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Singlet oxygen triggers chloroplast rupture and cell death in the zeaxanthin epoxidase defective mutant *aba1* of *Arabidopsis thaliana* under high light stress

Álvaro Sánchez-Corrionero[a,b,c], Inmaculada Sánchez-Vicente[b], Sergio González-Pérez[a], Ascensión Corrales[a,b], Anja Krieger-Liszkay[d], Óscar Lorenzo[b], Juan B. Arellano[a,*]

[a] Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de merinas 52, Salamanca 37008, Spain
[b] Departamento de Botánica y Fisiología Vegetal, Instituto Hispano-Luso de Investigaciones Agrarias (CIALE), Facultad de Biología, Universidad de Salamanca, C/Río Duero 12, Salamanca 37185, Spain
[c] Department of Biotechnology, Center for Plant Genomics and Biotechnology, Universidad Politécnica de Madrid, Pozuelo de Alarcón 28223, Spain
[d] Institute for Integrative Biology of the Cell, Commissariat à l'Energie Atomique et aux Energies Alternatives Saclay, Institut des sciences du vivant Frédéric Joliot, Centre National de la Recherche Scientifique, Université Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette Cedex 91198, France

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ABSTRACT

The two *Arabidopsis thaliana* mutants, *aba1* and *max4*, were previously identified as sharing a number of co-regulated genes with both the *flu* mutant and Arabidopsis cell suspension cultures exposed to high light (HL). On this basis, we investigated whether *aba1* and *max4* were generating high amounts of singlet oxygen ($^1O_2$) and activating $^1O_2$-mediated cell death. Thylakoids of *aba1* produced twice as much $^1O_2$ as thylakoids of *max4* and wild type (WT) plants when illuminated with strong red light. $^1O_2$ was measured using the spin probe 2,2,6,6-tetramethyl-4-piperidone hydrochloride. 77-K chlorophyll fluorescence emission spectra of thylakoids revealed lower aggregation of the light harvesting complex II in *aba1*. This was rationalized as a loss of connectivity between photosystem II (PSII) units and as the main cause for the high yield of $^1O_2$ generation in *aba1*. Up-regulation of the $^1O_2$ responsive gene *AAA-ATPase* was only observed with statistical significant in *aba1* under HL. Two early jasmonate (JA)-responsive genes, *JAZ1* and *JAZ5,* encoding for two repressor proteins involved in the negative feedback regulation of JA signalling, were not up-regulated to the WT plant levels. Chloroplast aggregation followed by chloroplast rupture and eventual cell death was observed by confocal imaging of the fluorescence emission of leaf cells of transgenic *aba1* plants expressing the chimeric fusion protein SSU-GFP. Cell death was not associated with direct $^1O_2$ cytotoxicity in *aba1*, but rather with a delayed stress response. In contrast, *max4* did not show evidence of $^1O_2$-mediated cell death. In conclusion, *aba1* may serve as an alternative model to other $^1O_2$-overproducing mutants of Arabidopsis for investigating $^1O_2$-mediated cell death.

1. Introduction

Programmed cell death (PCD) is an important process for plant development and response to environmental cues (van Doorn et al., 2011). At a cellular level, plant PCD is a programmed process consisting of a succession of intricate and energy-demanding cellular events that requires interplay and movement of several plant organelles, particularly chloroplasts and mitochondria, and retrograde communication with the cell nucleus for the regulation of target genes (Reape et al., 2015; Wertman et al., 2012).

Among the list of reactive oxygen species (ROS) that activates PCD, singlet oxygen ($^1O_2$) has received special attention over the last two decades after the publication of a seminal series of studies on $^1O_2$-mediated cell death in the conditional *fluorescent* (*flu*) mutant of

Abbreviations: ABA, abscisic acid; ACD2, ACCELERATED CELL DEATH2; ACSC, Arabidopsis cell suspension cultures; *ch1*, *chlorina1*; Chl, chlorophyll; DEPMPO, 2-diethylphosphono-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide; EDS1, ENHANCED DISEASE SUSCEPTIBILITY1; EX, executer; JA, jasmonate; JAZ, JASMONATE ZIM-DOMAIN; *flu*, *fluorescent*; HL, high light; H_2O_2, hydrogen peroxide; HO·, hydroxyl radical; NPQ, non-photochemical quenching; O_2^-, superoxide anion radical; OXI1, OXIDATIVE SIGNAL INDUCIBLE1; PCD, programmed cell death; Pchlide, protochlorophyllide; PI, propidium iodide; PS, photosystem; RC, reaction centre; ROS, reactive oxygen species; SL, strigolactone; SOD, superoxide dismutase; SSU-GFP, chimeric fusion protein consisting of the precursor of the nucleus-encoded and chloroplast-localized small subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase and the green fluorescence protein; TEMPD-HCl, 2,2,6,6-tetramethyl-4-piperidone hydrochloride; WT, wild type

* Corresponding author.
E-mail address: juan.arellano@irnasa.csic.es (J.B. Arellano).

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Arabidopsis thaliana ecotype Landsberg *erecta* (Meskauskiene et al., 2001; op den Camp et al., 2003). *flu* is characterized by the inactivation of the FLU protein, a nucleus-encoded plastid protein with a key role in the feedback control of chlorophyll (Chl) biosynthesis that acts independently from the heme-dependent feedback loop. Dark-incubated seedlings of *flu* accumulate protochlorophyllide (Pchlide) in thylakoids and exhibit strong Pchlide fluorescence when they are exposed to blue light. After a dark-to-light shift, Pchlide in its triplet excited state reacts with O_2, generating $^1O_2$ that leads to cell death. In the *flu* mutant, the onset of cell death is not a necrotic event relying on direct damage by cytotoxic $^1O_2$ to essential cellular components; in contrast, it is a $^1O_2$ genetically-mediated event that requires the participation of two plastid nuclear-encoded proteins denoted EXECUTER1 (EX1) and EX2 (Lee et al., 2007). In mature plants, the *flu* mutant does not die, but the plant growth ceases and the leaves display necrotic lesions. Cell death progression is only observed if extensive light stress is applied to wild type (WT) plants. In this latter case, cell death cannot be exclusively attributed to the EX-dependent signalling pathway (Kim et al., 2012) and other $^1O_2$-mediated signalling pathways have been invoked (Carmody et al., 2016; Dietz et al., 2016; Pattanayak et al., 2012; Shumbe et al., 2016).

In WT plants, $^1O_2$ production mainly takes place in the reaction centre (RC) of photosystem II (PSII) (Arellano and Naqvi, 2016; Krieger-Liszkay, 2005). Oxidation products of PSII-RC β-carotene, like β-cyclocitral, certain lipid oxidation products (i.e. oxylipins) or specific regulatory proteins (i.e. WHIRLY or PTM proteins) have been proposed to be messengers of the $^1O_2$ signal out of chloroplasts and to mediate gene responses to $^1O_2$ (Dietz et al., 2016; Ochsenbein et al., 2006; Ramel et al., 2012, 2013b). Under most conditions other ROS (superoxide radical $O_2^{\cdot -}$, hydrogen peroxide H_2O_2, hydroxyl radical $HO^{\cdot}$) are formed together with $^1O_2$ at high light (HL) stress (Foyer and Noctor, 2003) and they cross-talk even with antagonistic effects (Laloi et al., 2007).

In an attempt to shed more light on $^1O_2$-mediated signalling in plants, Havaux and colleagues worked with an alternative $^1O_2$-producing physiological model, where $^1O_2$ was indeed photosensitized in PSII. The Arabidopsis *chlorina1* (*ch1*) mutant is devoid of light harvesting-chlorophyll *a/b*-protein because it holds a defect in the synthesis of Chl *b* (Ramel et al., 2013a). Light stress in *ch1* induced leaf bleaching, which was accompanied with both genetically regulated lipid oxidation and direct lipid oxidation by $^1O_2$. A good correlation between the transcriptional profiles of *ch1* and *flu* was found, except that the EX- and ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)-dependent signalling cascade was not responsible for cell death in *ch1*. Differences in the nature of the $^1O_2$ producers (Pchlide *vs* PSII) and the production of different messengers were presented as a plausible explanation (Ramel et al., 2013a). In line with the former study, Rose Bengal—a water-soluble artificial photosensitizer generating $^1O_2$—was observed to activate PCD in Arabidopsis cell suspension cultures (A-CSC), but only when (light-grown) cells had functional chloroplasts (Gutierrez et al., 2014). The inspection of the transcriptional profile changes in ACSC when treated with Rose Bengal did not reveal the up-regulation of *EDS1*. In a recent study, the single mutant *oxi1*, an Arabidopsis mutant deficient in the *OXIDATIVE SIGNAL INDUCIBLE 1* (*OXI1*) gene encoding for a Ser/Thr kinase, and the double mutant *ch1 oxi1* showed to be more resistant to HL stress and to exhibit a drastic decrease in $^1O_2$-mediated cell death when compared with Arabidopsis WT plants and the single mutant *ch1* (Shumbe et al., 2016). Based on this finding, the former authors proposed an alternative OXI1-dependent signalling to regulate $^1O_2$-mediated cell death in Arabidopsis. Interestingly, the mobilization of natural photosensitizers between plant organelles also induces cell death (Pattanayak et al., 2012). The ACCELERATED CELL DEATH2 (ACD2) can accumulate in mitochondria during pathogen infection where it catabolizes red-chlorophyll-catabolite, a $^1O_2$ photosensitizer that can migrate from chloroplasts to mitochondria. The activity of ACD2 was observed to minimize $^1O_2$ formation in mitochondria, avoiding the onset of cell death. The triple mutant of *acd2 exe1 exe2* did not display alterations in PCD phenotype when compared with the single mutant *acd2*; a fact that contrasts with the triplet mutant *flu exe1 exe2*, where $^1O_2$ was overproduced in chloroplasts and PCD was not activated (Kim et al., 2012). An explanation to this apparent controversy between different $^1O_2$-dependent signalling pathways has recently been offered by Apel and co-workers, who have proposed that the EX-dependent signalling pathway is initiated in grana margins under non-photoinhibitory conditions (Wang et al., 2016).

Together with the single *flu* and *ch1* mutants, other Arabidopsis (double) mutants as *npq1 lut2* and *vte1 npq1* were described to generate high levels of $^1O_2$, but cell death was not genetically activated (Alboresi et al., 2011; Triantaphylides et al., 2008). The *npq1 lut2* lacks zeaxanthin and lutein and $^1O_2$ signalling activated the expression of genes whose function was to protect chloroplasts from the damaging effects of ROS, resulting in a stress acclimation response. The *vte1 npq*1 has defects in the biosynthesis of α-tocopherol and zeaxanthin and cell death was observed when plants of this double mutant were exposed to HL stress; however, the analysis of the oxidation products of lipids suggested a direct destructive role by $^1O_2$.

As described above, genetically and non-genetically $^1O_2$-mediated responses vary notably from WT plants to mutants producing high yields of $^1O_2$ and between these mutants. One key element which may help to elucidate this problem may be the interaction of ROS with hormones. In two single mutants of Arabidopsis, *max4* and *aba1* that are deficient in strigolactones (SLs) and abscisic acid (ABA), respectively, a cluster of co-regulated genes was previously identified in a transcriptional profile analysis that included ACSC, the *flu*, *max4* and *aba1* mutants and Arabidopsis WT plants treated with several hormones (Gonzalez-Perez et al., 2011). On the basis of this transcriptional analysis, it was proposed that $^1O_2$ may be overproduced in *max4* and *aba1* (Gonzalez-Perez et al., 2011; Gutierrez et al., 2011). ABA and SL*s* exert their local and systemic regulatory roles in plant development and stress tolerance in conjunction with several types of ROS (Hou et al., 2016; Xia et al., 2015) and other plant hormones (Cutler et al., 2010; Ton et al., 2009; Xiong et al., 2014). ABA plays a critical role in plant responses to drought stress regulating stomatal closure and root growth and also promotes resistance to pathogen attack (Ton et al., 2009). *aba1* is defective in zeaxanthin epoxidase responsible for the transformation of zeaxanthin—the first xanthophyll precursors of ABA—into antheraxanthin and violaxanthin, exhibits a stunted phenotype and keeps widely open the stomata even under water stress conditions (Barrero et al., 2005; Mizokami et al., 2015). SL*s* biosynthesis is enhanced under nitrogen and phosphorous limited conditions and they modify the architecture of roots to optimize the nutrient uptake and increase symbiotic interactions (Xiong et al., 2014). *max4* is defective in a plastid-localized carotenoid-cleaving dioxygenase involved in the biosynthesis pathway of the branch-inhibiting SL*s* and shows an increased shoot branching phenotype that can be rescued after the exogenous application of SL*s* (Sorefan et al., 2003). In addition, *max4* is hypersensitive to drought stress and exhibits an increased leaf stomatal density and a slow response to ABA-induced stomatal closure in comparison with WT plants (Ha et al., 2014).

The aim of this study is to show whether *max4* and *aba1* have indeed higher yields of $^1O_2$ production and also whether $^1O_2$-mediated cell death is activated in these mutants. Here we present data on $^1O_2$ production detected by the spin probe 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPD-HCl) and on cell morphological changes and cell death after HL stress using the chimeric fusion protein SSU-GFP, consisting of the precursor of the nucleus-encoded and chloroplast-localized small subunit of ribulose-1,5-phosphate carboxylase/oxygenase (SSU) and the green fluorescence protein (GFP).

2. Material and methods

2.1. Construction of aba1 and max4 mutant lines carrying the transgene 35S:SSU-GFP

Seeds of the transgenic *Arabidopsis thaliana* Col-0 WT plants expressing the chimeric fusion protein SSU-GFP were kindly gifted by Prof. Apel (Kim et al., 2012). Seeds of the transgenic plant *Arabidopsis thaliana* Col-0;*35S:SSU-GFP*, the Arabidopsis mutants *aba1* (*aba1-101*) (Barrero et al., 2005) and *max4* (*max4-1*; ABRC stock) (Sorefan et al., 2003) and new Arabidopsis mutant lines (see below) were sterilized, stratified and placed in agar plates containing MS medium for germination as previously described (Fernandez-Arbaizar et al., 2012; Murashige and Skoog, 1962).

The transgenic *Arabidopsis thaliana* Col-0;*35S:SSU-GFP* was crossed with the *max4* and *aba1* mutants. The segregation and selection of the F2 generations of the new Arabidopsis mutant lines was based on their phenotype, but not on their antibiotic resistance markers because the transgenic *Arabidopsis thaliana* Col-0;*35S:SSU-GFP* and the *aba1* and *max4* mutants were all resistant to Basta (Bainbridge et al., 2005; Kim et al., 2012). Plant mutants of the F2 generation exhibiting green fluorescence emission from the reporter fusion protein SSU-GFP in their organs and holding the respective characteristic phenotypes of *aba1* and *max4* were selected. The phenotype of the F3 generation of the new Arabidopsis mutant lines expressing the chimeric fusion protein SSU-GFP is shown in the online resource (online resource, Fig. S1). Under the 35S promoter control, the *SSU-GFP* expression is constitutive and the chimeric fusion protein also accumulates in other plant organs like roots (online resource, Fig. S2).

2.2. Light stress conditions

The 9-day-old Arabidopsis WT plants and the *aba1* and *max4* mutant lines were illuminated at 20–22 °C with a 1000 μE m^{-2} s^{-1}m red light emitting diode centered on 650 nm during different time exposures. Red light, instead of white light, was used in all the light stress treatments. It was chosen to excite mainly Chl. After each light stress treatment, Arabidopsis leaves were collected and they were either used immediately for further experiments or kept at −80 °C until use.

2.3. Thylakoid isolation

Thylakoids were isolated according to standard procedures (Arellano et al., 1994). Oxygen evolution rates of thylakoids were measured polarographically using an oxygen electrode type Clark at saturating light conditions. The reaction mixture contained thylakoids with a concentration in Chl of 20 μg mL^{-1}, 1 mM $K_3[Fe(CN)_6]$ as electron acceptor and 10 mM NH_4Cl as uncoupler to destroy the pH gradient.

2.4. $^1O_2$ and $O_2\cdot^-$ radical detection by EPR spin trapping

Spin-trapping assays with TEMPD-HCl (Sigma-Aldrich, St. Louis, USA) to detect $^1O_2$ (Krieger-Liszkay et al., 2015; Ramel et al., 2013a) or the spin probe 2-diethylphosphono-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide (DEPMPO) to detect the formation of $O_2\cdot^-$ and HO· (Frejaville et al., 1995; Heyno et al., 2009) were carried out using thylakoid membranes at a concentration of 10 μg Chl mL^{-1}. The thylakoids were suspended in a buffer containing 0.3 M sorbitol, 50 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES pH 7.6. Samples were illuminated for 2 min with red light (RG 630) (1000 μE m^{-2} s^{-1}) in the presence of 100 mM TEMPD-HCl or in the presence of 50 mM DEPMPO. In the DEPMPO assay 50 μM diethylenetriaminepentaacetic acid was added and the thylakoids were uncoupled. EPR spectra were recorded at room temperature in a standard quartz flat cell using an ESP-300 X-band spectrometer (Bruker, Rheinstetten, Germany). The following parameters were used: microwave frequency, 9.73 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 G; microwave power, 63 mW in TEMPD assays; receiver gain, 2×10^4; time constant, 40.96 ms and number of scans 4. For control experiments, D_2O and NaN_3 were used to enhance or to quench $^1O_2$ production and superoxide dismutase (SOD) (50 U) to disproportionate $O_2\cdot^-$.

2.5. *Chl*fluorescence measurements

Chl Fluorescence emission spectra at 77-K were measured in a PTI spectrofluorometer (Model QM-2000-4). Thylakoids were diluted in a solution mixture containing the storage buffer and glycerol in ratio of 1:2 (v/v). Thylakoids were excited at 435 nm and the intensity of the fluorescence emission was monitored in the range between 650 nm and 800 nm. Excitation and emission slits were adjusted at 5.0 nm and 2.5 nm, respectively. The absorbance of thylakoids was kept below 0.05 at the Q_y region. The 77-K Chl fluorescence emission spectra of thylakoids were fitted to six Gaussian curve components: F680 (peak, 681 nm; half band width, 10.1 nm), F685 (peak, 685 nm; half band width, 9.3 nm), F695 (peak, 693 nm; half band width, 9.2 nm), F700 (peak, 700 nm; half band width, 15.8 nm), F720 (peak, 720 nm; half band width, 21.9 nm) and F735 (peak, 735 nm; half band width, 23.4 nm). The amplitude of F680 and F700 were used to determine aggregation of LHCII in thylakoids (Yamamoto et al., 2013).

2.6. Target transcripts to evaluate ROS and JA responsive genes after light stress conditions

The transcripts At5g64870, At3g28580, At5g01600, encoding for NODULIN-like protein, the AAA-type ATPase family protein and FERRITIN1, respectively, were chosen based on previous studies, where their fast or slow up-regulation or the cross-talk between $^1O_2$- and H_2O_2-dependent signallings of stress responses were investigated in ACSC, Arabidopsis WT plants or mutants (Gonzalez-Perez et al., 2011; Kim et al., 2012; Laloi et al., 2007; op den Camp et al., 2003; Ramel et al., 2013a). The transcripts At1g19180 and At1g17380 encoding for JASMONATE ZIM-DOMAIN1 protein (JAZ1) and JAZ5, respectively—two repressor proteins of the jasmonate (JA) signalling (Chini et al., 2007; Thines et al., 2007)—were also included. The transcript At2g19760 (*PROFILIN1*) is a member of an Arabidopsis constitutive multigene family (Cao et al., 2016; Mussar et al., 2015) and it was selected as house-keeping gene and used as internal reference gene to account for experimental variations. *PROFILIN1* expression has shown to be unaffected in previous light stress studies, unlike the usually selected *ACTIN2* (Laloi et al., 2007). The primers designed to amplify the selected transcripts are shown in the online resource Table S1.

RNA isolation and relative quantification of mRNA expression were performed as previously described (Gonzalez-Perez et al., 2011; Livak and Schmittgen, 2001). The quantitative PCR analysis was applied to plants that were exposed to light stress conditions for 30 min. Relative mRNA abundance was calculated using the comparative delta-Ct method (Livak and Schmittgen, 2001) and expressed in relative values normalized to the value at time 0 (normal growth light conditions). Three biological replicates were analysed in this study and three technical replicates were performed for each sample. Data are mean values of three independent biological replicates ± SD.

2.7. Analysis of the cellular distribution of SSU-GFP by confocal microscopy

The green and red fluorescence emission of the SSU-GFP protein and chlorophyll-binding proteins was monitored with a confocal microscope (model DM IRB; Leica Microsystems) following excitation of the SSU-GFP and chlorophyll-binding proteins at 488 nm and 633 nm, respectively, with an argon laser and a Triplet Dichroic 488/543/633 excitation beam splitter. Confocal images were taken using the

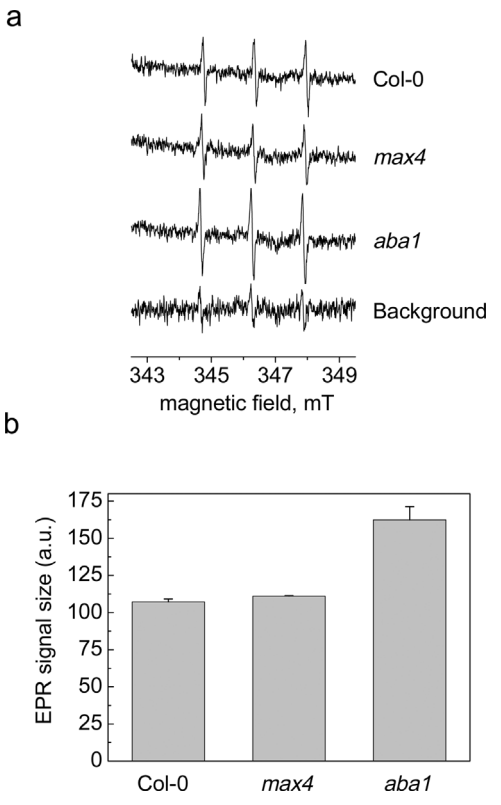

Fig. 1. Singlet oxygen detection by EPR spectroscopy using TEMPD-HCl as spin probe in thylakoids of Arabidopsis WT plants and the *max4* and *aba1* mutants. Thylakoids were illuminated with HL (1000 μE m^{-2} s^{-1}) for 2 min. The background signal of TEMPD-HCl illuminated in the suspension buffer, but in the absence of thylakoid, is shown for comparison. a) Typical spectra of the spin adduct are shown. b) Data from two independent preparations and 3 repetitions per preparation. The data represent the mean ± SD.

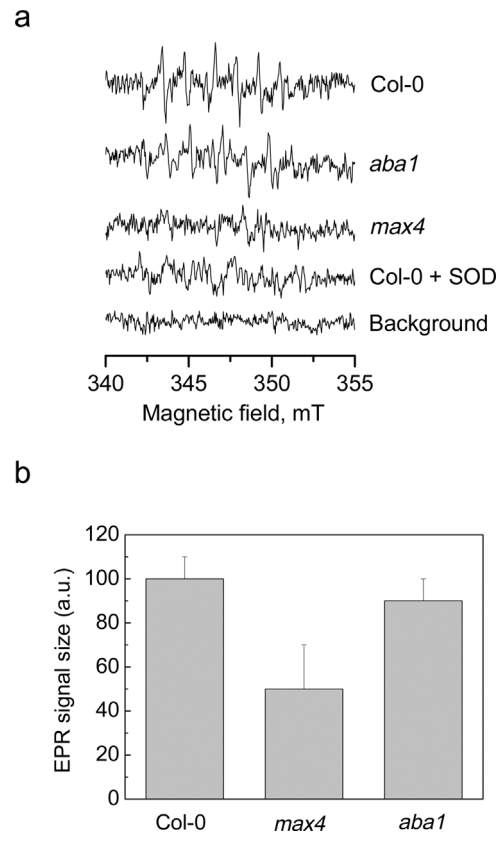

Fig. 2. ROS detection by spin trapping EPR using DEPMPO as spin probe in thylakoids of Arabidopsis WT plants and the *max4* and *aba1* mutants. Thylakoids were illuminated with HL (1000 μE m^{-2} s^{-1}) for 2 min. SOD (50 U) was added to thylakoids of Arabidopsis WT as a control experiment and the background signal of DEPMPO illuminated in the suspension buffer, but in the absence of thylakoid, was shown for comparison. a) Typical spectra of the spin adducts are shown. b) Data from two independent preparations and 3 repetitions per preparation. The data represent the mean ± SD.

objective HC PL APO 63x/1.20 W CORR CS. The leaves of the transgenic plants were mounted on a slide with water and covered with a coverslip. The fluorescence emission of the SSU-GFP protein and chlorophyll-binding proteins was collected at 500–600 nm and 680–700 nm, respectively. Cell death was also examined by confocal microscopy using the nucleic acid binding dye propidium iodide (PI). Nine-day-old seedlings of the transgenic plants were dipped in a solution of 50 μM PI for few seconds. The excess of PI was removed by rinsing the seedlings in distilled water. The seedlings were mounted on slide as above and the fluorescence emission of PI was collected at 600–630 nm using a wavelength excitation at 543 nm. Control and HL stressed plants of three different experiments were used to take confocal images with similar results.

3. Results

Fig. 1 shows light-induced $^1O_2$ generation in thylakoids from Arabidopsis WT plants and the *aba1* and *max4* mutants. The generation of $^1O_2$ was measured using the spin probe TEMPD-HCl and illuminating thylakoids for 2 min at HL (1000 μE $m^{-2}s^{-1}$). Thylakoids of *max4* displayed an EPR signal in amplitude similar to that of WT thylakoids, showing that in both cases the photochemical production of $^1O_2$ was rather equal. On the contrary, the amplitude of the EPR signal of thylakoids of *aba1* was approximately 50% larger than that observed in thylakoids from WT and *max4* (Fig. 1a). To demonstrate that the EPR signals could be attributed to $^1O_2$ production generated by illuminating thylakoid membranes, control experiments have been performed previously with the addition of either NaN_3, a quencher of $^1O_2$, or D_2O, that increases the lifetime of $^1O_2$ (Krieger-Liszkay et al., 2015). In addition to $^1O_2$, the formation of $O_2^{\cdot -}$ and HO· was measured using DEPMPO as spin trap (Frejaville et al., 1995; Heyno et al., 2009).

Illumination of thylakoids of *aba1* and WT plants gave signals of the superoxide adduct of comparable size while *max4* showed a significantly smaller signal (Fig. 2). Taken together, the EPR measurements show that thylakoids from *aba1* generate more $^1O_2$ under HL than those from WT plants and *max4*. To exclude that the differences in $^1O_2$ generation between thylakoids from *aba1* and from the two other samples were caused by a loss of water-splitting activity, the activity of the photosynthetic electron transport chain was measured using 1 mM $K_3[Fe(CN)_6]$ as electron acceptor. In addition, to exclude difference in the proton motif force between the thylakoids from the three genotypes, the measurements were also done in the presence of 10 mM NH_4Cl as uncoupler. As shown in Table 1, the oxygen evolution activity of the thylakoids was not affected in the mutants.

To test whether changes in the antenna of *aba1* would be responsible for the higher yield of $^1O_2$ production in this mutant, 77-K Chl fluorescence spectra were measured (Fig. 3). In brief, the 77-K Chl fluorescence emission spectra of plant thylakoids display three

Table 1
Oxygen evolution (μmol O_2 mg Chl^{-1} h^{-1}) and F700/F680 fluorescence ratio of isolated thylakoid membranes.[a]

	(−) NH_4Cl	(+) NH_4Cl	F700/F680
Col-0	61 ± 4	180 ± 10	0.9 ± 0.1
max4	68 ± 5	184 ± 07	1.0 ± 0.1
aba1	65 ± 3	200 ± 10	0.54 ± 0.05

[a] Data were obtained with two independent preparations and 3 repetitions per preparation. The data represent the mean ± SD.

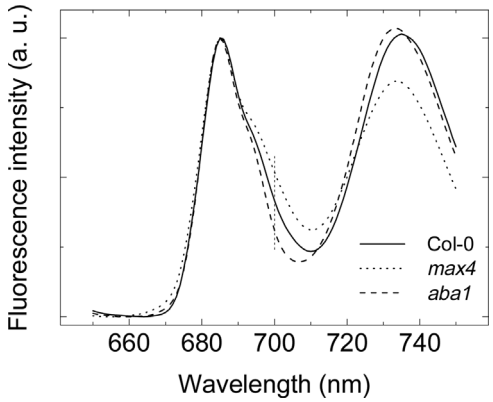

Fig. 3. Representative 77-K Chl fluorescence emission spectra of thylakoids from Arabidopsis WT plants and the *max4* and *aba1* mutants. The fluorescence emission spectra were normalized to the maximum of the LHCII-PSII band to better compare the F700 region (vertical dashed line), representing aggregated trimers of LHCII. Thylakoids were excited at 435 nm. See the text for further details.

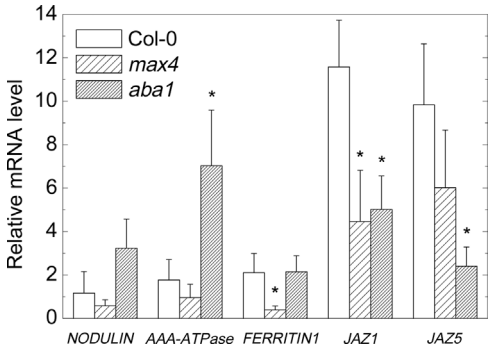

Fig. 4. Fold changes in the expression of selected markers responding to $^1O_2$ (*NODULIN* and *AAA-ATPase*), H_2O_2 (*FERRITIN1*) and JA signalling (*JAZ1* and *JAZ5*) in 9-day-old Arabidopsis WT plants and the *max4* and *aba1* mutants. Plants were illuminated with HL (1000 μE m^{-2} s^{-1}) for 30 min. Data are expressed in relative values normalized to the value at time 0 (growth light controls). Results shown are mean values ± SD of three measurements from three independent biological samples. * p-value < 0.05.

characteristic peaks at 685 nm, 695 nm and 735 nm, the former two peaks attributed to the Chl *a/b* light harvesting complex II (LHCII) and PSII, and the latter to PSI (Siefermann-Harms, 1988). Interestingly, LHCII aggregation induces changes in the fluorescence emission spectra of thylakoids around 700 nm (F700). This fluorescence band is related to the formation of excitonically coupled Chl molecules with a key role in non-photochemical quenching (NPQ) (Muller et al., 2010; Ruban et al., 2012). *aba*1 is known to exhibit a decreased stability of the major LHCII and a weakened association between the major LHCII and minor LHCII complexes (Lokstein et al., 2002; Tardy and Havaux, 1996). The normalized 77-K Chl fluorescence emission spectra indicated that the LHCII-PSII/PSI ratio was very similar in the three types of thylakoids under study, although it was often found slightly lower in thylakoids of *aba1* (Fig. 3). The curve fitting analysis with six Gaussian curve components was performed to determine the amplitude of F700, representing aggregated trimers of LHCII, and F680, representing free trimeric and monomeric forms of LHCII (Horton et al., 1991; Yamamoto et al., 2013). The F700/F680 ratio showed that LHCII aggregation was lower in *aba1* irrespective of the thylakoid batch (Table 1 and online resource, Fig. S3). This fluorescence spectral feature in thylakoids of *aba1* reinforces the view that PSII's ability to form aggregates trimers of LHCII is limited.

Next we investigated by quantitative PCR analysis whether the higher yield of $^1O_2$ in *aba1* affected the expression level of ROS-mediated responsive genes. The effect of HL stress on the early gene expression level of several specific markers for $^1O_2$ (*AAA-ATPase*) and H_2O_2 (*FERRITIN1*) and of *NODULIN* (op den Camp et al., 2003) was followed in WT plants and in the *max4* and *aba1* mutants. We also included two early JA-responsive genes, *JAZ1* and *JAZ5*, in the analysis. The encoded JAZ1 and JAZ5 proteins are negative regulators of the JA signalling (Chini et al., 2007; Thines et al., 2007) that are rapidly induced in response to *Pseudomonas syringae* infection (Demianski et al., 2012; Ishiga et al., 2013) or HL stress in ACSC where an acclimation response was observed (Gonzalez-Perez et al., 2011). Fig. 4 shows how the ROS markers were discreetly up-regulated in Arabidopsis WT plants after 30 min of HL stress, implying that, together with $^1O_2$ production, other ROS (i.e. $O_2\cdot^-$/H_2O_2) were formed (see Figs. 1 and 2) (Asada, 1999; Li et al., 2009). The selected transcripts did not exhibit up-regulation in *max4*; on the contrary, the transcriptional expression of *FERRITIN1* decreased with statistical significance. Thylakoids of *max4* held similar features to those of control thylakoids when $^1O_2$ production was examined, but different ones when $O_2\cdot^-$ production was examined (Fig. 2). This contrasts with the results for *aba1*, where *AAA-ATPase* was up-regulated with statistical significance. This result correlated well with the enhanced $^1O_2$ production in thylakoids of *aba1* under HL (Fig. 1). The up-regulation of *FERRITIN1* in *aba1* was similar to that in Arabidopsis WT plants, suggesting that in *aba1*, together with $^1O_2$, other ROS were formed. *JAZ1* and *JAZ5* were remarkably up-regulated in WT plants, showing that a rapid JAZ repressor-mediated defence response was active and the JA signalling attenuated in WT plants. They were also up-regulated in the *max4* and *aba1* mutants, but the level of expression was significantly lower. The lack of SLs and ABA in the *max4* and *aba1* pointed that the proper gene expression regulation of these two negative regulators of JA signalling required intact synthesis of both hormones and cross-talk of SLs and ABA with JA.

The effect of $^1O_2$ photoproduction on chloroplast dynamics and integrity was investigated in the transgenic plant lines of the three genotypes expressing the reporter fusion protein SSU-GFP. Changes in the cellular localization of this chimeric fusion protein after HL at different time exposures were followed by confocal microscopy. The analysis of the confocal micrographs of the green and red fluorescence emissions of SSU-GFP and the chlorophyll-binding proteins coming out from chloroplasts revealed that these organelles remained intact during the first two hours of light treatment (Fig. 5), implying that the level of $^1O_2$ produced during this period of time was not high enough to induce any visible damage to chloroplasts in any of the three transgenic plant lines. The confocal micrographs also showed that chloroplasts were usually localized in a region of the cytoplasm close to the plasma membrane forming in some cases short ring-like strings of beads.

According to the EPR analysis, thylakoids of *aba1* were more prone to overproducing $^1O_2$ and differences between this mutant and Arabidopsis WT plants and *max4* would thus have been observed if the HL stress had persisted. Prolongation of the HL stress to approximately 14 h was required to visualize both chloroplast aggregation and chloroplast rupture in different leaf cells of the transgenic plant line of *aba1*, while there was no evidence of subcellular changes in leaf cells of the transgenic plant lines of Arabidopsis WT and *max4* (Fig. 6). Dynamic movement of mitochondria and chloroplasts has been described as an event that precedes PCD in *acd2* and Arabidopsis WT plants challenged with *Pseudomonas syringae* (Yao and Greenberg, 2006). Figs. 5 and 6 show that chloroplasts form ring-like strings of beads in non-stressed *aba1* and even in WT plants and *max4* after the 14-h HL treatment. In contrast, evidence of either chloroplast aggregation or chloroplast rupture was observed in different batches of leaf cells of *aba1* after 14-h HL treatment, corresponding with different stages of the initiated cell death program in *aba1*. In the early stage, chloroplasts moved from the plasma membrane region towards a central region of the cell where they formed aggregates, but where they could still be distinguished as individual bright fluorescence emitting spots. In this stage, cell death was not yet prominent in *aba1* based on the scarce number of cell nuclei emitting PI fluorescence.

Chloroplast rupture followed chloroplast aggregation in *aba1* at later stage. The green and red fluorescence emissions of the SSU-GFP

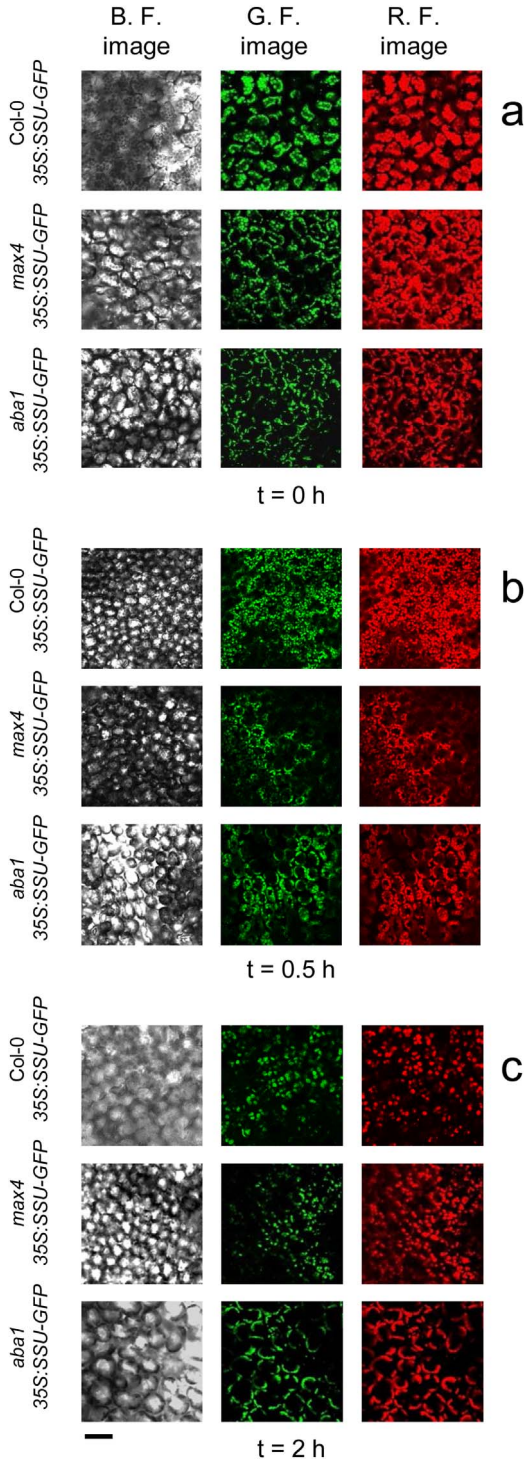

Fig. 5. Representative confocal micrographs of leaf cells of the transgenic plant lines of *Arabidopsis thaliana* Col-0 and the *max4* and *aba1* mutants expressing the chimeric fusion protein SSU-GFP after short HL stress periods. Plants were illuminated with normal light conditions (a) or with HL (1000 μE m^{-2} s^{-1}) for 30 min (b) or 2 h (c). Columns 1–3 contain bright field (B. F.), SSU-GFP green fluorescence (G. F.) emission at 500–600 nm and red Chl autofluorescence (R. F.) emission at 680–700 nm images. Scale bar: 50 μm.

protein and chlorophyll-binding proteins were not any more confined in aggregated chloroplasts, like bright fluorescence emitting spots. In contrast, the green and red fluorescence emissions became rather dull and spread through the whole cell cytoplasm, implying that disintegration of chloroplasts had occurred. Lightening of the cell wall and an increase in space between the cell wall and the cytoplasm were also observed in *aba1* (Fig. 6). Fluorescence emission of PI was observed as singular bright spots in cell nuclei of *aba1* after 14 h of HL stress (Fig. 6). Unexpectedly, we also observed that the cell nuclei of *aba1* accumulated Chl molecules; however, we do not know at present whether this has any physiological relevance or whether it is simply due to a non-specific increase in the nuclear membrane permeability, allowing other cell components to cross the nuclear membrane. The lack of evidence of chloroplast aggregation/chloroplast rupture or fluorescence emission of PI coming out from cell nuclei in the transgenic plant lines of Arabidopsis WT and *max4* indicated that $^1O_2$ production was not high enough to reach any suitable threshold to initiate cell death after 14 h of HL stress.

4. Discussion

The *max4* and *aba1* mutants of Arabidopsis are defective in SLs and ABA, respectively. In a meta-analysis conducted by some authors of the present study (Gonzalez-Perez et al., 2011), it was unexpectedly determined that the transcriptional profiles of *max4* and *aba1* held a number of co-regulated genes with both ACSC and *flu* when seedlings of this mutant were shifted from dark to low/moderate light conditions (op den Camp et al., 2003). On the basis of the meta-analysis, it was suggested that both, *max4* and *aba1*, generate higher yields of $^1O_2$ than the WT and that thereby biological processes associated with plant defence responses were activated (*i.e.*, response to abiotic or biotic stimuli, innate immune response or cell death). The photoproduction of $^1O_2$ is constitutive in chloroplasts, but changes in the activity or architecture of thylakoid photosynthetic complexes were suggested to be responsible for the potential enhancement of this type of ROS in *max4* and *aba1* in the absence of SLs and ABA (Gonzalez-Perez et al., 2011; Gutierrez et al., 2011).

SLs have been observed to have a positive effect on the expression of some light harvesting-associated genes and a negative effect on the Chl content when they lack in SL-deficient tomato mutants (i.e. *Sl-ORT1*) (Mayzlish-Gati et al., 2010). In a series of SL mutants of Arabidopsis, it was established that the leaves had functional chloroplasts and, in contrast to *Sl-ORT1*, the Chl content did not vary substantially with regard to the Chl content in leaves of Arabidopsis WT plants (Ueda and Kusaba, 2015). The PSII F_v/F_m ratios in these mutants were also similar to those in leaves of Arabidopsis WT plants and, interestingly, they were capable of retaining the values of the initial F_v/F_m ratio, even when the leaves were incubated in the dark for seven days, delaying leaf senescence, an effect that was not observed in leaves of Arabidopsis WT plants under the same dark conditions. The capacity of oxygen evolution was similar in thylakoids of Arabidopsis WT plants and the *max4* and *aba1* mutants showing that the photosynthetic electron transport in thylakoids was not compromised when these plant mutants were grown under normal conditions (Table 1). This is in line with a previous study where the electron transport rates in Arabidopsis WT plants and *aba1* were shown to be indistinguishable under normal growth conditions that are commonly used for Arabidopsis WT plants (70–300 μE m^{-2} s^{-1}), but progressively different from each other under growth conditions with light intensities higher than 500 μE m^{-2} s^{-1} (Pogson et al., 1998). The 77-K Chl fluorescence emission spectra of thylakoids of Arabidopsis WT plants and the *max4* and *aba1* mutants showed two main bands and the characteristic emission peaks described for LHCII-PSII and PSI (Siefermann-Harms, 1988) without any spectral feature that could hint accumulation of free pigments. However, the Gaussian curve fitting of the 77-K Chl fluorescence emission spectra revealed that LHCII aggregation was lower in *aba1* based on the F700/F680 ratio. This notably differs from *max4*, whose F700/F680 ratio was even higher than that in Arabidopsis WT plants. Lokstein and co-workers have reported previously a decreased stability of LCHII trimers and a lower ratio of PSII to PSI relative to Arabidopsis WT plants in *aba1* that was translated into both a disruption of the higher-order arrangement of LHCII-PSII and probably less connectivity between PSII units (Lokstein et al., 2002). The *aba1*

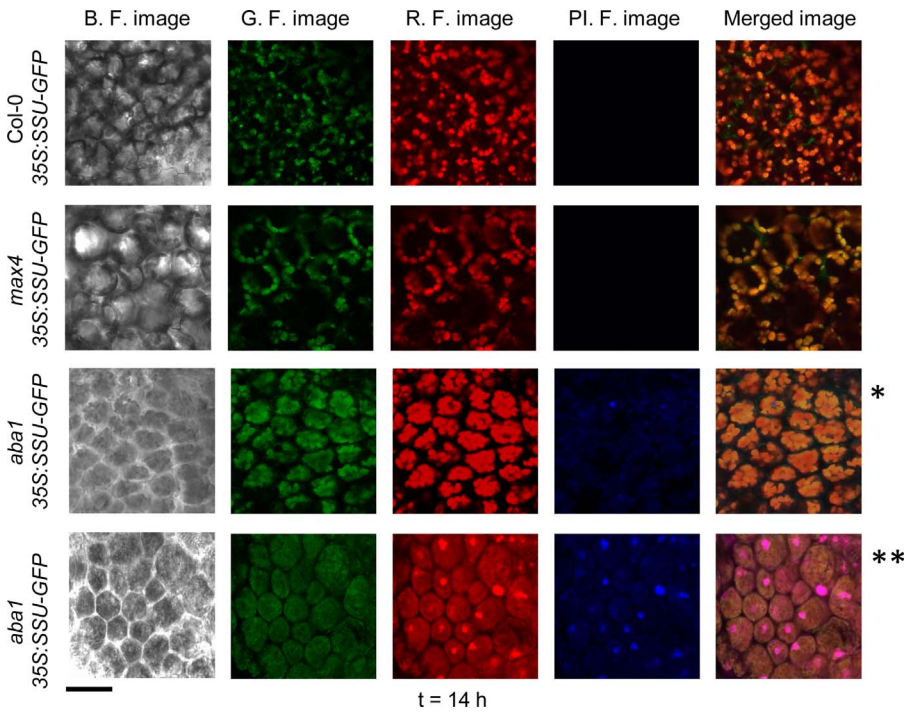

Fig. 6. Representative confocal micrographs of leaf cells of the transgenic plant lines of *Arabidopsis thaliana* Col-0 and the *max4* and *aba1* mutants expressing the chimeric fusion protein SSU-GFP after long HL stress periods. Plants were illuminated with HL (1000 μE m^{-2} s^{-1}) for 14 h. Columns 1–5 contain bright field (B. F.), SSU-GFP green fluorescence (G. F.) emission at 500–600 nm, red Chl autofluorescence (R. F.) emission at 680–700 nm, PI fluorescence (PI. F.) emission at 600–630 nm and merged fluorescence images. Leaves of *Arabidopsis thaliana* Col-0 and *max4* do not show evidence of $^1O_2$-mediated cell death. Two sets of confocal images were taken for leaves of *aba1*. The first set (*) corresponds with early $^1O_2$-mediated cell death events (chloroplast aggregation). The second set (**) shows $^1O_2$-mediated cell death at a later stage (chloroplast rupture). Scale bar: 25 μm.

mutant retains minor amounts of LHCII trimers in thylakoids and the PSII F_v/F_m ratio is lower in comparison with Arabidopsis WT plants (Lokstein et al., 2002; Tardy and Havaux, 1996). Furthermore, the induction of NPQ in *aba1* was observed to be faster, but the maximum level of NPQ was smaller than in Arabidopsis WT plants (Pogson et al., 1998). The loss of PSII's ability to form LHCII aggregates (or the red-shifted fluorescence band, F700) implicates that the Chl-Chl charge transfer mechanism, whereby the excessive excitation energy is rapidly deactivated to the ground state (Muller et al., 2010; Ruban et al., 2012), is inefficient in LHCII of *aba1* and, consequently, triplet excited state Chl production is enhanced. Taken together, the above results suggest that the functioning or architecture of PSII remains in *max4,* but is altered in *aba1*, and that the production of $^1O_2$ in *aba1* can be ascribed to less connectivity between PSII units and lower LHCII aggregation, which diminish NPQ.

HL stress is responsible for the photoproduction of $^1O_2$ in PSII, but it is also responsible for the over-reduction of the photosynthetic electron transport chain in thylakoids, which leads additionally to the production of reduced ROS ($O_2^{\cdot-}/H_2O_2$). Any of these types of ROS can cause oxidation of biomolecules, but they can also act as signalling molecules (Foyer and Noctor, 2009; Noctor and Foyer, 2016). Particularly, the oxidation of β-carotene of PSII by $^1O_2$ leads to the accumulation of β-cyclocitral, a messenger involved in the $^1O_2$-mediated signalling pathway in plants (Ramel et al., 2012), in addition to the EX- and EDS1-dependent signalling pathway described in the *flu* mutant (op den Camp et al., 2003). At the same time, the Mehler reaction is also responsible for the activation of oxidative signals that regulate gene expression (Foyer et al., 2012). *AAA-ATPase* is responsive to $^1O_2$ generated in PSII (Chan et al., 2016). The up-regulation of *AAA-ATPase* has been described in the *flu* mutant after a shift from dark to low/moderate light irradiance and Arabidopsis WT plants after a shift from moderate temperature/low light irradiance to low temperature/HL irradiance (Kim et al., 2012; op den Camp et al., 2003). Under the same conditions, *FERRITIN1*, a H_2O_2 specific marker, did not display up-regulation in the *flu* mutant, but it did in Arabidopsis WT plants. Since the rupture of chloroplasts was observed in the *flu* mutant, but not in Arabidopsis WT plants, the former authors interpreted that $^1O_2$ and $O_2^{\cdot-}/H_2O_2$ act primarily as signalling molecules without causing extensive photo-oxidative damage (Kim and Apel, 2013; Kim et al., 2012). In our study, the HL stress induced a discreet up-regulation of *AAA-ATPase* and *FERRITIN1* in Arabidopsis WT plants that went together with a notable up-regulation of the two JA-signalling repressor genes *JAZ1* and *JAZ5*. Additionally, the transgenic plants of Arabidopsis WT plants did not exhibit either changes in the cellular localization of the SSU-GFP protein or chloroplast aggregation/rupture in comparison with *aba1*, even when the HL stress persisted for 14 h. These results ought not to surprise us when compared with other HL stress studies, where Arabidopsis WT plants were illuminated for 2 days at 1000 μE m^{-2} s^{-1} and there was no evidence of leaf photobleaching or severe photodamage to PSII (Ramel et al., 2013a) or where Arabidopsis WT plants were subjected to a low temperature/HL treatment for seven days and chloroplasts rupture (and subsequent cellular death) was only observed four days after the beginning of the stress treatment (Kim et al., 2012). It is worthwhile remarking that *AAA-ATPase* was not up-regulated in the former study, where the light shift went from growth light conditions (180 μE m^{-2} s^{-1}) to HL stress (1000 μE m^{-2} s^{-1}), but it was up-regulated in the latter study, where the light shift went from low light (15 μE m^{-2} s^{-1}) to moderate/HL conditions (270 μE m^{-2} s^{-1}) with a 30-min dark incubation in between. The light shift applied in our study went from growth light conditions (80–100 μE m^{-2} s^{-1}) to HL stress (1000 μE m^{-2} s^{-1}) and did not cause cellular or transcriptional changes that could suggest severe photobleaching, chloroplast rupture or $^1O_2$-mediated cell death in Arabidopsis WT plants. These results are also in agreement with other studies where *AAA-ATPase* was shown not to be responsive to HL stress in Arabidopsis WT plants (Carmody et al., 2016). In contrast to WT plants, the gene expression of *AAA-ATPase* was clearly up-regulated in *aba1*, where $^1O_2$ production was ~50% larger in comparison with WT (and *max4*). *JAZ1* and *JAZ5* were considerably less up-regulated than in WT plants. This is consistent with the study by Shumbe and co-workers (Shumbe et al., 2016), who observed a significant down-regulation of JA-signalling synthesis transcripts and an up-regulation of several negative regulators of JA-signalling (including *JAZ5*) in *oxi1* under HL leading to a reduction in photo-induced oxidative damage and cell death. The drastically reduced cell death observed in *oxi1* under HL was attenuated after exogenous JA application. ABA antagonizes JA-(ethylene) signalling in Arabidopsis WT plants and evidence for the up-regulation of JA-(ethylene) responsive defence genes was demonstrated in *aba1* (and *aba2*) (Anderson et al., 2004). In

another study using *JAZ2*, *JAZ6* and *JAZ7*-silenced tomato plants, it was proposed that these members of the JAZ family function as negative regulators of disease-associated cell death to *Pseudomonas syringae* pv. *tomato* DC3000 (Ishiga et al., 2013). All this suggests that the changes in the gene expression levels of the JAZ family of repressor proteins are not high enough to counteract the progression of $^1O_2$-mediated cell death in *aba1* under HL stress.

AAA-ATPase and *FERRITIN1* were not up-regulated in *ch1* after two days of HL stress. A result that can be attributed to different experimental conditions or to direct photodamage by $^1O_2$, which, in turn, could mask the $^1O_2$- and EX-dependent signalling pathway (Kim et al., 2012; Ramel et al., 2013a). Similarly, *AAA-ATPase* and *FERRITIN1* were not up-regulated in *npq1 lut2*, although in this case an acclimation response, instead of cell death, was triggered by $^1O_2$. Interestingly, the expression level of *JAZ1* and *JAZ5* in *npq1 lut2* was comparable with, or higher than, that in WT plants (Alboresi et al., 2011). The inspection of the transcriptional profile of *oxi1* and *ch1 oxi1* indicated that *AAA-ATPase* and *FERRITIN1* were not up-regulated either. This contrasts with the results observed in *aba1*. Together with *AAA-ATPase*, up-regulation of *FERRITIN1* is discreetly observed in *aba1*, which was additionally accompanied with $O_2\cdot^-$ formation in thylakoids. This implicates that the regulation of gene expression by reduced ROS (i.e. $H_2O_2/O_2\cdot^-$) is also present in *aba1* and follows $^1O_2$ production. Reduced ROS are known to have an antagonistic effect on the $^1O_2$-mediated signalling cascade in plants (Laloi et al., 2007), delaying stress responses as cell death. In the cell morphological analysis, evidence of $^1O_2$-mediated cell death in *aba1* was only observed after 14 h of HL treatment. In the first stage, chloroplast moved toward a central region of the cell, where they formed aggregates. Chloroplast (and mitochondrial) aggregation has been proposed to be part of some form of cellular autophagy (Wertman et al., 2012). Lightening of the cell wall and cytoplasmic retraction are also cell morphological events that take place during PCD (Reape et al., 2015; Wertman et al., 2012). These events were also present in *aba1* after 14 h of HL stress, although cytoplasmic retraction was not extensive. The reason why cytoplasmic retraction was not extensive is not known at present and we could speculate that $^1O_2$-mediated cell death in *aba1* was switched on due to the HL stress treatment, but the continued stress overwhelmed the cells causing them to die outright before $^1O_2$-mediated cell death could be completed and maximal retraction occurred or, alternatively, they were still in the process of cytoplasmic retraction. This is possibly a matter of future research.

The information on the cross-talk between SLs and JA signalling is scarce (Xiong et al., 2014); however, it is known that SLs induce ubiquitination of D53 in rice—a repressor of the SLs signalling—that interacts with another transcription corepressor denoted as TPL-related (TPR) in rice. D53 ubiquitination is proposed to release downstream transcription factors from TPL/TPR as MYC with a crucial role in the JA signalling (Kazan and Manners, 2013). Our results showed how the expression level of *JAZ1* and *JAZ5* decreased in *max4* in comparison with WT plants, but the changes in *JAZ1* and *JAZ5* were not accompanied by concomitant up-regulation of the ROS markers. This, together with the fact that the activity or architecture of thylakoid photosynthetic complexes seems to remain unperturbed in *max4*, indicates that the only changes in the transcriptional expression of *JAZ1* and *JAZ5* were unable to trigger cell death in *max4* under HL.

5. Conclusions

The results show, first, that *aba1*, but not *max4*, is a $^1O_2$ overproducer under HL stress and, second, that *aba1* and *max4* display a low expression level of *JAZ1* and *JAZ5* in comparison to WT plants. Consequently, *aba1* and *max4* respond differently to HL stress. $^1O_2$ does not prompt rapid and direct photooxidative damage to plant cell in *aba1*; instead of this, it acts primarily as a signalling molecule that switches on, in conjunction with other reduced ROS formed during HL stress, a delay in the rupture of chloroplasts and the eventual cell death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.06.009.

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