/F_m in Col-0 were slightly lower than usually observed in unstressed plants (0.78, usually around 0.83), although no indication for stress in these plants was observed. This is probably due to the integration over the whole rosette rather than measuring perfectly oriented leaf blades and this should affect the measurements of all plants in an equal manner. Spontaneous lesion formation is observed in *dnd1-1* and *cpr5-1* in an age-dependent manner. Plants without visible lesions were used, but microscopic lesions might still explain the decreases in F_v/F_m.

Closure of stomata in response to the external application of SA has been observed previously (Manthe *et al.*, 1992; Chen *et al.*, 1993; Rao *et al.*, 1997; Shirasu *et al.*, 1997; Lee, 1998; Mateo *et al.*, 2004). However, many of these reports have to be interpreted with caution, since it is not stated whether appropriate measures were taken to buffer the acidity of free SA, which in our experiments was harmful to *Arabidopsis* leaves already at 0.5 mM SA (data not shown). Interestingly, *dnd1-1* and *cpr5-1* also displayed higher dark respiration rates and lower maximum CO_2 assimilation. However, accumulation of photoassimilates as starch strongly decreased in *dnd1-1*, *cpr1-1*, and *cpr6-1*, but not *cpr5-1*, possibly indicating changes in assimilate partitioning (Fig. 4).

SA and short-term acclimation to high light

Oxidative stress is generated by high light intensities and *Arabidopsis* plants acclimated to HL were shown to contain higher levels of SA and GSH (Karpinski *et al.*, 2003; Ogawa *et al.*, 2004). It is shown here that increased or reduced levels of SA impaired photosynthesis in a different way. Mutants with high foliar SA content had lower maximum and operating efficiency of PSII when compared with wt (Fig. 2). However, they were able to increase GSH content further and to improve the operating efficiency of PSII during acclimatory responses to sHL (Fig. 2). Consistent with the compatibility of high SA levels with acclimation to HL, HL/LL biomass ratios were bigger for *dnd1-1*, *cpr1-1*, *cpr5-1*, and *cpr6-1*. Biomass accumulation of these lines in HL was still lower than in wt (Fig. 1). The inhibitory effects of foliar SA, glutathione, and H_2O_2 accumulation observed in LL seem to be less pronounced in HL when the metabolism is adjusted to higher levels of these compounds.

Decreased levels of SA and H_2O_2 observed in *sid2-2* and NahG plants slightly impaired PSII operating efficiency and enhanced thermal energy dissipation in LL (Fig. 2). However, no impairment of growth in LL or HL or reduced levels of starch were observed under the conditions tested here (Figs 1, 4). NahG and *sid2-2* plants were unable to increase photosynthetic electron transport efficiency or GSH levels to the same extent as wt plants during exposure to sHL. This suggested that wt levels of SA and/or H_2O_2 are required to induce proper light acclimation responses (Figs 2, 5). H_2O_2 was shown to be an essential element in local and systemic acclimation to light in *Arabidopsis* (Karpinski *et al.*, 1999). The lower H_2O_2 content in *sid2-2* and NahG may account for their impaired capability to acclimate rapidly to HL (Fig. 2). Stronger accumulation of anthocyanins in *sid2-2* and NahG also indicated that HL exposure manifested a more severe stress for these plants.

Photosynthetic electron transport characteristics, H_2O_2, glutathione biosynthesis and changes in leaf water status have been implicated in the signalling pathway regulating *Apx2* expression (Karpinski *et al.*, 1997, 1999; Fryer *et al.*, 2003; Ball *et al.*, 2004). A lower threshold for *Apx2* induction was consistently observed in mutants with elevated SA levels (see supplementary Fig. 2 at JXB online).

It can be concluded that controlled SA levels are important for optimal photosynthetic performance and growth under different light conditions.

SA levels are paralleled by H_2O_2

Applications of H_2O_2 and SA have shown that these two compounds induce each other and could thus form a feed-forward loop (Leon *et al.*, 1995; Bi *et al.*, 1995; Rao *et al.*, 1997; Shirasu *et al.*, 1997). It is, however, noteworthy that treatment of leaves with H_2O_2 did not produce all the SA-mediated events (Rao *et al.*, 1997). It is shown

here that endogenous contents of SA are correlated to the levels of foliar H_2O_2 independent of the mutation or transgene causing the alterations in SA content (Fig. 5A). Surprisingly, this correlation was observed for both increased and decreased levels of SA, with lines deficient in SA accumulating only 50% of H_2O_2 in LL compared with wt. These results confirm a tight physiological link between SA and H_2O_2, but also demonstrate the existence of mechanisms to break the feed-forward loop and limit SA and H_2O_2 accumulation. The precise source(s) of H_2O_2 remain to be elucidated. However, increased photosynthetic and photorespiratory ROS production are supported by SA-induced photoinhibition and closure of stomata (Figs 2, 5A; Manthe *et al.*, 1992; Lee, 1998; Mori *et al.*, 2003, Mateo *et al.*, 2004). Enhanced activity of SODs in *dnd1-1* and *cpr6-1* may also participate in the formation of H_2O_2 (Fig. 6). Obviously, ROS-producing mechanisms involved in the oxidative burst during the hypersensitive response, i.e. membrane-bound NADPH oxidases, cell wall peroxidases, and amine oxidases have to be considered as well (Desikan *et al.*, 1996; Blee *et al.*, 2001; Bolwell *et al.*, 2002; Torres *et al.*, 2003). In addition, SA was found to bind and inhibit catalases, but the relevance of this effect *in vivo* was discussed controversially (Chen *et al.*, 1993, 1997; Sanchez-Casas and Klessig, 1994; Conrath *et al.*, 1995; Ruffer *et al.*, 1995; Durner and Klessig, 1996). A higher CAT activity was detected in mutants with elevated SA levels, supporting the concept that SA induces increased production of H_2O_2 rather than reduced scavenging by CAT activity (Fig. 6).

Confirming the assumption that photosynthesis and photorespiration constitute the main ROS source in mutants with constitutively elevated SA levels, spontaneous lesion formation in *dnd1-1* and *cpr5-1* is enhanced at low humidity (i.e. when stomata are closed) and in high light intensities (Jirage *et al.*, 2001). These results suggest that basal foliar levels of SA and H_2O_2 in wt plants are adjusted to a level that allows optimal growth and at the same time fast and efficient induction of defence responses.

Interdependence of foliar SA and glutathione

Through its high intracellular concentration, glutathione constitutes one of the major components of the antioxidant defence system. Glutathione is also the major determinant of the cellular redox status in plants (Blokhina *et al.*, 2003; Foyer and Noctor, 2005; Mullineaux and Rausch, 2005). Higher GR activity observed in the lines with high SA levels indicated an increased oxidative pressure on the glutathione pool already in LL (Fig. 6B). Increased NADPH consumption by GR to maintain the adequate foliar redox-state in the lines with high SA levels might be diverted from primary metabolic processes and thus contribute to the reduced biomass accumulation. Reduced GR activity, despite a more oxidized glutathione pool in NahG, could indicate impaired perception or transduction of redox-related signals.

Translation but not transcription of γ-glutamylcysteine synthetase (*Gsh1*), the rate-limiting enzyme for glutathione biosynthesis in LL conditions, is induced during oxidative stress, whereas in weak light (<100 μmol m^{-2} s^{-1}) photosynthetic electron transport rates seem to limit glutathione synthesis (Creissen *et al.*, 1999; Xiang and Oliver, 1998; Noctor *et al.*, 2002; Ogawa *et al.*, 2004). Increased levels of glutathione detected in *Arabidopsis* lines with constitutive SA accumulation suggest a physiological coupling between SA and glutathione levels, potentially mediated by ROS (Figs 5, 7). This coupling is furthermore confirmed by the higher GSH content observed in the SA-accumulating mutants *mpk4-1* and *npr1-1* and after feeding of SA to wt plants (Fig. 7C; Petersen *et al.*, 2000; PM Mullineaux, unpublished results). A positive correlation between SA and GSH levels was also observed in the analysis of Ni tolerance of several Brassicaceae species (Freeman *et al.*, 2005).

Constitutive SA accumulation in the *cpr* and *dnd1* mutants affected only the glutathione pool size, but not its redox status, whereas feeding of SA to leaves induced a transient oxidation of the glutathione pool. Similar changes in the glutathione pool size and its redox status were observed after feeding *Arabidopsis* plants with SA or functional analogues through the root, although this happened with slower kinetics (Mou *et al.*, 2003). Mou *et al.* concluded that a SA-induced shift towards reduced glutathione was required to induce Npr1-dependent *Pr1* gene expression. Our observation of normal glutathione redox status in mutants with constitutive *Pr1* gene expression and similar findings by Senda and Ogawa (2004) in the *lesion simulating disease 1* (*lsd1*) mutant indicate that increased concentrations of foliar glutathione could be sufficient to induce Npr1-dependent signalling. This could explain why *dnd1-1* and *cpr6-1* have a higher *Pr1* transcript induction when compared with *cpr5-1,* which has a slightly lower glutathione content (Fig. 5B; see supplementary Fig. 2 at JXB online).

Predominant cytosolic localization of glutathione synthase (GSH2), catalysing the final step of glutathione biosynthesis, in Brassicaceae could result in differences in the redox status of cytosolic and plastidic glutathione pools in situations with high rates of glutathione synthesis (Noctor *et al.*, 2002; Mullineaux and Rausch, 2005). A more reduced cytosolic glutathione pool would favour the reduction and activation of cytosolic Npr1 (Mou *et al.*, 2003). A further induction of *Pr1* expression was observed after exposure to sHL and the consequent increase in glutathione levels in SA and H_2O_2 accumulating lines (Fig, 5B; see supplementary Fig. 2 at JXB online). It is proposed that the tight physiological coupling of SA and glutathione levels is required to cope with oxidative stress brought about by environmental challenges that induce SA synthesis, and to mount appropriate defence responses.

Cross dependence between SA, glutathione and ROS: implications for plant resistance and signal transduction

High levels of SA seem to be tightly linked to high glutathione and H_2O_2 content. Similarly, feeding of GSH to leaves caused an increase in SA and plants engineered to accumulate glutathione suffered from oxidative stress (Fig. 5; Creissen *et al.*, 1999). Interestingly, low levels of SA in NahG and *sid2-2* were accompanied by approximately 50% lower H_2O_2 levels, but only by a slight reduction of the glutathione content in NahG. Considering the tight and fast mutual interdependence of SA, glutathione and H_2O_2, caution is necessary when assigning physiological and signalling functions to one of the three molecules. Reports on the influence of SA on flowering induction and growth at low temperatures will require reconsideration with respect to the results presented in this article (Martinez *et al.*, 2004; Scott *et al.*, 2004). Similarly, increased disease resistance and retarded growth in mutants with constitutive SA accumulation is very likely to be a combinatorial effect of signals triggered by SA, H_2O_2, glutathione, and potentially additional yet unidentified compounds. The functions of SA and H_2O_2 in pathogen resistance have been analysed to a large extent, whereas glutathione and redox signalling was mainly considered in abiotic stress responses. Early findings that GSH elicits specific defence responses in legume cell cultures were not followed up in more detail (Wingate *et al.*, 1988; Lamb and Dixon, 1997). It was recently observed that plants with only 30–50% of wt glutathione levels due to impaired biosynthesis (*cad2-1* and *rax1-1*) were more susceptible to avirulent pathogen challenge (Ball *et al.*, 2004). As mentioned above, other studies place changes in pool size or redox status of glutathione downstream of SA in specific defence signalling cascades (Mou *et al.*, 2003; Senda and Ogawa, 2004). Sophisticated approaches will be required to dissect the effects of SA, H_2O_2 and glutathione and to unravel the mechanisms connecting the levels of these three compounds. The tight coupling of SA to H_2O_2 and glutathione as well as the further results presented in this article strongly suggest that the role of SA in plants is not limited to pathogen defence signalling, but that SA is also influencing light acclimation processes and the regulation of the redox homeostasis of the cell.

Supplementary data

Supplementary data can be found at JXB online.

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