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

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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 ($^{1}\text{O}_2$) has received special attention over the last two decades after the publication of a seminal series of studies on $^{1}\text{O}_2$-mediated cell death in the conditional fluorescent (flu) mutant of...
**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 O$_2^*$ 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 O$_2^*$ to essential cellular components; in contrast, it is a O$_2^*$-mediated apoptotic 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 O$_2^*$-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, O$_2^*$ 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-cyclocryptan, certain lipid oxidation products (i.e. oxylinps) or specific regulatory proteins (i.e. WHIRLY or PTM proteins) have been proposed to be messengers of the O$_2^*$ signal out of chloroplasts and to mediate gene responses to O$_2^*$ (Dietz et al., 2016; Ochsneben et al., 2006; Ramel et al., 2012, 2013b). Under most conditions other ROS (superoxide radical O$_2^*-$, hydrogen peroxide H$_2$O$_2$, hydroxyl radical HO·) are formed together with O$_2^*$ 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 O$_2^*$-mediated signalling in plants, Havaux and colleagues worked with an alternative O$_2^*$-producing physiological model, where O$_2^*$ was indeed photosensitized in PSII. The Arabidopsis chlor1a1(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 O$_2^*$. 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 O$_2^*$ 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 O$_2^*$—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 _ox1_, an Arabidopsis mutant deficient in the OXIDATIVE SIGNAL INDUCIBLE 1 (OX11) gene encoding for a Ser/Thr kinase, and the double mutant ch1 _ox1l_ showed to be more resistant to HL stress and to exhibit a drastic decrease in O$_2^*$-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 OX11-dependent signalling to regulate O$_2^*$-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 O$_2^*$ photosensitizer that can migrate from chloroplasts to mitochondria. The activity of ACD2 was observed to minimize O$_2^*$ formation in mitochondria, avoiding the onset of cell death. The triple mutant of _acd2 exc1 exc2_ did not display alterations in PCD phenotype when compared with the single mutant _acd2_; a fact that contrasts with the triple mutant _flu exc1 exc2_, where O$_2^*$ was overproduced in chloroplasts and PCD was not activated (Kim et al., 2012). An explanation to this apparent controversy between different O$_2^*$-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 O$_2^*$, but cell death was not genetically activated (Alboresi et al., 2011; Triantaphylides et al., 2008). The _npq1 lut2_ lacks zeaxanthin and lutein and O$_2^*$ 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 npq1_ 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 O$_2^*$.

As described above, genetically and non-genetically O$_2^*$-mediated responses vary notably from WT plants to mutants producing high yields of O$_2^*$ 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 striglolactones (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 O$_2^*$ may be overproduced in _max4_ and _aba1_ (Gonzalez-Perez et al., 2011; Gutierrez et al., 2011). ABA and SLs 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 precursor 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). SLs 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 SLs and shows an increased shoot branching phenotype that can be rescued after the exogenous application of SLs (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 O$_2^*$ production and also whether O$_2^*$-mediated cell death is activated in these mutants. Here we present data on O$_2^*$ production detected by the spin probe 2,2,6,6-tetramethyl-1-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 3SS: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-0:3SS: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-Arbaiarz et al., 2012; Murashige and Skoog, 1962).

The transgenic Arabidopsis thaliana Col-0:3SS: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-0:3SS: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 3SS 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 μmol m⁻² s⁻¹ 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 K₃[Fe(CN)]₆ as electron acceptor and 10 mM NH₄Cl as uncoupler to destroy the pH gradient.

2.4. ¹⁸O₂ and O₂•⁻ radical detection by EPR spin trapping

Spin-trapping assays with TEMPD-HCl (Sigma-Aldrich, St. Louis, USA) to detect ¹⁸O₂ (Krieger-Líszkay 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 O₂•⁻ 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 MgCl₂, 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 X 10⁶; time constant, 40.96 ms and number of scans 4. For control experiments, D₂O and Na₂S₂O₄ were used to enhance or to quench ¹⁸O₂ production and superoxide dismutase (SOD) (50 U) to disproportionate O₂•⁻.

2.5. Chlorophyll 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 Qₑ 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 ¹⁸O₂ and H₂O₂-dependent signalings 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 Triple Dichroic 488/543/633 excitation beam splitter. Confocal images were taken using the...
showing that in both cases the photochemical production of 1O2 was displayed an EPR signal in amplitude similar to that of WT thylakoids, rather equal. On the contrary, the amplitude of the EPR signal of thylakoid membranes, control experiments have been performed previously with the addition of either NaN3, a quencher of 1O2, or D2O, that increases the lifetime of 1O2 (Krieger-Liszkay et al., 2015). In addition to 1O2, the formation of O2•− and HO• was measured using DEPMPO as spin trap (Frejaville et al., 1995; Heyno et al., 2009).

3. Results

Fig. 1 shows light-induced 1O2 generation in thylakoids from Arabidopsis WT plants and the max4 and aba1 mutants. Thylakoids were illuminated with HL (1000 μmol m−2s−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.

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 1O2 under HL than those from WT plants and max4. To exclude that the differences in 1O2 generation between thylakoids from aba1 and from the other samples were caused by a loss of water-splitting activity, the activity of the photosynthetic electron transport chain was measured using 1 mM K3[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 NH4Cl 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 1O2 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

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<tr>
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<th>(−) NH4Cl</th>
<th>(+) NH4Cl</th>
<th>F700/F680</th>
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<tr>
<td>Col-0</td>
<td>61 ± 4</td>
<td>180 ± 10</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>max4</td>
<td>68 ± 5</td>
<td>184 ± 07</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>aba1</td>
<td>65 ± 3</td>
<td>200 ± 10</td>
<td>0.54 ± 0.05</td>
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* Data were obtained with two independent preparations and 3 repetitions per preparation. The data represent the mean ± SD.
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 (Sierermann-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). aba1 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 1O2 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 1O2 (AAA-ATPase) and H2O2 (FERRITINI) 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 ACS 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 1O2 production, other ROS (i.e. O2-/H2O2) 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 FERRITINI decreased with statistical significance. Thylakoids of max4 held similar features to those of control thylakoids when 1O2 production was examined, but different ones when O2-/ 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 1O2 production in thylakoids of aba1 under HL (Fig. 1). The up-regulation of FERRITINI in aba1 was similar to that in Arabidopsis WT plants, suggesting that in aba1, together with 1O2, 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 1O2 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 1O2 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 1O2 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
and red Chl autocontain bright conditions (a) or with HL (1000 μE m⁻² s⁻¹) for 14 h of HL stress. Plants were illuminated with normal light protein SSU-GFP after short HL stress periods. Plants were illuminated with normal light

Arabidopsis thaliana

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 1O₂ 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 1O₂ 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 1O₂ 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 Fv/Fm 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 Fv/Fm 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 LHCHI trimers and a lower ratio of PSI to PSI relative to Arabidopsis WT plants in aba1 that was translated into both a disruption of the higher-order arrangement of LHCHI-PSII and probably less connectivity between PSI units (Lokstein et al., 2002). The aba1
The up-regulation of cyclocitral, a messenger involved in the 1O2-mediated signalling of reduced ROS (O2−/H2O2) transport chain in thylakoids, which leads additionally to the production of H2O2, can cause oxidative damage (Kim and Apel, 2013; Kim et al., 2012). In our study, the functioning or architecture of PSII remains in max4 but is altered in aba1, and that the production of 1O2 in aba1 can be ascribed to less connectivity between PSII units and lower LHCCI aggregation, which diminish NPQ.

HL stress is responsible for the photoproduction of 1O2 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 (O2−/H2O2). 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 1O2 leads to the accumulation of β-cyclocitrinal, a messenger involved in the 1O2-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 1O2 generated in PSI (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 H2O2 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 1O2 and O2−/H2O2 act primarily as signalling molecules without causing extensive phototoxicative 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 μm 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 μm m−2 s−1) to HL stress (1000 μm m−2 s−1), but it was up-regulated in the latter study, where the light shift went from low light (15 μm m−2 s−1) to moderate/HL conditions (270 μm 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 μm m−2 s−1) to HL stress (1000 μm m−2 s−1) and did not cause cellular or transcriptional changes that could suggest severe photobleaching, chloroplast rupture or 1O2-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 O2− 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 oxz1 under HL leading to a reduction in photo-induced oxidative damage and cell death. The drastically reduced cell death observed in oxz1 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 1O2-mediated cell death in aba1 under HL stress. AAA-ATPase and FERRITIN1 were not up-regulated in aba1 after two days of HL stress. A result that can be attributed to different experimental conditions or to direct photodamage by 1O2 which, in turn, could mask the 1O2- 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 1O2. 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 axi1 and ch1 axi1 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 discretely observed in aba1, which was additionally accompanied with O2- formation in thylakoids. This implicates that the regulation of gene expression by reduced ROS (i.e. H2O2/O2-) is also present in aba1 and follows 1O2 production. Reduced ROS are known to have an antagonistic effect on the 1O2-mediated signalling cascade in plants (Laloi et al., 2007), delaying stress responses as cell death. In the cell morphological analysis, evidence of 1O2-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 also been proposed to be part of some form of cellular autophagy (Wertman et al., 2012). Lightening of the cell wall and cyttoplasmic 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 cyttoplasmic retraction was not extensive. The reason why cyttoplasmic retraction was not extensive is not known at present and we could speculate that 1O2-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 1O2-mediated cell death could be completed and maximal retraction occurred or, alternatively, they were still in the process of cyttoplasmic 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 TPR-related (TPR) in rice. D53 ubiquitination is proposed to release downstream transcription factors from TRL/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 1O2 over-producer 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. 1O2 does not prompt rapid and direct photodamage 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

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Arabidopsis cell suspension culture under high-light conditions. Plant Physiol. 156, 1439–1456.


