A mutation affecting ASCORBATE PEROXIDASE 2 gene expression reveals a link between responses to high light and drought tolerance

JAN BART ROSSEL¹, PHILIPPA B. WALTER¹, LUKE HENDRICKSON¹, WAH SOON CHOW², ANDREW POOLE³, PHILIP M. MULLINEAUX⁴ & BARRY J. POGSON¹

¹ARC Centre of Excellence in Plant Energy Biology, School of Biochemistry and Molecular Biology, The Australian National University, Canberra, A.C.T. 0200, Australia, ²Photobioenergetics Group, Research School of Biological Sciences, The Australian National University, P.O. Box 475, Canberra, A.C.T. 2601, Australia, ³Commonwealth Scientific and Industrial Research Organization (CSIRO) Plant Industry, Canberra, Australia and ⁴Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, UK

ABSTRACT
Molecular analyses of plants have revealed a number of genes whose expression changes in response to high light (HL), including the $\text{H}_2\text{O}_2$ scavenger, ASCORBATE PEROXIDASE 2 (APX2). We carried out a screen in Arabidopsis thaliana for lesions that alter HL-induced expression of APX2 to identify components in abiotic stress signalling pathways. High light was used as it can be instantaneously applied or removed and accurately measured. We identified a number of $\text{alx}$ mutations causing altered APX2 expression. Here we describe the gain-of-function mutant, $\text{alx}8$, which has constitutively higher APX2 expression and higher levels of foliar abscisic acid (ABA) than wild type. In fact, exogenous ABA increased APX2 expression and the APX2 promoter contains ABA response elements. Furthermore, we have shown that HL stress increases ABA in wild-type plants, implicating ABA in the regulation of HL-inducible genes. The $\text{alx}8$ mutant is drought tolerant, exhibits improved water-use efficiency and a number of drought-tolerance genes are upregulated. Additionally, $\text{alx}8$ demonstrates the complexity of ABA-dependent and ABA-independent transcriptional networks as some components in both pathways are upregulated in $\text{alx}8$. This study provides evidence for common steps in drought and HL stress response pathways.

Key-words: ABA; abscisic acid; mutant screen; photoprotection; stress response networks.

INTRODUCTION
Under environmental conditions that do not negatively affect plant growth, plants have evolved several mechanisms to protect themselves against the adverse effects of reactive oxygen species (ROS) formed during cellular metabolism (Asada 1999). One of these mechanisms includes photoprotective compounds such as carotenoids and vitamin E (α-tocopherol). Another line of defence consists of an antioxidant system that provides protection against ROS produced during normal cellular metabolic activity and photosynthesis. This defence mechanism consists of enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Niyogi 1999). The ascorbate peroxidases use ascorbic acid (vitamin C) as their substrate to catalyse the conversion of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$. When a plant is exposed to light energy in excess of the photochemical capacity of the photosystems, this can lead to an imbalance between the production and removal of ROS. In addition to excess light, a range of abiotic stresses such as $\text{O}_3$, drought, salt, toxic metals and temperature can lead to an increased production of ROS (Conklin & Last 1995; Richards et al. 1998; Shinozaki & Yamaguchi-Shinozaki 2000), in part by limiting photosynthetic capacity. ROS are extremely detrimental to plant survival as they can induce the oxidation of lipids, proteins and DNA that are necessary for the proper functioning of the chloroplast and the cell as a whole (Foyer, Descourvieres & Kunert 1994). However, ROS not only damage cells, but also induce protective mechanisms.

A rapid transcriptional response to high light stress has been observed for two members of the ascorbate peroxidase gene family, APX1 and APX2, both of which are targeted to the cytosol (Karpinski et al. 1997). APX2 transcription is thought to be, at least in part, regulated by the redox state of the plastoquinone (PQ) pool and to a lesser degree by the elevated ROS levels. A reduced PQ pool (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DBMIB treatment) increases APX2 expression whereas an oxidized PQ pool (3-(3,4-dichlorophenyl)-1,1-dimethyleurea; DCMU treatment) inhibits expression, even under high light (Karpinski et al. 1999). Other stresses such as wounding and heat stress and glutathione metabolism have also been shown to induce APX2 expression (Ball et al. 2004; Chang et al. 2004; Panchuk, Volkov & Schoffl 2002). More recently,
APX2 expression was shown to be induced in plants sprayed with abscisic acid (ABA) and lower in the ABA-insensitive mutants, abi1–1 and abi2–1 (Fryer et al. 2003). These findings suggest multiple levels of control exert a transcriitional effect on APX2 expression. With the exceptions of glutathione metabolism (Ball et al. 2004) and the heat shock transcription factor 3 (Panchuk et al. 2002) little is known about the regulatory cascade in which ROS, redox and ABA mediate APX2 expression.

Elevated ROS is one of the signals that could link abiotic stress signalling pathways, but redox and ABA may potentially contribute. To date there have been no reports on whether ABA is elevated in response to high light nor is it understood if, or to what extent, high light (HL) and drought stress pathways are inter connected. We postulated that identifying key regulators and signalling components involved in HL-mediated oxidative stress may lead to cross protection against drought, as many HL regulated genes are also induced under drought conditions (Kimura et al. 2003). We undertook a ‘phenotype to gene’ forward genetics approach to identify a class of mutations, alx, that can be used to study the regulation of APX2 expression and thereby determine how HL pathways are regulated (Ros sel, Cuttriss & Posgon 2004) and whether these pathways overlap with the drought response. Our approach consisted of screening for aberrant expression of APX2 in progeny of chemically mutagenized plants. Altered APX2 expression was detected by luminescence (Rossel et al. 2004) due to the expression of the luciferase reporter gene under the control of the APX2 promoter (Karpinski et al. 1999). Analysing the alx8 mutation, described here, has advanced our understanding of the interactions between ABA, HL and drought tolerance stress networks.

MATERIALS AND METHODS
Plant material and growth conditions
For growth in tissue culture media, Arabidopsis thaliana ecotype Columbia (Col) seeds were surface-sterilized (3 min in 25% bleach, 0.05% Tween 20; 5 min 70% ethanol; washed four times in sterile water), plated on Murashige & Skoog (MS) 1% agar, 2% sucrose plates and vernalized for 3 d before transfer to a growth cabinet and grown at 21 °C under 24 h light (100 μmol photons m⁻² s⁻¹). For growth in soil, a soil mix was made (three parts soil/one part vermiculite) and soaked with 0.5x Hoaglands Fertilizer (Hoag lands & Arnon 1950). Seeds were vernalized for 3 d and transferred to the growth room at 21 °C on a 16 h/8 h day/night cycle at 100 μmol photons m⁻² s⁻¹.

APX2-luciferase mutant screen and CCD imaging
Arabidopsis seed already transformed with the APX2 promoter-luciferase reporter gene (APX2:LUC) construct (Karpinski et al. 1999) were mutagenized with ethylmethane sulfonate and M2 plants screened in pools derived from 500 M1 parents as described previously (Ball et al. 2004). The mutant screen was carried out using a luminescence counter to count photons emitted per seedling in 96-well microtitre plates as described previously (Rossel et al. 2004). To confirm the reproducibility of the altered APX2 expression, four-week-old Arabidopsis plants containing APX2:LUC were imaged using a cooled CCD camera (Model DV 435, Andor Technology, Tokyo, Japan). Prior to imaging, the plants were sprayed with a 1 mM D-luciferin (BIOSYNTH, Staad, Switzerland) solution containing a few drops of Tween 80 and left in growth light conditions for 5 min. Images obtained using the Andor DV435 CCD camera were analysed using Image-Pro software (Media Cybernetics; Carlsbad, CA, USA).

Gene expression analysis by real time RT-PCR
Real time RT-PCR analyses were performed on the RotorGene 2000 (Corbett Research, Sydney, Australia). RNA was extracted from single leaves of 24 ± 4 d-old plants with the Qiagen RNeasy plant kit (Qiagen; Santa Clarita, CA, USA) as per the manufacturer’s instructions. Real time RT-PCR was carried out using the QuantiTect™ SYBR® green real time-PCR kit (Qiagen) as per the manufacturer’s instructions. Primers used for real time RT-PCR were as follows. APX2, 5′-ACCGGCTCATTTTTGACAC-3′ and 5′-AGTACGTGGTCTGGCAACAGCTCCAC-3′; APX1, 5′-CCCTCCATTCATGGAGCAGC-3′ and 5′-TCGAAAGTTCCAGCAACATGCCTA-3′; RD24, 5′-CACCAAGGCACAAACCTGTTC-3′ and 5′-GCAGAGAGACGGAGTTGTC-3′; DRE2A, 5′-AGTACGTGGTCTGGCAACAGCTCCAC-3′ and 5′-TCGAGCTGAAACAGAGAGACGGAGTTGTC-3′; sHSP, 5′-CCCTGGATGGAAGAAG-3′ and 5′-TAGGCCACCGTAACAGTCAA-3′; CYCLOPHILIN, 5′-TCTTCCTCTTCGGA-3′ and 5′-TCTTCCTCTTCGGA-3′; STZ, 5′-AGTACGTGGTCTGGCAACAGCTCCAC-3′ and 5′-TCGAGCTGAAACAGAGAGACGGAGTTGTC-3′.

Measurement of photosynthetic and photoprotective parameters
Leaf photosynthetic parameters were measured on 24 ± 4 d-old plants using an open circuit, LI-6400 infrared...
gas exchange system with the leaf chamber chlorophyll fluorometer attachment (LI-6400-40 LCF, Lincoln, NE, USA). Concurrent measurements of leaf carbon assimilation ($A$), stomatal conductance ($g_s$), transpiration ($E$), intercellular airspace CO$_2$ concentration ($C_i$), leaf temperature and leaf to air vapour pressure difference ($vpd_L$ – calculated from leaf temperature), and fluorescence yields were monitored in this system. Artificial illumination was supplied to the leaf from a red–blue LED light source attached to the sensor head. Gas-exchange parameters were calculated as described previously (von Caemmerer & Farquhar 1981) with revisions (von Caemmerer & Quick 2000).

Pulse-amplitude modulated leaf chlorophyll fluorometry was undertaken using either the LI-6400-40 LCF or the PAM 101 (H. Walz, Effeltrich, Germany). Fluorescence parameters were measured in accordance with published methods (Bilger, Schreiber & Bock 1995) using accepted nomenclature (van Kooten & Snel 1990). After a dark acclimation period of 30–45 min, the intrinsic quantum efficiency of open photosystem II (PSII) reaction centres was measured:

$$\Phi = \frac{F_m - F_o}{F_m}$$

Subsequent to illumination, the utilization of photons absorbed by the PSII antennae in photosynthetic electron transport and thermal dissipation was assessed from the quantum efficiency ($\Phi$) and flux ($J$) of photochemical energy dissipation [$\Phi_{PSII}$, $J_{PSII}$; Genty, Briantais & Baker (1989)], light-dependent [$\Phi_{NPQ}$, $J_{NPQ}$; Genty et al. (1996)] and light-independent thermal dissipation and fluorescence ($\Phi_{EQR}$, $J_{EQR}$) energy dissipation (Hendrickson et al. 2004) with $\Phi_{Q}$ + $\Phi_{NPQ}$ + $\Phi_{PSII}$ = 1. The rates of energy dissipation $J$ were calculated assuming 0.5 as the proportion of absorbed photons utilized by PSII reaction centres (Melis, Spangfort & Andersson 1987) and an average leaf absorbance of 0.85. An additional estimate of the extent of thermal antennae non-photochemical quenching (NPQ) was also measured after one hour treatment at 1000 µmol photons m$^{-2}$ s$^{-1}$ (Schreiber, Bilger & Neubauer 1994).

Photosynthetic response to irradiance

After 1 h dark acclimatization, leaves were illuminated at 500 µmol photons m$^{-2}$ s$^{-1}$ photosynthetically available radiation (PAR) at 25 °C, until the rate of net CO$_2$ fixation and fluorescence yield reached steady state (after ~30 min) at 350 µbar CO$_2$ and 210 mbar O$_2$ in N$_2$. PAR was then increased to 1200 µmol photons m$^{-2}$ s$^{-1}$ and then reduced in a step-wise manner.

Abscisic acid measurements

Leaves harvested from 24 ± 4-d-old plants were immediately frozen in liquid nitrogen and freeze dried. Approximately 0.5 g of freeze dried leaf material from three individual plants was ground up in a mortar and transferred to a 50 mL falcon tube containing 20 mL 80% methanol and an internal ABA standard ([1$^\text{H}$]ABA). The tube was then transferred to an end-over-end shaker at 4 °C and allowed to shake overnight to extract and measure ABA, following previously described methods (Jacobsen et al. 2002).

RESULTS

Identification of an alx drought-tolerant mutant

One-week-old mutagenized APX2:LUC seedlings were screened for altered APX2 expression before and after HL treatment. The altered APX2 expression was assayed by counting photons emitted from the seedlings at low light (LL), after 1 h of HL and 15 h at LL post 1 h HL using a luminescence counter. Because the location of each plant was defined by the microtitre plate and the assay was in vivo and non-destructive, the temporal kinetics for individual plants could be determined. Thirteen mutations with altered expression of APX2 were identified and one of the gain-of-function mutations, alx8, was subjected to detailed analysis.

The gain-of-function mutant seedling, alx8, had increased APX2:LUC activity when expressed as photon counts per seedling under both LL and HL (data not shown). To confirm that the increased APX2:LUC expression occurred in mature alx8 plants as well as in seedlings, we imaged luciferase activity in vivo in four-week-old plants using a cooled CCD camera before and after 1 h of HL (Fig. 1). Figure 1 clearly indicates greater luciferase activity in alx8 than wild type in low light and high light treatments, with high light having the highest luciferase activity (Fig. 1a–d). Real time RT-PCR analyses confirmed that endogenous APX2 expression in alx8 was increased in conjunction with the increased APX2:LUC activity (Fig. 1b,d), as endogenous APX2 expression in alx8 was many fold higher than wild type APX2:LUC plants exposed to low and high light (Fig. 1e).

The response of the alx8 mutant to other abiotic stress-inducing agents, such as NaCl, the herbicide methyl viologen and water deficit, was tested. The alx8 mutant did not appear to show markedly altered tolerance to either NaCl or methyl viologen (data not shown). However, when alx8 and wild-type plants were subjected to water deficit, the drought tolerance of alx8 was clearly visible, as leaves remained turgid, green and viable after more than 10 d of withholding water, whereas wild-type leaves had wilted and died (Fig. 2). In fact, of the 13 alx mutations identified, four exhibited drought tolerance (data not shown).

Genetics of alx8

The alx8 plants exhibited an altered leaf shape and slower growth (Fig. 2). Segregation analysis was performed on F2 plants derived from crosses between wild type and alx8 to confirm that drought tolerance, APX2:LUC activity, and altered leaf shape and slower growth cosegregated.
The phenotypes in the F1 generation were all recessive. F2 plants were scored as wild type or altered leaf shape and the presence of the APX2:LUC transgene confirmed. In all plants analysed, altered leaf shape cosegregated with higher luciferase activity, as did wild-type leaves and low luciferase activity. Similarly, drought tolerance and altered leaf shape cosegregated with a probability of <0.001, as did drought sensitivity and wild-type leaf shape with a probability of <0.005. Thus, altered leaf shape, drought tolerance and high APX2:LUC activity in F2 alx8 plants all cosegregated, which is consistent with a single locus.

**Increased water-use efficiency (WUE) in alx8**

To determine the physiological basis for the drought tolerance and increased APX2 expression in alx8, the photosynthetic irradiance (PAR) responses of wild type and alx8
were compared using simultaneous leaf gas exchange and chlorophyll fluorescence (Figs 3 & 4). Both wild-type and alx8 leaves had similar photosynthetic light saturation points, but wild-type leaves exhibited 39% higher net rates of photosynthetic CO₂ fixation than alx8 at light saturation (Fig. 3a).

Rates of energy dissipation via photochemistry and light-dependent xanthophyll-mediated thermal dissipation were similar for wild type and alx8 (Fig. 3d,e). The response of linear electron transport, \( J_{\text{PSII}} \), to photosynthetic irradiance, \( \text{PAR} \), followed a hyperbolic relationship saturating at \( \sim 300 \mu\text{mol photons m}^{-2}\text{s}^{-1} \) and was similar between wild type and alx8 (Fig. 3d). Thus, high \( \text{APX2} \) expression could not be explained by a difference in photosynthetic capacity in alx8, as \( J_{\text{PSII}} \) was similar to that of wild type. Thermal dissipation exhibited a linear relationship with light intensity above \( 200 \mu\text{mol photons m}^{-2}\text{s}^{-1} \) and did not differ between wild type and alx8 (Fig. 3e). Correspondingly, the efficiency of light-dependent \( \Delta \text{pH} \) and xanthophyll-mediated dissipation processes (\( \Phi_{\text{NPQ}} \)) increased as an energy sink with increasing light intensity (Fig. 4). The fraction of absorbed irradiance dissipated by the various pathways was identical for wild type and alx8 at all light irradiances, implying that the excess irradiance not utilized
Table 1. Genetic analysis of F2 progeny of alx8, Luc+ × wild type

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<tr>
<th>Leaf shape and luciferase activity</th>
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<td>Luciferase activity*</td>
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*Plants grown in LL were tested by PCR for presence of the APX2:LUC transgene, scored as altered or wild type for leaf shape, and then qualitatively assessed as nil to low or high luciferase activity by CCD imaging (Fig. 1).

bStatistical analysis was undertaken with a null hypothesis of independent assortment for each set of pairwise comparisons. The chi-squared (χ²) and probability (P) values were highly significant indicating cosegregation and thus linkage of the traits.

cWater was withheld from plants, which were qualitatively assayed for the proportion of wilted and dying leaves. In most instances, this clearly distinguishes between wild type and alx8 plants (Fig. 2), with some variability being caused by uneven residual water content between pots.

Figure 3. Irradiance responses of the photosynthetic parameters for both wild-type (■) and alx8 (○). The parameters measured were: (a) net CO₂ assimilation rate (A); (b) stomatal conductance (gs); (c) intercellular CO₂ partial pressure (Ci); (d) linear electron transport rate through PSII (JPSII); (e) ΔpH and xanthophyll-mediated thermal dissipation rate, JNPQ, and (f) vapour pressure difference between the leaf and air (vpdL). Measurements were made at 24 °C and 350 µbar CO₂ in air. Each data point is the mean ± SE of three to five leaves.
High light stress and drought tolerance

by carbon assimilation in alx8 was dissipated by other processes (Fig. 4).

Interestingly, with respect to the drought tolerance, alx8 leaves exhibited 56% lower stomatal conductance and a 12% lower CO₂ partial pressure in the intercellular airspaces than the wild-type plant at equivalent PAR, despite similar leaf temperature and vapour pressure (Fig. 3b,c). The combined reduction in net CO₂ fixation and an even greater reduction in stomatal conductance led to increased water-use efficiency (WUE) in alx8 as compared to wild-type plants at irradiances from 40 to 1200 µmol photons m⁻² s⁻¹ (Fig. 5). WUE increased with PAR up to a peak at 300 µmol photons m⁻² s⁻¹ and then declined slightly at higher light irradiances (Fig. 5).

Changes in abscisic acid levels and APX2 expression in alx8 in drought and HL

As ABA is the key hormone for regulating stomatal aperture and thus WUE (Roelfsema & Prins 1995; Pei et al. 1997; Leymarie, Lasceve & Vavasseur 1998) we analysed the ABA content in alx8. In alx8 leaves, ABA levels were threefold higher than in wild-type leaves in LL and twofold higher than wild-type leaves in HL (Fig. 6a). Furthermore, ABA levels were increased by HL in both wild-type and alx8 plants. In wild-type plants ABA levels doubled after exposure to high light for an hour.

To determine whether a constitutive increase in the osmotic stress-inducible ABA biosynthetic enzyme, 9-cis-epoxycarotenoid dioxygenase (NCED3) (Xiong et al. 2002), may be responsible for the increased foliar ABA content in alx8, we measured the mRNA abundance using real time RT-PCR analysis. However both wild-type and alx8 exhibited similar NCED3 mRNA abundance levels (Fig. 6b).

To corroborate whether ABA is involved in the transcriptional network affecting APX2 regulation, wild-type APX2:LUC plants were treated with ABA after which APX2:LUC expression was visualized using a cooled CCD camera system. ABA (50 µM) was applied to the plants by
feeding through a petiole. Within a short time (<30 min) after ABA application, APX2:LUC expression could be visualized throughout the plant (Fig. 7a). Furthermore, analysis of 500 base pairs upstream of the APX2 transcriptional start site (PlantCARE; http://intra.psbg.ugent.be:8080/PlantCARE/) (Rombauts et al. 1999) predicted a cis-acting element involved in abscisic acid responsiveness (ABRE) (data not shown). These findings thus provided one explanation towards the increased level of APX2:LUC expression observed in alx8.

In light of the induction of APX2 by exogenous ABA and HL, we investigated its regulation in response to drought. This is in contrast to HL where even 1 h of HL was sufficient to induce APX2 in alx8.

Expression of abiotic stress response genes in alx8

The findings that APX2 expression and drought tolerance were altered in alx8 suggested a link between the high light and drought stress transcriptional networks. Real time RT-PCR analyses were performed to determine whether other abiotic stress and/or drought responsive genes were transcriptionally affected in alx8 (Fig. 8). The genes analysed were APX1, GST6, a small HSP (At2g29500) and ZAT10 (At1g27730), which are induced by high light or ROS (Chen, Chao & Singh 1996; Rossel, Wilson & Pogson 2002) and the drought and/or cold-responsive genes: DREB2A, RAP2.6 and RD29A (Fowler & Thomashow 2002; Liu et al. 1998). In fact, all were significantly induced by HL, including the three drought responsive genes (Fig. 8) and all were predicted by PlantCARE to contain an ABRE element within their promoter (data not shown).

APX2 expression in alx8 was >10-fold higher than wild-type at LL and >fivefold higher again than wild-type HL levels. Similarly, DREB2A, ZAT10, and RAP2.6 were upregulated in alx8 compared to wild-type levels at LL and HL. sHSP and RAP2.6 were at similar levels to wild type in alx8 for LL, but the induction by HL in alx8 was >15-fold and >240-fold higher than wild-type levels, respectively. In contrast, the expression profile of the ROS responsive genes, APX1 and GST6, and the ABA responsive gene, RD29A, were relatively similar in alx8 compared to wild-type levels, as was NCED3 (Fig. 6).

DISCUSSION

Increased drought tolerance, water-use efficiency, ABA content and mRNA levels of stress response genes in alx8

We used high light as a definable and controllable model system to deliver abiotic stress allowing us to identify mutations affecting the transcriptional response networks that mediate APX2 expression. By undertaking this screen in high humidity we selected for a different class of mutations to the regulator of APX2 (rax) mutations (Ball et al. 2004). That is, Ball and colleagues targeted mutations specific to the HL response, while our screen enabled the identification of mutations that constitutively activated oxidative stress response pathways, including the drought response, providing that such an activation induced APX2. The rationale has now been validated both by this study and the recent observations that exogenous ABA can stimulate APX2 expression (Fryer et al. 2003). Consequently, it has been possible to identify mutants in high light-mediated
stress response that also affect ABA-dependent abiotic stress signalling pathways, such as drought.

We have shown that ABA levels increase in wild-type plants in response to 1 h high light (Fig. 6a); a result not reported previously. Also, APX2 is constitutively upregulated in alx8 plants that have constitutively increased endogenous ABA levels. Thus, ABA contributes to the induction of at least one photoprotective gene, which is consistent with earlier studies suggesting that exogenous ABA might play a role in protecting against photodamage (Ivanov et al. 1995; Sharma, Sankhalkar & Fernandes 2002), and that ABA induces APX2 expression (Fryer et al. 2003). In fact, analysis of the promoters of 28 genes upregulated by a heat-filtered high light microarray experiment (Rossel et al. 2002) revealed that 13 contained ABRE elements. The role of ABA in HL stress may also explain the observations that the majority of HL-regulated genes are also upregulated by drought (Kimura et al. 2003). Thus, although the main focus on the regulation of APX2 and other HL genes has been the relative contributions of the redox poise of the chloroplast, particularly the PQ pool, glutathione and ROS, ABA clearly affects APX2 gene expression during HL. Likewise, the simplest explanation for the drought-induced increase in APX2 transcript abun-
dance in wild-type plants is a rise in ABA levels. The increase in APX2 expression in alx8 may be directly mediated by the increased ABA per se as the APX2 promoter contains an ABA-specific response element. Alternatively, the increase in APX2 in alx8 could result from an increase of ROS as ABA was shown to induce the production of H2O2 in guard cells promoting stomatal closure (Pei et al. 2000). However, given that GST6 expression which is H2O2 responsive (Chen et al. 1996), was not altered in alx8 and APX2 is not strongly responsive to H2O2 the latter is an unlikely explanation.

One process that might have been expected to result in higher APX2 expression would be alterations to the rate and efficiency of photosynthetic electron transport. However, as alx8 expressed similar Fv/Fm values to wild type, and the rate of energy dissipation via photochemistry and light-dependent xanthophyll-mediated thermal dissipation were not significantly different, there is no evidence of impaired intrinsic quantum efficiency of PSII in dark-adapted leaves. Indeed, as the fraction of absorbed irradiance utilized or dissipated by these pathways was each more or less identical between alx8 and wild type at all light irradiances this would imply that the excess irradiance not consumed by carbon assimilation in alx8 was consumed by other photochemical processes. The most probable of these processes is photorespiration, as CO2 at the chloroplast is lower in alx8 (Fig. 3c). The lower Ci would automatically increase the rate of photorespiration due to the catalytic specificity of ribulose-1,5-biphosphate carboxylase/oxygenase (Portis 1992). The reduction in Ci by stomatal closure and reduced A in alx8 therefore alters the balance between carboxylation and oxygenation favouring photorespiration.

Figure 8. Analyses of relative mRNA abundance of stress responsive genes. Using real time RT-PCR mRNA abundance was analysed in wild-type (wt, open bars) and alx8 (striped bars) plants grown in low light (100 µmol photons m⁻² s⁻¹) and then exposed to 1 h high light (wt-HL, alx8-HL, 1000 µmol photons m⁻² s⁻¹). Error bars indicate standard error across three biological replicates. Relative mRNA abundance was expressed as fold change normalized against wild-type LL.
the changes observed in gene expression could be linked to this.

**Drought and light stress networks are affected in alx8**

Although not all of the stress response genes analysed in this study were equally affected by the *alx8* mutation their expression patterns could be divided into three groups: those that have high expression levels under wild-type and HL (*APX2, ZAT10* and *RAP2.6*); those that have basal expression at LL, but are much more strongly induced by HL compared to wild-type (*sHSP* and *DREB2A*); and those whose expression is similar or marginally different to wild type (*APX1, GST6* and *RD29A*). Thus, although all are upregulated by HL in the wild type, the clustering into three groups in *alx8* is consistent with different pathways mediating their induction in response to high light.

Although there is a level of transcriptional integration between a wide variety of stress response networks, evidenced in genes that have overlapping transcriptional responses to various stresses such as wounding, pathogen, cold, drought, salt and high light stress (Cheong et al. 2002; Kreps et al. 2002; Rossel et al. 2002; Rabbani et al. 2003), it is important to take into account the multiple pathways within a network. In fact, a model that sought to integrate the multiple pathways involved in salt, drought and cold responses has been developed (Zhang, Creelman & Zhu 2004). A feature of this model was the ABA-independent and dependent pathways, with *DREB2A, RAP2.6* and *ZAT10* being ABA-independent, and *RD29A* and now *APX2* as ABA-dependent, yet while *APX2* was upregulated in *alx8, RD29A* was not. So, while a contributing factor to the drought tolerance and improved water-use efficiency is the strongly reduced stomatal conductance, which may largely be attributed to the increased levels of ABA (Bradford 1983; Harris et al. 1988; Ober & Setter 1992), analysis of ABA-dependent and ABA-independent stress-induced genes indicate that *alx8* affects genes in both networks.

The complex traits of *alx8* all cosegregate and are largely consistent with a lesion in an early step in HL and drought stress signalling networks. First, *alx8* affects components of both ABA-dependent and ABA-independent pathways (Fig. 8), including *DREB2A*, which is a transcription factor that regulates a suite of drought stress response genes (Zhang et al. 2004). Second, ABA itself is elevated and this is an early step in the ABA network. Third, while drought tolerance can be conferred by overexpression of latter steps in either of these pathways, they do not result in all of the *alx8* traits, such as elevated ABA and altered leaf shape. That is, overexpression of ABA-dependent transcription factors, *CBF4* (C-repeat dehydration-responsive element binding factor or *DREB1*), *ABF3* and *ABF4* (abscisic acid response element binding factor), improved drought tolerance without altering leaf morphology (Haake et al. 2002; Kang et al. 2002).

The data further exemplifies that abiotic stress-response pathways can be viewed as complex networks with interactions beyond elevated ROS. That is, the responsiveness of *APX2* to exogenous ABA, the coincident increase in ABA and *APX2* gene expression in *alx8* and the elevated ABA content in HL-treated wild-type plants are evidence of a role for ABA in the HL transcriptional response network. Furthermore, we identified a number of mutations confering drought tolerance by screening for altered HL responses. In fact, the drought tolerance of *alx8* is more striking than the large increase in transcription of HL-inducible photoprotective genes and demonstrates an interaction between high light and drought stress networks.

**ACKNOWLEDGMENTS**

J.B.R. was supported by an A.N.U. International Postgraduate Research Award. Additional support was provided by Australian Research Council Grants, DP0343160 (B.J.P. and W.S.C.) and CE0561495 (B.J.P.).

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Received 15 April 2005; accepted for publication 7 June 2005