PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

rstb.royalsocietypublishing.org

Research

Cite this article: Gorecka M, Alvarez-Fernandez R, Slattery K, McAusland L, Davey PA, Karpinski S, Lawson T, Mullineaux PM. 2014 Abscisic acid signalling determines susceptibility of bundle sheath cells to photoinhibition in high light-exposed *Arabidopsis* leaves. *Phil. Trans. R. Soc. B* **369**: 20130234.
http://dx.doi.org/10.1098/rstb.2013.0234

One contribution of 20 to a Theme Issue 'Changing the light environment: chloroplast signalling and response mechanisms'.

Subject Areas:
molecular biology, physiology, plant science, cellular biology, genetics

Keywords:
abscisic acid, *APX2*, bundle sheath cells, high light, photoinhibition, reactive oxygen species (ROS)

Author for correspondence:
Philip M. Mullineaux
e-mail: mullin@essex.ac.uk

†These authors contributed equally to this study.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rstb.2013.0234 or via http://rstb.royalsocietypublishing.org.

Royal Society Publishing

Abscisic acid signalling determines susceptibility of bundle sheath cells to photoinhibition in high light-exposed *Arabidopsis* leaves

Magdalena Gorecka[1,†], Ruben Alvarez-Fernandez[2,†], Katie Slattery[2], Lorna McAusland[2], Phillip A. Davey[2], Stanislaw Karpinski[1], Tracy Lawson[2] and Philip M. Mullineaux[2]

[1]Department of Genetics, Breeding and Plant Biotechnology, Warsaw University of Life Sciences, Nowoursynowska Street 159, Warszawa 02-776, Poland
[2]School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK

The rapid induction of the bundle sheath cell (BSC)-specific expression of *ASCORBATE PEROXIDASE2* (*APX2*) in high light (HL)-exposed leaves of *Arabidopsis thaliana* is, in part, regulated by the hormone abscisic acid (ABA) produced by vascular parenchyma cells. In this study, we provide more details of the ABA signalling that regulates *APX2* expression and consider its importance in the photosynthetic responses of BSCs and whole leaves. This was done using a combination of analyses of gene expression and chlorophyll *a* fluorescence of both leaves and individual BSCs and mesophyll cells. The regulation of *APX2* expression occurs by the combination of the protein kinase SnRK2.6 (OST1):protein phosphatase 2C ABI2 and a Gα (GPA1)-regulated signalling pathway. The use of an *ost1-1/gpa1-4* mutant established that these signalling pathways are distinct but interact to regulate *APX2*. In HL-exposed leaves, BSC chloroplasts were more susceptible to photoinhibition than those of mesophyll cells. The activity of the ABA-signalling network determined the degree of susceptibility of BSCs to photoinhibition by influencing non-photochemical quenching. By contrast, in HL-exposed whole leaves, ABA signalling did not have any major influence on their transcriptomes nor on their susceptibility to photoinhibition, except where guard cell responses were observed.

1. Introduction

The ability of plants to respond and acclimate to changes in light intensity requires a complex signalling network, which is subjected to fine spatial and temporal control [1,2]. In *Arabidopsis thaliana* (*Arabidopsis*) leaves subjected for up to 60 min to moderate increases in light intensity, i.e. typically less than 10-fold of the growth photosynthetically active photon flux density (PPFD), hereafter referred to as high light (HL), photoinhibition is largely reversible [1,3]. Within 10 min of HL exposure, accumulation of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) occurs in chloroplasts of bundle sheath cells (BSCs) [3,4]. Leaves subjected to HL and high humidity do not accumulate H_2O_2 [3]. This humidity dependency is also evident for the HL-mediated induction of BSC-specific *ASCORBATE PEROXIDASE2* (*APX2*) [5] and led to the identification of abscisic acid (ABA) as a regulator of *APX2* expression [3,4,6]. In HL-exposed leaves, vascular parenchyma, adjacent to BSCs in *Arabidopsis*, is the source of the ABA for the induction of *APX2* [3,7]. *APX2* induction in HL also requires signalling sourced from redox events around linear photosynthetic electron transport [6,8,9], H_2O_2 sourced from the chloroplast and plasma membrane [3,6] and a decreased cellular redox status determined by the thiol antioxidant glutathione [5].

The control of *APX2* expression in BSCs involves at least one positive and one negative ABA-directed signalling pathway [3,4]. The pathway that positively regulates *APX2* expression has been shown to involve the SUCROSE NONFERMENTING1 (SNF1)-related protein kinase SnRK2.6, also called OPEN STOMATA1 (OST1) [3,10,11]. The activity of OST1 and the other main foliar subclass III SnRK2 isoforms (SnRK2.2 and SNRK2.3) [10,11] is negatively regulated by the 2C class of protein phosphatases (PP2Cs) [12,13] of which there are five isoforms present in adult leaves [14]. Of the PP2Cs so far tested, ABI1 and ABI2 (ABI stands for ABA INSENSITIVE), as the dominant negative mutants *abi1-1* and *abi2-1* [15], impact upon *APX2* expression [4]. This group of PP2C and SNRK2 isoforms along with their family of 14 cognate ABA START protein receptors are regarded as comprising a 'core' ABA-signalling pathway [13].

Heterotrimeric G protein signalling exerts a negative regulation of *APX2* induction in HL [3] of which the Gα and Gβ subunits GPA1 and AGB1, respectively, play a prominent role. The rice (*Oryza sativa*) GPA1 homologue regulates the production of H_2O_2 sourced from superoxide anion, which is produced from O_2 in a reaction catalysed by a RESPIRATORY BURST (NADPH) OXIDASE HOMOLOGUE (RBOH) at the plasma membrane [16]. In *Arabidopsis*, there are two major RBOH isoforms expressed in the leaf, RBOHD and RBOHF [17]. A double null mutant of these two genes shows inhibition of *APX2* expression in HL [6]. In guard cells, ABA-induced stomatal closure is mediated by RBOHF and RBOHD [18] and may be linked to GPA1-mediated signalling [19,20]. It is not clear how the H_2O_2 from either the plasma membrane or the chloroplast in BSCs or guard cells can act as a signal to the nucleus by traversing a reducing cytosolic environment [21]. One mechanism in guard cells has been proposed in which oxidized GLUTATHIONE PEROXIDASE3 (GPX3) inhibits the activity of ABI2 and possibly ABI1 [22].

In this study, we set out to answer three questions regarding the role of ABA signalling in HL responses of *Arabidopsis*. First, could more detail be added to the core ABA signalling directing *APX2* expression in BSCs? Second, is ABA signalling important for BSC responses to HL and not just the regulation of *APX2*? Third, is ABA signalling important for the immediate responses to HL of the leaf as a whole or is this confined to BSCs?

2. Material and methods

(a) *Arabidopsis* genotypes

All mutants used in this study have been described previously: *abi1-1* and *abi2-1* [15], *abi1-2*, *abi2-2*, *abi2-2/abi1-2/hab1-1* (*abi2-2T*) and *abi1-2/hab1-1/pp2ca1-1* (*pp2ca1T*) [14], *hab1-1* and *hab2-2* [23], *ost1-1* [10], *snrk2.2/snrk2.3* [11], *gpx3-1* [22], *gpa1-4* [24], *rbohD*, *rbohF* [17], *ABA DEFICIENT2* (*ABA2*) over-expressing line 4-3 (ABA2OE) [25]), *apx2-1* [26] and *apx2-2* (SALK_057686), which was an independent isolate of an *APX2* knockout mutant described previously [27]. Accession Col-0 was used as wild-type control in all cases, except for *ost1-1*, *abi1-1* and *abi2-1* for which accession Landsberg *erecta* (Ler) was used. The genotypes of all mutants were confirmed for this study.

A double mutant *gpa1-4/ost1-1* was made by crossing *gpa1-4* (Col-0; ♂) with *ost1-1* (Ler, ♀). The cross was taken to be successful by confirming in F_1 plants the presence of the heterozygous T-DNA insertion event of *gpa1-4* using the polymerase chain reaction (PCR), conditions and primers on genomic DNA as previously described [24]. F_2 progeny were first screened for a homozygous *gpa1-4* mutation and among these a homozygous *ost1-1* mutation was searched for by PCR of genomic DNA using primers ost1-1_F2 and ost1-1_R2 to *SNRK2.6/OST1* (5′-CTGATTATAGA TAGGGGAAACA-3′ and 5′-CTGATTATAGATAGGGGAAACA-3′ respectively) to generate a 800 bp amplicon. The amplicon was subjected to dideoxy sequencing using the BIG DYE Terminator v. 3.1 Ready Reaction Mix (Applied Biosystems) according to the manufacturer's instructions using the primer ost1-1_R3 (5′-TCACAAATAAA TCAACAAATGC-3′) and the sequence generated on an ABI3100 DNA analyser (Applied Biosystems). The mutation G (wild-type) to A (*ost1-1*) [10] at location chr4:16273850 (in AT4G33950.2) was scored from the DNA sequence data to recover two *gpa1-4* homozygous individuals that were also homozygous for the *ost1-1* mutation. These plants were each self-crossed to generate F_3 progeny, the homozygous *ost1-1* mutation confirmed and homozygous *gpa1-4* was confirmed as null by the absence of *GPA1* transcript by reverse transcriptase (RT)-PCR as previously described [3] using primers: 5′-CATAGAACTGTCGGGGAAATTGTGAATCATCAC CAGCC-3′ and 5′-GAAACAACAACGGCGAAGAGTTTTTTGC TTTCAGGGTTCT-3′. The F_3 progeny were used in the experiments described here. From the same screening, two wild-type F_2 individuals were chosen based on the absence of the above mutations and harbouring heterozygous loci for Ler and Col-0 using the cleaved amplified polymorphic sequence (CAPS) marker ATMYB3R (www.arabidopsis.org). These lines (F_3) were used as wild-type controls for the *APX2* expression analyses in this study on *ost1-1/gpa1-4*.

(b) Plant growth conditions

Plants were grown under 8 h photoperiods, 22 ± 1°C, 60% relative humidity and a PPFD of 150 (±15) μmol m^{-2} s^{-1} as described previously [28], hereafter termed low light (LL) conditions. Unless stated otherwise, all plants were used from 35 to 40 days post-germination.

(c) High light exposures and chlorophyll *a* fluorescence measurements and imaging

For *APX2* gene expression experiments, plants were exposed to a PPFD of 1500 μmol m^{-2} s^{-1} from a white light emitting diode (LED) array (Isolight; Technologica Ltd, Colchester UK). Leaf surface temperature reached 27°C after 5 min exposure at 5 cm from the LED array and was constant thereafter for up to 6 h. After 6 h HL exposure using this LED source, the maximum photosystem II (PSII) quantum efficiency (Fv/Fm) [29] was 0.59 ± 0.03 (s.d., $n = 8$) from a LL value of 0.81 ± 0.01, measured using a PAM-2000 portable fluorometer (Walz GmbH, Effeltrich, Germany) according to the manufacturer's instructions. Exposure of plants to 27°C for 30 min under LL induced *APX2* expression by 1.4-fold compared with plants at growth temperature (±0.4 s.d.; $n = 3$). By contrast, under the isolight the induction was 61.5-fold (±12.2 s.d.; $n = 3$). Thus, in this experimental system the predominant response studied was of *Arabidopsis* to HL and not the rise in temperature.

ABA-signalling mutants were examined for their whole leaf response to increasing PPFD from 200 to 1400 μmol m^{-2} s^{-1} in 200 μmol m^{-2} s^{-1} increments every 5 min. This was carried out on a chlorophyll *a* fluorescence (Cf) imaging system (Fluorimager, Technologica Ltd, Colchester, UK) in which the protocol had been pre-programmed into the instrument. Whole rosette Cf images were collected at each PPFD and the images were processed manually to collect numerical data from fully expanded leaves (see electronic supplementary material, figure S3*a*) for the Cf parameters Fq′/Fm′, Fv′/Fm′, Fq′/Fv′ and NPQ [29]. qL [29] was calculated post-measurement from the images of Fq′/Fm′, Fo′ and F′. The raw data from each leaf and each plant and

treatment were fed *via* Excel into a program in R which calculated and plotted responses of the Cf parameters against PPFD and provided the data for statistical analysis. The R script is available upon request.

(d) High-resolution imaging

Three-week-old plants were subjected to HL for up to 1 h or kept in LL, then a single leaf was placed onto the microscopic slide, overlaid with a coverslip and Cf image data were collected under actinic light. Cf data from six mesophyll cells and six BSCs per leaf were analysed. In each experiment, three plants were analysed per treatment per cell type. Parameters were measured in a Micro-FluorCam (Photon System Instruments, Czech Republic). Calculations for Fq′/Fm′, Fv′/Fm′ and Fq′/Fv′ were done according to Baker [29].

For the study of *apx2* mutants, 4-week-old plants were kept in LL or exposed to HL for 1 h. Then plants were kept in the dark for 30 min and a single leaf was placed in the measuring head of Imaging-PAM, version MINI equipped with Head IMAG-MIN/B (Walz GmbH) and Cf parameters collected over a range of PPFDs at 5 min intervals. Two areas from each leaf were taken for analysis, first from the mid-vein region and second from the leaf lamina. Three independent plants were taken for each treatment.

(e) Effect of a step change in photosynthetically active photon flux density on CO_2 assimilation rate and stomatal conductance

Measurements were made using infrared gas exchange analysis on individual fully expanded leaves of 6-week-old plants following the methods of Lawson *et al.* [30]. Briefly, leaves were first equilibrated to a PPFD of 100 μmol m^{-2} s^{-1}, ambient [CO_2] of 400 μmol mol^{-1} and 50% humidity at 22°C. Following stabilization, light was increased to 1000 μmol m^{-2} s^{-1} and the responses of CO_2 assimilation rate (A) and stomatal conductance (g_s) were recorded every minute until the leaf had stabilized to the new environment. The rate of change in g_s was determined during the first 15 min following a change in PPFD.

(f) RNA extraction, analysis of *APX2* expression by quantitative real time polymerase chain reaction and microarray analysis

RNA extraction, cDNA synthesis and quantitative (q) real time PCR were carried out on fully expanded leaves as previously described [28] using a SYBR green kit (Bioline Reagents Ltd. London, UK) according to the manufacturer's instructions. *APX2* primers used for qPCR were 5′ GATATTGCCGTTAGG CTTCTTGACCCT 3′ and 5′ GAAGAGCCTTGTCGGTTGGTAG TT 3′. *CYCLOPHILIN* (*CYC*; AT2G29960) was used as reference gene. The *CYC* primers were 5′ TCTTCCTCTTCGGAGCCATA 3′ and 5′ AAGCTGGGAATGATTCGATG 3′.

Microarray analysis was conducted using Agilent 4×44k arrays (G2519F-021169) exactly as previously described [28]. Comparisons were conducted between fully expanded leaves of mutant and wild-type plants exposed to HL. All microarray data were submitted to EMBL-EBI under the following codes: ABA2OE (E-MTAB-2048), *abi2-2/abi1-2/hab1-1* (*abi2-2T*, E-MTAB-2047), *rbohF* (E-MTAB-2049), *gpa1-4* (E-MTAB-2050), *ost1-1* (E-MTAB-2051) and *snrk2.2/snrk2.3* (*snrk2D*, E-MTAB-2052).

(g) Imaging H_2O_2 in leaves using Amplex Red Ultra

Infiltration of Aplex Red Ultra (ARU) into detached leaves followed by exposure to HL, its penetration properties, specific reaction with H_2O_2, imaging of the resulting resorufin fluorescence and digital processing of false-coloured images are described in detail by Galvez-Valdivieso *et al.* [3].

3. Results

(a) High light-induced *APX2* expression is controlled by two separate ABA-directed signalling pathways

APX2 expression under HL was compared between mutant and wild-type controls for a range of ABA-signalling mutants (figure 1; see Material and methods) representing a more detailed analysis of the pathways described previously [3,4,6]. Exposure of *abi2-2* to HL resulted in a *ca* 10-fold increased expression relative to Col-0 for *APX2* (figure 1*a*), while in HL-exposed *abi2-2/abi1-2/hab1-1* plants *APX2* expression was increased *ca* 80-fold (figure 1*a*). No other PP2C mutant showed any major effect on HL-induced *APX2* expression (figure 1*a*). The lack of effect on HL-responsive *APX2* expression of *abi1-2* or in *abi1-2/hab1-1/pp2ca1-1* contrasts with earlier observations using the dominant negative alleles of *ABI1* and *ABI2*, *abi1-1* and *abi2-1* [4,15]. These observations were confirmed here although the inhibition was the strongest with *abi2-1* (figure 1*b*).

Among the protein substrates for ABI2 and ABI1 are the major foliar SNRK2 isoforms, SNRK2.2, SNRK2.3 and SNRK2.6 (OST1) [12,31]. As previously shown [3], *ost1-1* plants were strongly inhibited for HL-induced *APX2* expression and *gpa1-4* showed enhanced *APX2* expression (figure 1*c*). HL-induced *APX2* expression in *ost1-1/gpa1-4* was in between the parental mutant values (figure 1*c*). As the expression phenotype of either single mutant was not evident in the double mutant, it was concluded that there are two separate signalling pathways, which nevertheless could act antagonistically on each other in controlling *APX2* expression.

In *Arabidopsis*, a source of extracellular H_2O_2 for signalling for ABA and HL is from RBOHD and RBOHF isoforms (see Introduction). *APX2* expression was inhibited only in *rbohF* plants (figure 1*d*). Examination of resorufin fluorescence to visualize H_2O_2 accumulation in HL-exposed leaves of *rbohD* and *rbohF* showed diminished and enhanced levels of veinal H_2O_2, respectively (figure 1*f*; electronic supplementary material, figure S1). Under the same conditions, the *rbohD/rbohF* double mutant accumulated less H_2O_2 (figure 1*f*; electronic supplementary material, figure S1).

(b) Mutants in ABA signalling that positively regulate *APX2* expression are affected in photosynthetic efficiency in HL-exposed BSCs.

BSCs of Col-0 plants exposed to HL for up to 60 min suffered progressively more photoinhibition, here shown as a decline in the Cf parameter Fq′/Fm′, than neighbouring mesophyll cells (figure 2*a*). Therefore, the Cf parameters Fq′/Fm′, Fq′/Fv′ and Fv′/Fm′ were measured before and after 60 min HL in a set of ABA-signalling mutants primarily involved in regulating *APX2* expression. In Col-0 and Ler plants, HL-exposed BSC chloroplasts again showed a larger decline in Fq′/Fm′and also Fv′/Fm′ than those from mesophyll cells (figure 2*b*,*c*). Fq′/Fv′ was no different between the two cell types (figure 2*d*). In comparison with their wild-type controls, *abi2-2*, *abi2-2/abi1-2/hab1-1*, *ost1-1* and *gpa1-4* did not show significantly lower Fq′/Fm′ and Fv′/Fm′ values in

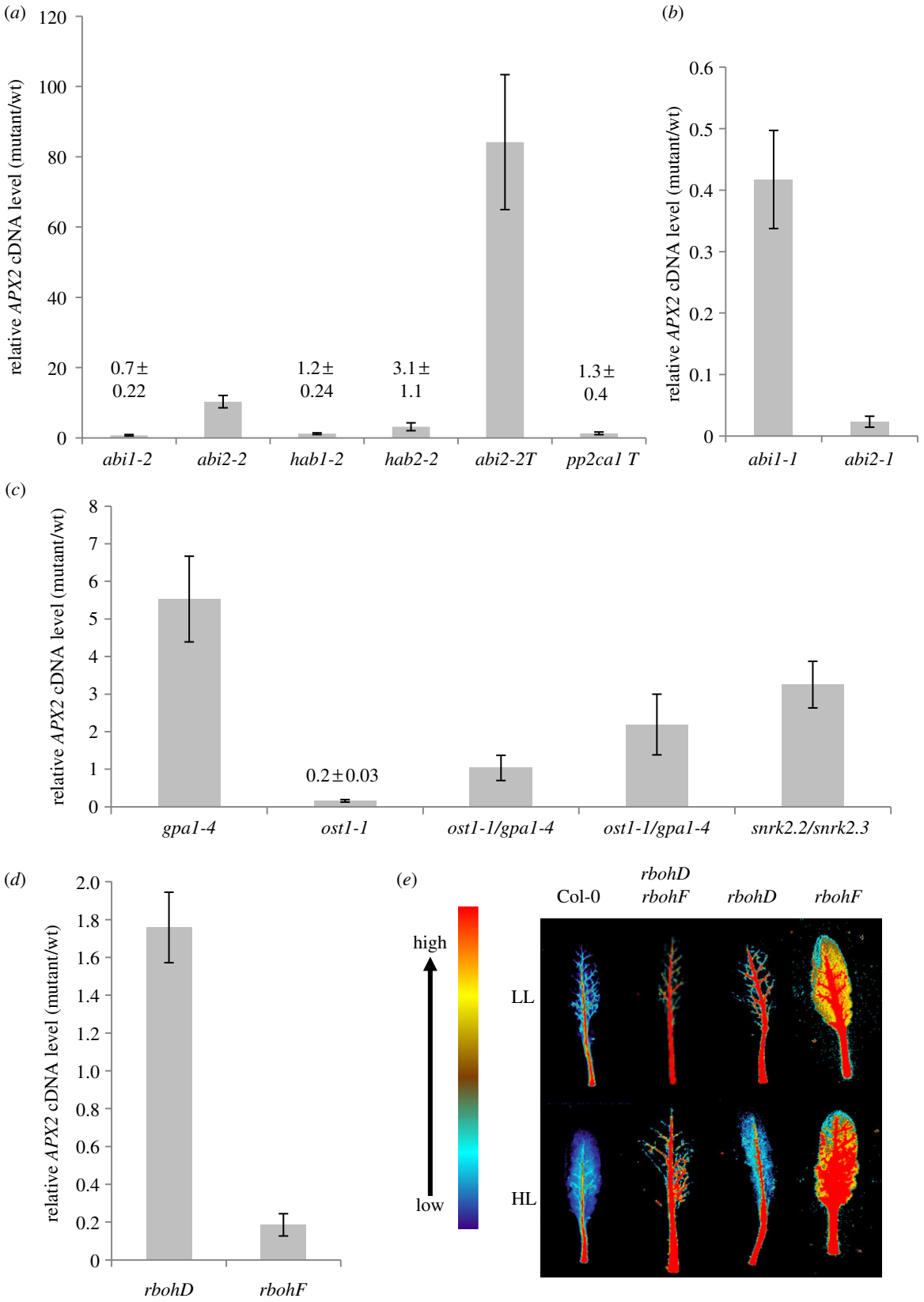

Figure 1. Expression of *APX2* and ARU staining for extracellular H_2O_2 in *Arabidopsis* leaves subjected to HL. (*a*) *APX2* cDNA levels from HL-exposed single and triple PP2C mutants compared with HL exposed Col-0. HL exposures (1500 µmol m^{-2} s^{-1} PPFD for 30 min) were conducted on 5-week-old short day-grown rosettes and RNA extracted from fully expanded leaves. cDNA levels were determined by qPCR using a SYBR Green-based assay (see Material and methods) with *CYC* as the reference gene. Values are means (± s.e.) of two experiments each with six plants used ($n = 12$). (*b*) Relative *APX2* cDNA levels in *abi1-1* and *abi2-1*. Experimental conditions were as in (*a*), except that these are data (means ± s.e.) from one experiment of six plants ($n = 6$). (*c*) Relative *APX2* cDNA levels in *ost1-1/gpa1-4*, *ost1-1*, *gpa1-4* and *snrk2.2/snrk2.3*. The HL conditions, sample size and qPCR were as in (*a*). Two individual lines of the double mutant were analysed. Wild-type controls were Ler for *ost1-1*, Col-0 for *gpa1-4* and *snrk2.2/snrk2.3* and an F_3 Ler/Col-0 hybrid (see Material and methods). (*d*) *APX2* cDNA levels in *rbohD* and *rbohF* relative to Col-0. The HL conditions, sample size and qPCR were as in (*a*). (*e*) Resorufin fluorescence in HL-exposed detached leaves of *rbohD*, *rbohF* and *rbohD/rbohF*. ARU (40 µM) was infiltrated into leaves (see Material and methods) and false-coloured image produced digitally against a scale of the fluorescence emission. (Online version in colour.)

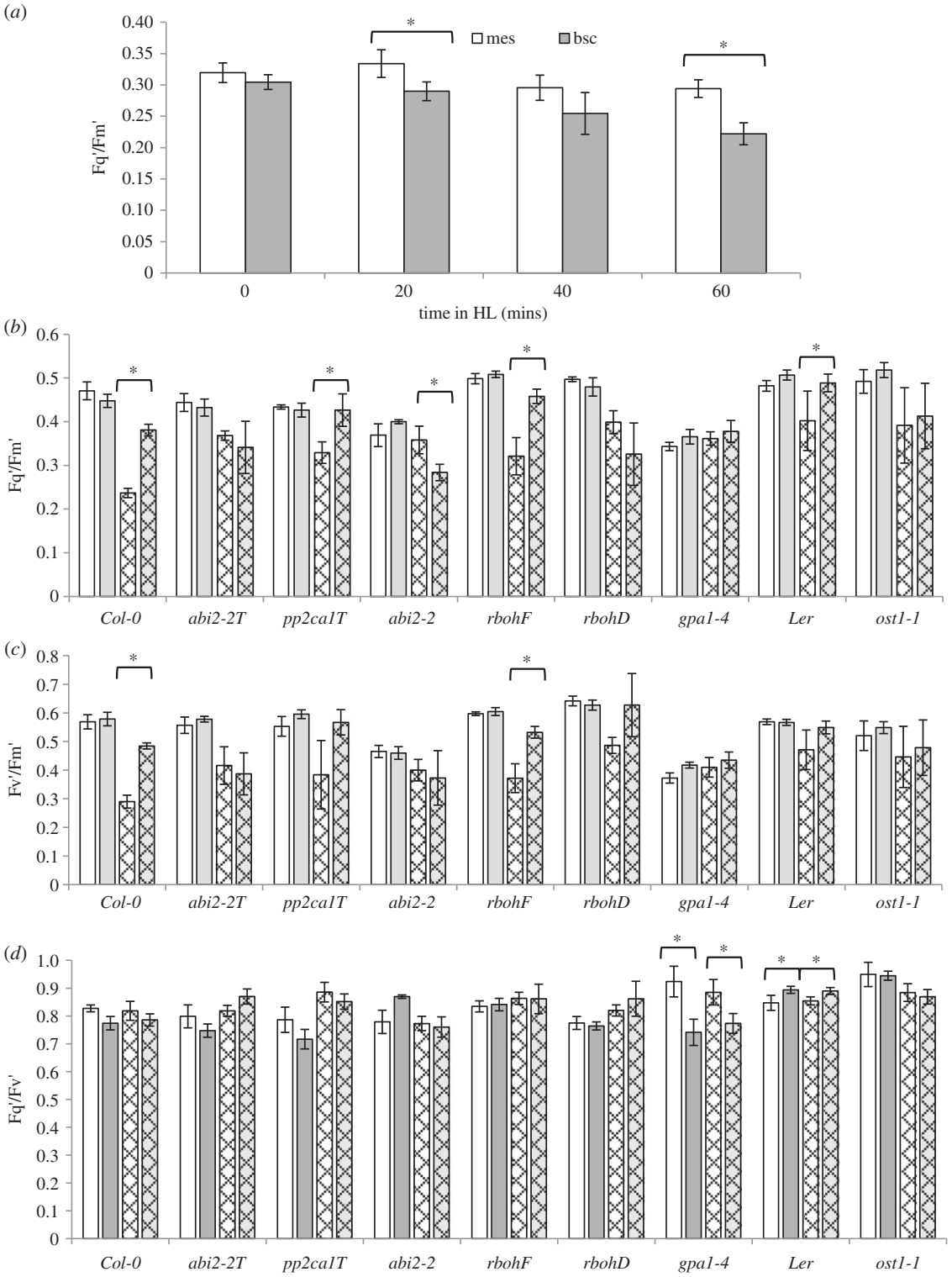

Figure 2. Cell-specific chlorophyll fluorescence (Cf) parameters under HL and LL. (*a*) Plants were exposed to HL (1500 μmol m^{-2} s^{-1} PPFD) for 0, 20, 40 and 60 min, and then individual detached leaves were placed under a high-resolution Cf imaging system (see Material and methods) and Fq′/Fm′ determined for six different cells of BSC (grey bars) and mesophyll (white bars) per leaf from three plants ($n = 18$). (*b–d*) Fq′/Fm′ (*b*), Fv′/Fm′ (*c*) and Fq′/Fv′ (*d*) Cf parameters (mean ± s.e.) from HL-exposed bundle sheath and mesophyll cells (white checked bars and grey checked bars, respectively) and their corresponding LL controls (white bars and grey bars) of wild-type and ABA-signalling mutants. Plants were exposed to HL for 60 mins as in (*a*), and then individual detached leaves were imaged as in (*a*). Cf data were collected for six different cells of each type per leaf from three plants ($n = 18$). From these data, Fq′/Fm′ (*b*), Fv′/Fm′ (*c*) and Fq′/Fv′ (*d*) were calculated. In all panels *a–d*, pairs of columns marked with an asterisk (*) mean that the comparison is significant at $p \leq 0.001$ (Student's *t*-test). The words *abi2-2T* and *pp2ca1T* are for the *abi2-2/abi1-2/hab1-1* and *pp2ca1-1/abi1-2/hab1-1* mutants, respectively.

BSCs compared with mesophyll cells. It was concluded that ABA/ROS signalling, primarily associated with ABI2/OST1 [13] and GPA1, could influence the susceptibility of BSC chloroplasts to photoinhibition. By contrast, mesophyll cells were less affected in these mutants (figure 2*b–d*). In contrast, *rbohF*, in which HL-induced *APX2* expression was inhibited (figure 1*d*), had no effect on BSC or mesophyll responses to HL compared with wild-type (figure 2*b–d*).

From the above observations, it was reasoned that the degree of expression of *APX2* might be a determinant of

Table 1. Stomatal conductance (g_s), rates of stomatal closure and maximum rates of photosynthesis (*A*sat) in response to 15 min at a PPFD of 1000 μmol m^{-2} s^{-1}. Values are the means ± s.d. ($n = 3$). Those values marked * are significantly different from their control genotypes at $p \leq 0.05$ (Student's *t*-test).

genotype	g_s (0 min) (mmol m^{-2} s^{-1})	g_s (15 min) (mmol m^{-2} s^{-1})	rate of change of g_s (mmol m^{-2} s^{-1})	*A*sat (15 min) (μmol m^{-2} s^{-1})
Col-0	207.5 ± 77.0	308.0 ± 92.2	6.7 ± 1.1	11.8 ± 0.4
Ler	309.1 ± 64.2	431.5 ± 96.6	8.2 ± 2.7	15.3 ± 1.5
abi2-1	75.4 ± 15.0*	102.3 ± 23.2*	1.8 ± 0.5*	7.1 ± 1.1*
abi2-2	153.6 ± 22.3	209.3 ± 38.8	3.7 ± 1.4*	10 ± 1.4
abi1-1	202.2 ± 86.0	288.7 ± 136.2	5.8 ± 3.6	12.1 ± 3.1
abi1-2	533 ± 251.6	642.9 ± 253.9	7.3 ± 0.5	21.1 ± 4.5
hab1-1	91.7 ± 26.8	109 ± 88.8	1.2 ± 4.2	8.7 ± 1.5
abi2-2/abi1-2/ hab1-1	32.2 ± 4.4	44.0 ± 2.8*	0.8 ± 0.1*	4.5 ± 2.2*
pp2ca1/abi1-2/ hab1-1	29.8 ± 4.8	43.7 ± 8.9*	0.9 ± 0.4*	5.3 ± 1.6*
ost1-1	214.8 ± 43.0	243.9 ± 38.3	1.9 ± 0.6*	10.1 ± 1.3*
rbohD/rbohF	60.8 ± 14.6*	125.2 ± 33.8*	4.3 ± 1.3*	7.3 ± 0.2*
gpa1-4	82.1 ± 15.9	111.8 ± 13.4	2.0 ± 0.9*	11.1 ± 2.4

the susceptibility of BSCs to photoinhibition. To test this proposal, Fq′/Fm′, NPQ and qL were measured in the leaf lamina and mid-vein sections of two null alleles of *APX2*, *apx2-1* and *apx2-2* (see Material and methods) exposed to HL. No differences in response of these mutants to HL were observed compared with Col-0 (see electronic supplementary material, figure S2).

(c) Abscisic acid signalling in whole leaf high light responses

ABA-signalling mutants were examined for their whole leaf response to HL (see Material and methods). Whole rosette Cf images were collected and the images were processed to collect data from fully expanded leaves for Fq′/Fm′, Fv′/Fm′, Fq′/Fv′, NPQ and qL (Material and methods). An example is shown in the electronic supplementary material, figure S3*a* and the type of data plots obtained is shown in the electronic supplementary material, figure S3*b*. The combined data from all analyses of 13 ABA-signalling mutants can be found in the electronic supplementary material, figure S4. This large dataset was condensed by carrying a multivariate statistical analysis using principal component analysis (PCA) to look for patterns or trends within groups of mutants and wild-type accessions (see electronic supplementary material, figure S3c,d). PCA showed that only minor effects occurred at the whole leaf level in mutants after variation among wild-type controls was taken into account. Some of these whole leaf effects could be a consequence of increased stomatal conductance (g_s), which have been observed during the first approximately 15 min of HL exposure [3,4]. Stomatal conductance, g_s, started and attained different values in many of the mutants and thus had altered carbon assimilation rates (*A*sat; table 1). The rates of change in g_s in response to 15 min HL were in many cases affected by the mutations (table 1).

Microarray analyses were conducted on whole leaf RNA prepared from HL-exposed wild-type leaves compared with the following ABA-signalling mutants: *ost1-1*, *snrk2.2/snrk2.3*, *gpa1-4*, *abi2-2/abi1-2/hab1-1* and *rbohF*. Apart from effects on the transcript levels of genes in the null mutants, no significant differences were detected in the mutants' transcriptomes compared with their wild-type controls. The data have been lodged with the European Bioinformatics Institute ArrayExpress (see Material and methods). It was concluded that HL-responsive genes expressed in major leaf tissues, for example the mesophyll, were not influenced by the same ABA-signalling pathways shown to affect *APX2* expression in BSCs.

(d) Does abscisic acid have any role to play in the immediate response of leaves to high light?

The above lack of any major effect of HL-associated ABA-signalling pathways on whole leaf responses contrasts with 816 genes identified from published data as commonly responsive to ABA and HL treatments [3]. However, these data were from plants treated with 10–100 μM ABA. We reasoned that this may have overestimated the number of ABA-regulated genes in HL-exposed leaves in which the levels only double [3]. Plants that overexpress the short-chain dehydrogenase gene *ABA2*, which codes for a key enzyme of ABA biosynthesis, show twofold increased levels of ABA [25]. The plants overexpressed *ABA2* under LL and HL conditions (figure 3*a*). Under HL conditions, *APX2* was 200-fold more expressed than in Col-0 (figure 3*b*). BSCs of HL-exposed ABA2OE plants showed less inhibition of Fq′/Fm′ compared with neighbouring mesophyll cells (figure 3*c*; $p \leq 0.001$) acting through Fv′/Fm′ ($p \leq 0.001$; figure 3*d*) and no effect on Fq′/Fv′ ($p > 0.001$; figure 3*e*). In contrast, the exposure of leaves to HL from 200 to 1400 μmol m^{-2} s^{-1} revealed that the ABA2OE had no differences in any Cf parameter compared with Col-0 (see electronic supplementary material, figure S4). Microarray analysis of total leaf RNA from HL-exposed ABA2OE plants showed no significant differences in gene expression compared with HL wild-type plants. The data are available for inspection from ArrayExpress (see Material and methods). It was concluded that changes in ABA levels

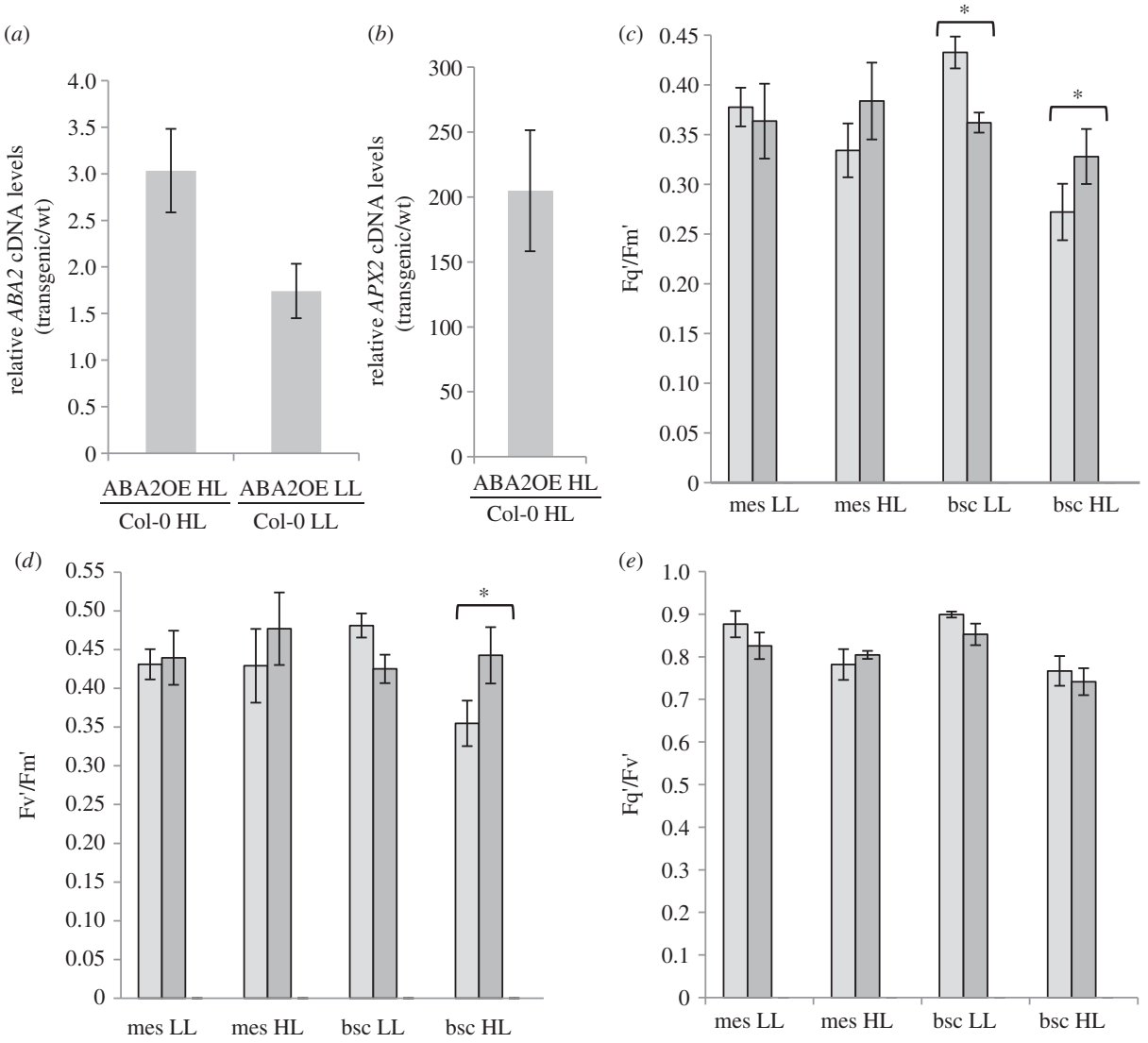

Figure 3. *APX2* and *ABA2* expression and response of BSCs and mesophyll cells to HL in ABA2OE and Col-0 plants. (*a*) *ABA2* cDNA levels in ABA2OE plants relative to Col-0 leaves from HL-exposed or LL plants normalized with respect to *CYC* expression (see legend figure 1*a* and Material and methods). Values are means (±s.e.) from four plants from two experiments ($n = 8$). (*b*) *APX2* cDNA levels in ABA2OE plants relative to Col-0 normalized to *CYC* expression. Values are means (±s.e.) from four plants from three experiments ($n = 12$). (*c*) Fq′/Fm′ values (mean ± s.e.; $n = 18$) for mesophyll (mes) and BSCs (bsc) of Col-0 (light grey bars) and ABA2OE (dark grey bars) plants subjected HL and LL as in the legend of figure 2. (*d*), as for (*c*), but Fv′/Fm′ values. (*e*), as for (*c*), but Fq′/Fv′ values. Pairs of columns marked with an asterisk (*) denote a significant difference ($p \leq 0.001$) as in figure 2.

associated with HL exposure [3] do not impact significantly on the transcriptomes of major leaf tissues.

4. Discussion

(a) *APX2* is controlled by two distinct but interacting ABA-signalling routes

Positive regulation of *APX2* expression by ABA is achieved by signalling primarily involving OST1 (SnRK2.6) and the PP2C isoform ABI2 (figure 1*a–c*). Of the remaining four foliar PP2C isoforms, HAB1, HAB2 and PP2CA were ruled out as regulators of *APX2* expression (figure 1*a*). However, the role of ABI1 is more ambiguous. The *abi1-1* and *abi2-1* mutations are in the phosphatase catalytic site (G180D and G168D, respectively), which causes reduced enzyme activity with a standard heterologous substrate [15]. However, genetically this causes a hypermorphic phenotype [12,32] often resulting in quantitatively indistinguishable characteristics associated with *abi1-1* and *abi2-1*, and consequently many functions of ABA they affect [15], including *APX2* expression (figure 1*b*) [5]. This degree of redundancy in the phenotype of *abi1-1* and *abi2-1* was not reflected in the null mutant *abi1-2* and *abi2-2* alleles in which there would have been an absence of the respective PP2C isoform (figure 1*a* and table 1) [14]. Thus from considering *APX2* expression in *abi2-1* and *abi2-2* (figure 1*a*,*b*), ABI2 can clearly be determined to regulate the expression of this gene. However, *abi2-2/abi1-2/hab1-1* showed markedly more stimulation of HL-responsive *APX2* expression than *abi2-2* (figure 1*a*), which suggests that ABI1 acts in a secondary role to, or cooperates with, ABI2 to regulate *APX2* expression in BSCs.

In a number of different experimental systems probing the role of ABA, the interaction of class A PP2Cs with SnRK2 class III isoforms shows a high degree of overlap [13,31]. By contrast, OST1 (SnRK2.6) retains a dominant role in HL-induced positive control of *APX2* expression and may be primarily confined to an interaction with ABI2 in this context. This could be because the ABI2:OST1 interaction is

BSC-specific in HL-exposed leaves and has to respond to small changes in ABA levels [3]. That an ABI2:OST1 combination may dominate the positive regulation of *APX2* may also reflect a more prominent role of H_2O_2 in modulating ABA signalling in BSCs than in other leaf tissues. ABI2 reversibly reacts, via redox-active cysteine residues, with H_2O_2, inhibiting its activity [33]. *In vivo*, this redox regulation of ABI2 may be conveyed by oxidized GPX3 [22]. However, no effect of *gpx3-1* on *APX2* expression was observed (data not shown). Nevertheless, it remains possible that another BSC-specific GPX isoform could fulfil a redox transduction role to ABI2 in this tissue. A putative reversible oxidation of ABI2 in BSCs is attractive because it would augment or amplify the regulation by ABA [13], thus enhancing a signalling response to HL despite a possible small increase in basal ABA levels [3]. In support of this suggestion, treatment of LL-grown leaves with 10 µM ABA, in the absence of any increase in H_2O_2, takes several hours to induce *APX2* expression [4].

There was a clear loss of *APX2* induction in HL-exposed *rbohF* leaves (figure 1*d*). One substrate of OST1 is RBOHF [34], which might connect plasma membrane-sourced ROS production to the OST1:ABI2 signalling pathway in BSCs. In *rbohD* and *rbhohD/rbohF* leaves, extracellular H_2O_2 production was diminished (figure 1*e*). This suggests that the increased ROS production may arise from RBOHD and that in wild-type plants the enzyme is negatively regulated by RBOHF in veinal tissue. This suggestion is consistent with a recent model showing that RBOHF negatively regulates RBOHD through salicylic acid [35]. Furthermore, the connection of G-protein signalling into NADPH oxidase-sourced ROS production [16,19,20] suggests a way in which the GPA1- and OST1-mediated signalling pathways can be distinct but interact to achieve a response of BSCs to HL (figure 1*c*).

(b) Bundle sheath cell chloroplasts are more susceptible to photoinhibition than mesophyll chloroplasts under high light conditions

HL-exposed BSCs have lower PSII operating efficiency (Fq'/Fm') than mesophyll cells (figure 2*a*,*b*; $p < 0.001$; Student's *t*-test). The lowered maximum PSII operating efficiency (Fv'/Fm') of BSC chloroplasts in HL compared with LL conditions (figure 2*c*) suggests that increased non-photochemical quenching [29] but not photochemical quenching capacity (Fq'/Fv'; figure 2*e*) occurred. Nevertheless, these changes in non-photochemical quenching were not sufficient to prevent photoinhibition in HL-exposed BSCs compared with mesophyll cells (figure 2*a*,*b*). This suggests that BSC chloroplasts suffered photoinhibition in HL, whereas mesophyll chloroplasts did not. *Arabidopsis* leaves are classified as 'moderately heterobaric' [36] i.e. have some limitations to the lateral diffusion of CO_2 through to BSCs, which may also offer some resistance to inward CO_2 diffusion [36]. Therefore, under HL conditions restricted photosynthetic capacity in BSCs could promote photoinhibition caused by increased singlet oxygen ($^1O_2$) production in PSII antennae, while still not being sufficient to promote a significant alteration in photochemical quenching. This situation would quickly lead to the production of other ROS. For example, the reaction of $^1O_2$ with the high amount of ascorbate in the chloroplast produces H_2O_2 under physiologically relevant conditions [37]. This would explain the increased H_2O_2 observed specifically in HL-exposed BSC chloroplasts [3,4], despite this HL exposure not promoting substantial photoinhibition in the rest of the leaf [3,4,6].

(c) Abscisic acid signalling modulates photoinhibition in high light-exposed bundle sheath cells

The reduction in PSII operating efficiency in the BSC chloroplasts of some ABA-signalling mutants in HL was less, such that the distinction between mesophyll and BSCs was lost (figure 2*a*; $p > 0.001$; Student's *t*-test). There was an association of altered PSII operating efficiency of BSCs in HL (figure 2*a*) to stomatal responses to HL in the mutants (table 1). However, this was not as evident in mesophyll cells of the same mutants (figure 2*b*). Thus in HL, guard cells are influenced by ABA signalling but it is difficult to discern how this might influence BSC responses. It has been proposed that a transitory drop in leaf turgor in HL caused by increased stomatal conductance would induce ABA biosynthesis in the vascular parenchyma [3,4]. In which case, mutants with restricted stomatal opening in response to HL (table 1) would result in less induction of *APX2* and less protection from photoinhibition. This did not occur (figures 1–3). Alternatively, lateral CO_2 diffusion to BSCs would be even more restricted in some of these mutants and ABA2OE plants, which should have increased susceptibility to photoinhibition in their BSCs. Manifestly this situation did not arise (figures 2*a* and 3*c*). Thus, these factors are unlikely to explain the mutants' reduced susceptibility to photoinhibition of BSCs or the effects upon *APX2* expression.

Those ABA-signalling mutants that show reduced differences in photoinhibition between mesophyll and BSCs (figures 2*b* and 3*c*) were associated with altered *APX2* expression (figures 1*a*–*c*, 3*b*). However, the loss of *APX2* expression in *apx2-1* and *apx2-2* did not affect responses to HL (see electronic supplementary material, figure S2). This may reflect that *APX2* is a single component of an extensive BSC antioxidant network, which ABA and H_2O_2 may regulate at multiple points. It should be noted that, to our knowledge, the antioxidant network of BSCs of C_3 plants, for example *Arabidopsis*, is unknown, with APX2 being the only known specific component [3,5].

Finally, it should be noted that the ABA signalling primarily influenced non-photochemical quenching (figures 2*c* and 3*d*), although how this was achieved is not clear from this study. An impact on antioxidant processes that decreased susceptibility of BSCs to photoinhibition may have been expected to be reflected in altered photochemical quenching [3,29]. This may explain the effect of *gpa1-4* on this parameter (figure 1*d*) but no other ABA-signalling mutant was affected (figures 1*d* and 3*e*). Therefore, the impact on non-photochemical quenching suggests strongly that ABA signalling in HL-exposed BSCs plays roles beyond direct regulation of an antioxidant network and supports a more extensive response to HL than has hitherto been envisaged.

Acknowledgements. The authors are grateful to Drs Pedro Rodriguez, Julian Schroeder, Wan-Hsing Cheng, Jian-Kang Zhu, Miguel Torres, Jeffrey Leung, Alan Jones and Nottingham *Arabidopsis* Stock Centre for providing the mutants used in this study.

Funding statement. K.S. and L.M. acknowledge the support of a BBSRC and NERC research studentship, respectively. S.K. and M.G. acknowledge the support of the Welcome2008/1 programme operated within the framework of the Foundation for Polish Science, co-financed by the European Regional Development Fund and by the REGPOT project FP7-286093 WULS-PLANT HEALTH.

References

1. Mullineaux PM, Baker NR. 2010 Oxidative stress: antagonistic signaling for acclimation or cell death? *Plant Physiol.* **154**, 521–525. (doi:10.1104/pp.110.161406)
2. Kangasjärvi S, Nurmi M, Tikkanen M, Aro E-M. 2009 Cell-specific mechanisms and systemic signaling as emerging themes in light acclimation of C3 plants. *Plant Cell Environ.* **32**, 1230–1240. (doi:10.1111/j.1365-3040.2009.01982.x)
3. Galvez-Valdivieso G *et al*. 2009 The high light response in *Arabidopsis* involves ABA signaling between vascular and bundle sheath cells. *Plant Cell*. **21**, 2143–2162. (doi:10.1105/tpc.108.061507)
4. Fryer MJ, Ball L, Oxborough K, Karpinski S, Mullineaux PM, Baker NR. 2003 Control of *Ascorbate Peroxidase 2* expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of *Arabidopsis* leaves. *Plant J.* **33**, 691–705. (doi:10.1046/j.1365-313X.2003.01656.x)
5. Ball L *et al*. 2004 Evidence for a direct link between glutathione biosynthesis and stress defence gene expression in *Arabidopsis*. *Plant Cell* **16**, 2448–2462. (doi:10.1105/tpc.104.022608)
6. Bechtold U, Richard O, Zamboni A, Gapper C, Geisler M, Pogson B, Karpinski S, Mullineaux PM. 2008 Impact of chloroplastic- and extracellular-sourced ROS on high light-responsive gene expression in *Arabidopsis*. *J. Exp. Bot.* **59**, 121–133. (doi:10.1093/jxb/erm289)
7. Endo A *et al*. 2008 Drought induction of *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol*. **147**, 1984–1993. (doi:10.1104/pp.108.116632)
8. Karpinski S, Reynolds H, Karpinska B, Wingsle G, Creissen G, Mullineaux PM. 1999 Systemic signalling and acclimation in response to excess excitation energy in *Arabidopsis*. *Science* **284**, 654–657. (doi:10.1126/science.284.5414.654)
9. Jung H-S, Crisp PA, Estavillo GM, Cole B, Hong F, Mockler TC, Pogson BJ, Chory J. 2013 Subset of heat-shock transcription factors required for the early response of *Arabidopsis* to excess light. *Proc. Natl Acad. Sci. USA* **110**, 14 474–14 479 (doi/10.1073/pnas.1311632110)
10. Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J. 2002 *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14**, 3089–3099. (doi:10.1105/tpc.007906)
11. Fujii H, Zhu J-K. 2009 *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl Acad. Sci. USA* **106**, 8380–8385. (doi:10.1073/pnas.0903144106)
12. Vlad F *et al*. 2008 Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. *Plant Cell* **21**, 3170–3184. (doi:10.1105/tpc.109.069179)
13. Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. 2010 Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol*. **61**, 651–679. (doi:10.1146/annurev-arplant-042809-112122)
14. Rubio S *et al*. 2009 Triple loss of function protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. *Plant Physiol*. **150**, 1345–1355. (doi:10.1104/pp.109.137174)
15. Leung J, Merlot S, Giraudat J. 1997 The *Arabidopsis ABSCISIC ACID-INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* **9**, 759–771. (doi:10.1105/tpc.9.5.759)
16. Suharsono U, Fujisawa Y, Kawasaki T, Iwasaki Y, Satoh H, Shimamoto K. 2002 The heterotrimeric G protein alpha subunit acts upstream of the small GTPase Rac in disease resistance of rice. *Proc. Natl Acad. Sci. USA* **99**, 13 307–13 312. (doi:10.1073/pnas.192244099)
17. Torres MA, Dangl JL, Jones JDG. 2002 *Arabidopsis* $gp91^{phox}$ homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl Acad. Sci. USA* **99**, 517–522. (doi:10.1073/pnas.012452499)
18. Kwak JM *et al*. 2003 NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J*. **22**, 2623–2633. (doi:10.1093/emboj/cdg277)
19. Mishra G, Zhang W, Deng F, Zhao J, Wang X. 2006 A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. *Science* **312**, 264–266. (doi:10.1126/science.1123769)
20. Zhang Y *et al*. 2009 Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in *Arabidopsis*. *Plant Cell*. **21**, 2357–2377. (doi:10.1105/tpc.108.062992)
21. Mullineaux PM, Karpinski S, Baker NR. 2006 Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants. *Plant Physiol*. **141**, 346–350. (doi:10.1104/pp.106.078162)
22. Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P. 2006 An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell*. **18**, 2749–2766. (doi:10.1105/tpc.106.044230)
23. Saez A, Apostolova N, Gonzalez-Guzman M, Gonzalez-Garcia MP, Nicola C, Lorenzo O, Rodriguez PL. 2004 Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C *HAB1* reveal its role as a negative regulator of abscisic acid signalling. *Plant J*. **37**, 354–369. (doi:10.1046/j.1365-313X.2003.01966.x)
24. Jones AM, Ecker JR, Chen JG. 2003 A re-evaluation of the role of the heterotrimeric G protein in coupling light responses in *Arabidopsis*. *Plant Physiol.* **131**, 1623–1627. (doi:10.1104/pp.102.017624)
25. Lin P-C, Hwang S-G, Endo A, Okamoto M, Koshiba T, Cheng W-H. 2007 Ectopic expression of *ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1* in *Arabidopsis* promotes seed dormancy and stress tolerance. *Plant Physiol*. **143**, 745–758. (doi:10.1104/pp.106.084103)
26. Szechynska-Hebda M, Kruk J, Gorecka M, Karpinska B, Karpinski S. 2010 Evidence for light wavelength-specific photoelectrophysiological signaling and memory of excess light episodes in *Arabidopsis*. *Plant Cell*. **22**, 2201–2218. (doi:10.1105/tpc.109.069302)
27. Larkindale J, Vierling E. 2007 Core genome responses involved in acclimation to high temperature. *Plant Physiol*. **146**, 748–761. (doi:10.1104/pp.107.112060)
28. Bechtold U, Lawson T, Mejia-Carranza J, Meyer RC, Brown IR, Altmann T, Ton J, Mullineaux PM. 2010 Constitutive salicylic acid defences do not compromise seed yield, drought tolerance and water productivity in the *Arabidopsis* accession C24. *Plant Cell Environ*. **33**, 1959–1973. (doi:10.1111/j.1365-3040.2010.02198.x)
29. Baker NR. 2008 Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu. Rev. Plant Biol*. **59**, 89–113. (doi:10.1146/annurev.arplant.59.032607.092759)
30. Lawson T, Lefebvre S, Baker NR, Morison JIL, Raines CA. 2008 Reductions in mesophyll and guard cell photosynthesis impact on the control of stomatal responses to light and CO_2. *J. Exp. Bot.* **59**, 3609–3619. (doi:10.1093/jxb/ern211)
31. Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Sinozaki K. 2009 Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **106**, 17 588–17 593. (doi:10.1073/pnas.0907095106)
32. Moes D, Himmelbach A, Korte A, Haberer G, Grill E. 2008 Nuclear localization of the mutant protein phosphatase abi1 is required for insensitivity towards ABA responses in *Arabidopsis*. *Plant J*. **54**, 806–819. (doi:10.1111/j.1365-313X.2008.03454.x)
33. Meinhard M, Rodriguez PL, Grill E. 2002 The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. *Planta* **214**, 775–782. (doi:10.1007/s00425-001-0675-3)
34. Sirichandra C *et al*. 2009 Phosphorylation of the *Arabidopsis* AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Lett*. **583**, 2982–2986. (doi:10.1016/j.febslet.2009.08.033)
35. Chaouch S, Queval G, Noctor G. 2012 *AtRbohF* is a crucial modulator of defence-associated metabolism and a key actor in the interplay between intracellular oxidative stress and pathogenesis responses in *Arabidopsis*. *Plant J*. **69**, 613–627. (doi:10.1111/j.1365-313X.2011.04816.x)
36. Morison JIL, Lawson T. 2007 Does lateral gas diffusion in leaves matter? *Plant Cell Environ*. **30**, 1072–1085. (doi:10.1111/j.1365-3040.2007.01685.x)
37. Kramarenko GG, Hummel SG, Martin SM, Buettner GR. 2006 Ascorbate reacts with singlet oxygen to produce hydrogen peroxide. *Photochem. Photobiol*. **82**, 1634–1637. (doi:10.1562/2006-01-12-RN-774)