Abscisic acid (ABA) and phototropins act antagonistically to control stomatal movements. Here, we investigated the role of ABA in phototropin-directed chloroplast movements in mesophyll cells of Arabidopsis thaliana. We analyzed the expression of phototropins at mRNA and protein level under the influence of ABA. PHOT1 mRNA level was decreased by ABA in the dark while it was insensitive to ABA in light. PHOT2 mRNA level was independent of the hormone treatment. The levels of phototropin proteins were down-regulated by ABA, both in darkness and light. No impact of exogenous ABA on amplitudes and kinetics of chloroplast movements was detected. Chloroplast responses in wild type Arabidopsis and three mutants, abi4, abi2 (abscisic acid insensitive4, 2) and aba1 (abscisic acid1), were measured to account for endogenous ABA signaling. The chloroplast responses were slightly reduced in abi2 and aba1 mutants in strong light. To further investigate the effect, abi2 and aba1 mutants were supplemented with exogenous ABA. In the aba1 mutant, the reaction was rescued but in abi2 it was unaffected. Our results show that ABA is not directly involved in phototropin-controlled chloroplast responses in mature leaves of Arabidopsis. However, the disturbance of ABA biosynthesis and signaling in mutants affects some elements of the chloroplast movement mechanism. In line with its role as a stress hormone, ABA appears to enhance plant sensitivity to light and promote the chloroplast avoidance response.

Key words: abscisic acid, abscisic acid mutants, Arabidopsis thaliana, blue light, chloroplast movement, phototropins

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INTRODUCTION

Blue light-controlled chloroplast movements

Chloroplast redistribution is a common physiological phenomenon in plant cells (reviewed in Gabryś & Krzeszowiec, 2012). It can be defined as an intracellular directional movement that depends on the quality and quantity of light. In fact, chloroplasts perform two distinct movement responses, avoidance and accumulation (see Banaś et al., 2012 and ref. therein). These reactions have two known functions. The avoidance response plays a role in the protection of the photosynthetic apparatus in strong light (Kasahara et al., 2002; Sztatelman et al., 2010). In this case chloroplasts avoid cell regions exposed to strong light. They redistribute toward cell walls parallel to light direction and screen each other. The accumulation response plays a role in fine-tuning photosynthesis in weak and intermediate light – chloroplasts accumulate in the most illuminated area of the cell to maximize light absorption (Zurzycki, 1955). Light-induced chloroplast movements have been discussed in numerous reviews (Haupt & Scheuerlein, 1990; Haupt, 1999; Takagi, 2003; Wada et al., 2003; Gabryś, 2004; Wada & Suetsugu, 2004; Gabryś, 2012; Banaś et al., 2012; Gabryś & Krzeszowiec, 2012).

Chloroplast responses are induced by blue light in most higher plants (Gabryś, 2012). The blue light signal is perceived by phototropins, photoreceptors which are light-activated protein kinases. In Arabidopsis thaliana two phototropins have been identified: phot1 and phot2. Both phototropins control the accumulation response, whereas mainly phot2 directs the avoidance response (Jirálo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001). Apart from chloroplast redistribution they also control other acclimation responses: phototropism (Sai et al., 2001), leaf flattening (Inoue et al., 2008), nuclear avoidance movement (Iwabuchi et al., 2007) and stomatal opening (Kinoshita et al., 2001). The studies of chloroplast movements in higher plants are currently based on two main models, Arabidopsis thaliana (Trojan & Gabryś, 1996; Okawa et al., 2003; Suetsugu et al., 2005; Banaś & Gabryś, 2007; Krzeszowiec et al., 2007; Kadota et al., 2009) and Nicotiana tabacum (Nauš et al., 2008; Aneilska-Mazur et al., 2009; Augustynowicz et al., 2009; Eckstein et al., 2016).

Abscisic acid signaling

The plant hormone abscisic acid (ABA) plays an important role in the regulation of several developmental processes, from seed maturation to senescence. It is also a key factor in plant responses to environmental conditions, especially water stress. Many of these responses are dependent on endogenous ABA levels, which result from the interplay between ABA biosynthesis, degradation and the formation of inactive conjugates (Nambara & Marion-Poll, 2005). One of the key enzymes in ABA biosynthesis is zeaxanthin epoxidase encoded by the ABA1 gene in A. thaliana (Marin et al., 1996). It catalyses the conversion of zeaxanthin to violaxanthin and is also involved in the
xanthophyll cycle. The abs1 mutant shows reduced growth and significantly lower endogenous ABA content (Rock & Zeeman, 1991; Barrero et al., 2005).

Only recently has the PYR/PYL/RCAR-PP2C-SnRK2 (PYRABACTIN RESISTANCE/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR – PROTEIN PHOSPHATASE 2C – SUCROSE NON-FERMENTING1-RELATED PROTEIN KINASE 2) ABA signaling pathway been fully elucidated (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Umezawa et al., 2010). The binding of ABA by receptors belonging to the PYR/PYL/RCAR protein family promotes their interaction with type 2C protein phosphatases (PP2Cs). PP2Cs act as negative regulators of ABA signaling by dephosphorylating and inhibiting SnRK2 kinases. Upon ABA perception PP2C phosphatase activity is inhibited, which allows SnRK2 activation and further signal transduction. In A. thaliana PP2Cs are encoded by ABF1 and ABF2 (ABA INSENSITIVE4) genes (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998). The abf1 and abf2 mutants exhibit constitutive photophase activity and a consequence SnRK2 inactivation, resulting in plant insensitivity to ABA. abf1 and abf2 show reduced sensitivity to exogenous ABA and increased ABA accumulation under low water potential, but normal stomatal behaviour (Korzeń et al., 1984; Assmann et al., 2000; Verslues & Bray, 2006). Recently ABI2 has been shown to participate in ABA-mediated regulation of chloroplast gene transcription (Yamburenko et al., 2015).

Numerous interactions have been identified between ABA and other signaling pathways, including responses to other hormones, sugars and pathogens (Brady & McCourt, 2003; Rook et al., 2006; Acharya & Assmann, 2009; Cao et al., 2011). One of the proteins responsible for ABA cross-talk is ABI4 (ABA INSENSITIVE 4), an AP2/ERF transcription factor (Finkelstein et al., 1998). It participates, among others, in plastid retrograde signaling by regulating nuclear genes for photosynthesis and chlorophyll metabolism, and acts as an integrator of environmental signals (Leon et al., 2013; Wind et al., 2013; Zhang et al., 2013). The abf4 mutant exhibits ABA-insensitive germination but normal seed dormancy and stomatal responses (Finkelstein et al., 1994).

**Crosstalk between ABA and phototropins**

A link between the stress hormone ABA and phototropins was first established in stomatal movements. Blue light-controlled opening of the stomatal appara- tus starts with the photoexcitation of phototropins. The BLUS1 (BLUE LIGHT SIGNALING1) Ser/Thr protein kinase is a phototropin substrate and a primary regulator of stomatal opening (Takemiya et al., 2010). Subsequently, the signal is transduced to a plasma membrane H+-ATPase which establishes a proton-motive force allowing ion uptake. Mainly K+ is transported into the cell via KAT1 and KAT2, two voltage-gated K+ inward-rectifying channels activated upon hyperpolarization of the plasma membrane (Pilot et al., 2001; Sirichandra et al., 2009). Two more proteins are known to be involved in the signaling pathway: a 14-3-3 protein (Tseng et al., 2012) and a type 1 protein phosphatase (PP1) (Takemiya et al., 2010). The other key factor regulating guard cell movement is ABA (for review see Sirichandra et al., 2009) which induces stomatal closure. The core signaling module in stomatal closure is the ABA receptor complex PYR/PYL/RCAR-PP2C-SnRK2 (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). ABA blocks the plasma mem-
brane H+-ATPase acting via PP1 inhibition (Zhang et al., 2004; Takemiya & Shimazaki, 2010). Simultaneously, ABA inhibits the K+ influx channel, and activates S-type anion channels. The resulting depolarization of the plasma membrane activates K+ efflux from the cell that decreases the turgor of guard cells and causes stomatal closing (for review see Hubbard et al., 2010). The effect of water stress which activates the ABA pathway dominates over light in accordance with the physiological role of stomatal movements. Although ABA and blue light are the main regulators of guard cell movements, other signaling pathways modulate these responses. In particular, other hormones influence stomatal behaviour (Acharya & Assmann 2009 and ref. therein). Both auxins and cytokinins promote stomatal opening and inhibit ABA-induced stoma-
tal closure, although opposite effects can sometimes also be observed (Blackman & Davies, 1983; Lohse & Hedrich, 1992; Tanaka et al., 2006). Auxin acts by activating the plasma membrane H+-ATPase. Another potent activator of the H+-ATPase and stomatal opening is the fungal phytotoxic fusicoxin (Assmann & Schwarz, 1992; Kinoshita & Shimazaki, 2001). Jasmonates, salicylic acid and brassinosteroids usually act as positive regulators of stomatal closure (Mori et al., 2001; Suhita et al., 2003; Haubrick et al., 2006) whereas the effects of ethylene on stomatal aperture are strongly dependent on cross-talk with other hormones (Tanaka et al., 2005; Desikan et al., 2006).

ABA has been shown to influence chloroplast movements in A. thaliana guard cells (Königer et al., 2010). In WT plants the chloroplast arrangement was similar in darkness and low light, with most chloroplasts located in the central position. In high light most chloroplasts relocated towards the pore. After exogenous ABA treatment (10 μM) no influence of light on chloroplast distribution was observed. The mechanism underlying this effect remains unknown.

Recently Rojas-Pierce and coworkers (2014) established a link between ABA and chloroplast movements in A. thaliana mesophyll cells. This work provides evidence for the involvement of PMH (PLASTID MOVEMENT IMPAIRED1), a gene required for normal chloroplast relocations, in two ABA-related responses: regulation of seed germination and ABA accumulation in seedlings during water deficit. On the other hand, the authors have shown the impairment of chloroplast movements in some ABA biosynthesis and signaling mutants. Much earlier, some connections between xanthophyll homeostasis and chloroplast movement have been shown (Tlałka et al., 1999; DeBlasio et al., 2005), but the authors never referred to ABA metabolism in their discussions. Tlałka and others (1999) have shown a correlation between zeaxanthin level and chloroplast responses to strong blue light. DeBlasio and coworkers (2005) have shown impaired avoidance responses in the npq2-1 (non-photicchemical quenching2-1) mutant, also called abd-6 (abiicic acid-6), lacking zeaxanthin epoxidase and deficient in ABA biosynthesis. In the light of present findings, these results seem in line with the postulated role of ABA in chloroplast movements.

The aim of the this study was to elucidate the role of ABA in the control of chloroplast redistribution in the photosynthetic tissue of A. thaliana. In particular, we asked two questions: 1) does ABA affect the expression of phototropins? 2) what is the nature of the crosstalk between ABA and phototropin signaling pathways in the control of chloroplast movements in mesophyll tissue?
MATERIALS AND METHODS

Plant material. Arabidopsis thaliana wild-type (WT) Columbia seeds (Col-0 N60000) and mutants aba4-1 (abscisic acid insensitive4-1, CS8104), ab2-1 (abscisic acid insensitive2-1, CS23) and aba6-1 (abscisic acid6-1, CS3772) were obtained from Nottingham Arabidopsis Stock Center (Nottingham, UK). Plants were grown in JIFFY-7 pots (Jiffy International AS, Norway) in a growth chamber (Sanyo MLR 350H, Japan) with a 10/14 h light/dark photoperiod at 25°C, 80% relative humidity, and illumination by fluorescent lamps (FL40SS/W/37, Sanyo) with a photosynthetic photon flux density of 100 µmol m⁻² s⁻¹. 5 week-old plants were used for all experiments.

ABA treatments. Three types of ABA treatments were applied. (1) The short-term treatment consisted in an infiltration of dark-adapted leaves 1 h before the induction of chloroplast responses. The infiltration was performed in the dark room under very dim, green safe light. Detached leaves were infiltrated with a needle-less syringe. 100 µM ABA prepared in a buffer solution (10 mM HEPES, 0.5% Tween 20) was introduced under pressure into intracellular spaces. Control leaves were infiltrated with buffer alone. (2) The long-term treatment lasted 48 h. Plants were sprayed with 100 µM ABA (identical solution as that for the short-term treatment) or with buffer alone at 9 a.m. on two consecutive mornings. The spraying was done with hand-held spray bottles until the foliage was uniformly wetted. Plants were darkened overnight starting from 8 p.m. on the second day. One part of each plant was used the next morning for RNA isolation and the other part for chloroplast movement analysis. (3) In experiments involving mutants plants were sprayed with 100 µM ABA (solution and its application as in (2)) at 7 pm, and dark-adapted (and ABA-incubated) for at least 16 h before chloroplast movement measurement.

Analysis of the steady-state RNA level. Quantitative real-time PCR was performed to study the level of PHOT1 and PHOT2. After long-term ABA treatment, the plants used for RNA isolation were kept for 3 h either in the dark or in medium-intensity white light (100 µmol m⁻² s⁻¹) in the growth chamber.

Ten leaves from at least six individual plants were pooled before RNA extraction. RNA was isolated with a Spectrum Plant Total Kit (Sigma-Aldrich) and digested with DNaseI (Fermentas) during purification on the column. The RNA concentration was determined using a NanoPhotometer (Implen GmbH) and its integrity was checked by electrophoresis on 2% agarose gel. cDNA was prepared with the RevertAid M-MuLV Reverse Transcriptase Kit (Fermentas) using random hexamer primers. Real time reverse-transcription PCR was performed with SYBR Green JumpStart Taq ReadyMix (Sigma Aldrich) and a thermal cycler (Rotor-Gene 6000, Corbett Research, Australia). cDNA corresponding to 50 ng of RNA was used in a single reaction and all reactions were run in duplicates. The PCR conditions and primer sequences for PHOT1 and PHOT2 were based on Łabuz and others (2012), UBG10 was chosen as a reference gene because its expression was shown to be unaffected by ABA treatment and primer sequences were based on Czechowski and coworkers (2005). The specificity of the obtained products was verified on a dissociation curve at the end of each run and by 2% agarose gel electrophoresis.

Western blotting. The expression levels of phot1 and phot2 proteins were determined using Western blot analysis. Plants were subjected to long-term ABA and dark/light treatments in the same way as for mRNA level analysis. Each sample consisted of 2–3 leaves from an individual plant. Samples were ground in liquid nitrogen, weighed and suspended in an appropriate amount of protein extraction buffer (100 µl/10 mg sample; 0.1 M Tris, pH 8.8, 4% SDS, 2 mM phenylmethylsulphonyl fluoride (PMSF), 2% β-mercaptoethanol). Samples were incubated at 80°C for 3 min and centrifuged at 16000 × g, 4°C for 10 min (Sakamoto & Briggs, 2002). The obtained protein extract was mixed with appropriate amounts of SDS loading buffer, heated at 95°C for 10 min and separated by SDS-PAGE. All gels were run in triplicate: one for Coomassie staining and two for Western blot analysis (phot1 and phot2). Western blotting was performed using antibodies purchased from Agrisera: primary anti-PHOT1 (AS10 720; 1:200) and anti-PHOT2 (AS10 721; 1:2000) antibodies, and secondary goat anti-rabbit IgG–horseradish peroxidase antibodies (1:25 000). For a detailed description of anti-PHOT antibodies and Western blotting conditions see Łabuz et al., 2015. Protein detection was performed using Clarify Western ECL substrate (Bio-Rad) and densitometric analysis carried out using ImageJ software. Results are the mean values from 9 samples, normalized to the total amount of protein in each sample. To account for differences between blots, the normalized values were divided by the chemiluminescent signal/protein content ratio averaged over all lanes in a blot.

Photometric method. Chloroplast movements induced by continuous blue light (BL, 460 nm) were assessed using a double-beam photometer which records changes in red light transmittance (660 nm, 0.1 µmol m⁻² s⁻¹, modulated with the frequency of 800 Hz) through the leaf (Walczak & Gabryś, 1980). Leaf transmittance decreases in weak light (avoidance response) and increases in strong light (accumulation response) as a result of chloroplast redistribution. First, the initial transmittance level of leaves dark-adapted for at least 12 h was measured. Whenever possible, leaves with similar initial transmittance were chosen for one experiment. Subsequently, samples were illuminated with BL of different intensities, changed stepwise every 45 min. In short- and long-term ABA treatment experiments the following BL intensities were used: 1.4 µmol m⁻² s⁻¹ (weak BL), 18 µmol m⁻² s⁻¹ (medium BL) and 108 µmol m⁻² s⁻¹ (strong BL). In the dose-response experiments performed on mutants, additional intermediate BL fluence rates were applied: 3.6, 7.2 and 14.4 µmol m⁻² s⁻¹.

The following parameters were measured/calculated for each response: (1) amplitude – transmittance change after 45 minutes, (2) velocity – first derivative of the initial linear fragment of the transmittance curve.

Bright field microscopy of leaf cross-sections. Hand-cut cross-sections were prepared from fresh leaves of WT and mutant plants, untreated or subjected to long-term ABA treatment. Sections were infiltrated with water and visualized using a Nikon Eclipse TE 200 (Japan) inverted microscope equipped with a ×10 0.25 NA objective. The microscope was coupled to a QICAM (Evolution VF) cooled monochrome CCD digital camera and Image-Pro Plus software version 5.1 (Media Cybernetics Inc.).

ELISA experiments. WT A. thaliana plants were subjected to long-term ABA treatment. Subsequently, the plants were dark-adapted for 2 h, the samples were collected, washed in distilled water and immediately lyophilized. The tissue analyzed for ABA content was combined from 4 plants. Experiments were performed twice. ABA was extracted according to Janowiak and others (2002) and its concentration in leaves was quantified.
by ELISA according to the Walker-Simmons method (1987), using the MAC252 antibody.

**Statistical analysis.** Unpaired Student’s t-test was performed using GraphPad InStat version 3.10 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

**RESULTS**

**Analysis of phototropin expression patterns**

We investigated the influence of exogenously applied ABA on phototropin mRNA and protein levels in mature leaves of dark-adapted or irradiated Arabidopsis plants (Fig. 1A). Light down-regulated PHOT1 and up-regulated PHOT2 expression. The effect of ABA on PHOT1 expression depended on light conditions. While PHOT1 mRNA level decreased significantly upon ABA treatment in dark-adapted leaves, it did not change in leaves of plants illuminated with white light. By contrast, ABA had no significant effect on the expression of PHOT2, either in dark-adapted or illuminated leaves.

At the protein level no effect of light on phototropin expression could be observed, but at the same time the influence of ABA was more pronounced (Fig. 1B). PHOT1 was significantly down-regulated by ABA in both darkness and light, whereas PHOT2 only in darkness. In the case of both phototropins the effect of ABA was stronger in darkness.

**Figure 1.** The expression of phototropin1 and phototropin2 in leaves of WT *A. thaliana* at mRNA (A) and protein (B) levels. 5-week-old plants were pretreated with control buffer or 100 μM ABA, dark-adapted overnight and then either illuminated with 100 μmol m⁻² s⁻¹ white fluorescent light in the growth chamber for 3 h (light) or left in the dark (dark). (A) Each bar corresponds to the average of at least three measurements from a pool of ten leaves collected from at least six different plants. All qRT-PCR reactions were run in duplicates. (B) Each bar corresponds to the average of 9 measurements of leaves collected from individual plants. All error bars represent SE. Asterisks indicate the statistical significance of differences between control and ABA-treated plants (p-values calculated using Student’s t-test: *p=0.01–0.05, **p=0.001–0.01, ***p<0.001).

**Figure 2.** Amplitudes (left) and velocities (right) of transmittance changes reflecting chloroplast relocations induced by continuous blue light of increasing fluence rates (indicated on graph, each step 45 min) in WT *A. thaliana* leaves pretreated with 100 μM ABA for 1 h in the dark.

Results are means of 11 (control samples) or 12 (ABA-treated samples) independent experiments, error bars represent S.E. No statistically significant differences were detected between control and treated plants (all p-values calculated using Student’s t-test above 0.05).
Chloroplast movements – short-term ABA exposure

To investigate the interplay between ABA and light signaling pathways in chloroplast movements we performed a series of photometric studies. In the first experiment, the effects of short-term ABA exposure (100 μM, 1 h) were tested. The extreme BL fluence rates applied caused maximal accumulation (1.4 μmol m⁻² s⁻¹) and avoidance responses (108 μmol m⁻² s⁻¹) of chloroplasts, whereas irradiation with intermediate BL (18 μmol m⁻² s⁻¹) produced an incomplete avoidance response (Trojan & Gabryś, 1996). No significant differences in amplitudes or velocities were detected between ABA-treated and control leaves in any of the responses (Fig. 2).

Chloroplast movements – long-term ABA exposure

In the next experiment, the influence of long-term ABA treatment on chloroplast movements was investigated. The exposure to abscisic acid was extended to 48 h. During that time plants were sprayed twice with 100 μM ABA. Similarly as in the short-term experiment, ABA did not influence the amplitudes or velocities of any of the chloroplast responses (Fig. 3).

In order to exclude the possibility that ABA did not reach the mesophyll cells we measured its concentration in the leaf tissue using ELISA. The level of abscisic acid was 2.8 times higher in the treated plants than in the control ones (ABA-treated samples 4.0±0.15 nmol g⁻¹ DW versus control 1.4±0.33 nmol g⁻¹ DW), which confirms the effectiveness of ABA treatment. The experiment was performed twice with four plants per group. No differences in leaf anatomy and condition between control and ABA-treated plants could be observed (Fig. 4).

Chloroplast movements in ABA mutants

To further elucidate the interplay of ABA and blue light-controlled chloroplast movements, we investigated selected Arabidopsis ABA signaling and biosynthesis mutants: aba1-6, abi2-1 (abi insensitive, hereafter referred to as abi4, abi2) and aba1-6 (abscisic acid, hereafter referred to as aba1).

A dose-response analysis of chloroplast movements was performed in the described mutants, using 5 increasing BL intensities (Fig. 5). All mutants performed accumulation responses which were statistically indistinguishable from WT. At higher BL intensities (18–108 μmol m⁻² s⁻¹) only aba4 responses were comparable with those of WT plants. The avoidance responses of both abi2 and aba1 chloroplasts were significantly lower than in WT plants starting from the fluence rate of 36 μmol m⁻² s⁻¹. The p-values of differences between WT and mutant plants at 36, 72 and 108 μmol m⁻² s⁻¹ were respectively: p=0.0011, p=0.0194, p=0.0238 for aba2 and p=0.0491, p=0.0245, p=0.0104 for aba1. At 18 μmol m⁻² s⁻¹ abi2 exhibited a very weak accumulation response, in contrast to WT and the other mutants which performed an avoidance response at this light intensity. To check whether the results of chloroplast movement measurements could be affected by differences in leaf architecture, cross-sections of mutant leaves were prepared. Although some differences in cell size and leaf thickness were observed, no serious abnormalities were visible (Fig. 4).

Supplementation experiments

We carried out supplementation experiments to determine whether the addition of ABA restores avoidance responses in abi2 and aba1 mutants (Fig. 6). Mutant and WT plants were sprayed with 100 μM ABA and chloroplast movements measured after 16 h of dark adaptation. Only avoidance responses induced by 18, 36 and 108 μmol m⁻² s⁻¹ were tested since chloroplast accumulation was not impaired in the studied
mutants. The responses of WT and \textit{abi2} chloroplasts to all three BL intensities were unaffected by ABA treatment. By contrast, exogenous ABA treatment of \textit{aba1} plants visibly enhanced chloroplast responses. Although the resulting amplitudes were still visibly lower than those of untreated and ABA-treated WT plants, the differences between control and ABA-treated \textit{aba1} plants were statistically significant at 18 and 36 μmol m\(^{-2}\) s\(^{-1}\) (\(p=0.0394\), \(p=0.0157\)) and on the borderline of significance at 108 μmol m\(^{-2}\) s\(^{-1}\) (\(p=0.0529\)). Thus, the avoidance response in the \textit{aba1} mutant can be partially rescued by exogenous ABA while changes introduced by the \textit{abi2} mutation cannot be compensated in this way. Leaf cross-sections of ABA-sprayed plants were performed to check for potential adverse effects of this treatment. No differences in leaf anatomy and condition between control and ABA-treated WT and mutant plants could be seen (Fig. 4).

**DISCUSSION**

Considerable progress has been made in the studies of chloroplast movements since the first publication by Senn in 1908. In the last two decades the photoreceptors have been discovered (Christie \textit{et al.}, 1998; Jarillo \textit{et al.}, 2001; Kagawa \textit{et al.}, 2001). While the mechanisms of chloroplast movements became more comprehensible, numerous questions, particularly those concerning signal transduction, still remain unanswered. The complexity of the phenomenon on the one hand and the photoprotective role of the avoidance response on the other (Kasahara \textit{et al.}, 2002; Sztatelman \textit{et al.}, 2010) stimulated us to inquire how the stress hormone ABA is involved in the studied mechanism. A link between ABA and chloroplast movements has already been shown by Rojas-Pierce and coworkers (2014). We tried to further elucidate this question and investigate the potential mechanisms of ABA interactions with the chloroplast movement signaling pathway.

**Analysis of phototropin expression patterns**

The expression of \textit{PHOT1} and \textit{PHOT2} was strongly regulated by light, which is consistent with the results of Labuz and coworkers (2012), but almost unaffected by ABA (Fig. 1A). Only \textit{PHOT1} was downregulated by ABA in the dark. Interestingly, at the protein level the expression pattern of phototropins was completely different. The levels of phototropin proteins were unaffected by light, but decreased after ABA treatment, especially in the case of \textit{PHOT1} (Fig. 1B). The observed discrepancies between phototropin mRNA and protein levels point to a complex regulation of the expression of these photoreceptors. The effect of light on phototropin expression at transcript level and its subsequent lack at the protein level are surprising, but consistent with the findings of Sztatelman and coworkers (2016). On the contrary, the influence of ABA on phototropin expression is negligible at mRNA level, but prominent at the protein level. This suggests that other regulatory factors act downstream of light to regulate phototropin protein levels and ABA could be one of them. Moreover, the downregulation of phototropin protein levels by ABA seems in line with the antagonistic roles of phototropins and ABA in the control of stomatal movement.
Chloroplast movements – short-term and long-term ABA exposure

Neither short-term (1 h) nor long-term treatment (2 days) exogenous ABA treatment affected chloroplast responses to blue light (Fig. 2 and 3). An analysis of ABA levels in leaves confirmed that the hormone treatment was effective. This shows that exogenous ABA does not provide a direct signal to chloroplast relocation in WT *Arabidopsis* mesophyll cells. Moreover the results of long-term ABA treatment experiments show that the down-regulation of phototropin protein levels by ABA does not affect chloroplast movements.

Chloroplast movements in ABA mutants

Finally, we investigated the role of endogenous ABA in chloroplast movements using three different abscisic acid mutants, *abi4*, *abi2*, *aba1*. The most remarkable observation was that all mutants showed accumulation responses in weak light similar to wild type plants. Differences were found only in the avoidance responses activated by strong light (Fig. 4). The differential effects of the investigated mutations on the accumulation and avoidance reactions are in line with all former evidence showing that these two types of responses have distinct mechanisms (Grabańska & Malec, 2004; Krzeszowiec & Gabryś, 2007; Aggarwal *et al.*, 2013). The main difference between the weak- and the strong-light response lies in the accumulation response being controlled by phototropin1 and phototropin2 and the avoidance response mainly by phototropin2. Only recently has it been revealed that phosphoinositides and calcium ions play differential roles in signal transduction in phot1- and phot2-controlled pathways (Aggarwal *et al.*, 2013). Also some of the proteins involved in the signal transduction pathway (*JAC1*, *PMI2*, *WEB1*) were shown to play a major role only in one of the responses (Suetsgu *et al.*, 2005; Luesse *et al.*, 2006; Kodama *et al.*, 2010). Our results, demonstrating that the studied ABA mutants have typical accumulation and lesser avoidance responses, are consistent with all these facts.

The chloroplast responses of the *abi4* mutant were comparable with wild type plants, which is in line with the results of Rojas-Pierce and coworkers (2014). The ABA1 protein was shown to play a role in many different physiological processes e.g. seed development, germination and seedling establishment, root development and pathogen responses, and to be involved in ABA, sugar (i.e. trehalose), nitrogen, ascorbate and ROS signaling (reviewed in León *et al.*, 2013). The ABA1 transcription factor was also shown to play a central role in plastid-to-nucleus retrograde signaling (Koussevitzky *et al.*, 2007; León *et al.*, 2013). The lack of differences in accumulation and avoidance responses between *abi4* and wild type indicates that no protein encoded by genes regulated by ABA1 is involved in the chloroplast movement mechanism in *Arabidopsis*.

The remaining two mutants, *aba1* and, *abi2* had significantly lower amplitudes of chloroplast responses in strong light as compared with the wild type. A partial inhibition of chloroplast responses to strong BL in *aba1* was already shown by DeBlasio and coworkers (2005) and Rojas-Pierce and coworkers (2014). To understand whether this effect is due to the reduced endogenous ABA level in this mutant we supplemented the plants with exogenous ABA. This treatment significantly enhanced chloroplast movements, partially rescuing the mutant phenotype (Fig. 6). The obtained result clearly shows that physiological endogenous ABA levels are necessary for the normal functioning of chloroplast movements. On the other hand, the reduced chloroplast avoidance in *abi1* can be partly due to the impairment of the xanthophyll cycle which was shown to affect chloroplast movements (Tlałka *et al.*, 1999). However, taking into account the role of zeaxanthin epoxidase in both ABA biosynthesis and the xanthophyll cycle it is difficult to definitely distinguish between the impact of each of these processes on chloroplast movement.

Reduced chloroplast movements in *abi2* plants also point to the involvement of ABA in the positive regulation of chloroplast movements, and more specifically, to the role of ABA perception through the PYR/PYL/RCAR-PP2C-SnRK2 signalosome. A similar result was obtained for the *abi1-1* mutant, defective in the *ABI1* gene, which is a close homolog of *ABI2* (Rojas-Pierce *et al.*, 2014). Additionally, in our study *abi2* plants were subjected to exogenous ABA treatment but it failed to restore normal chloroplast movements. This provides indirect evidence that the abnormal chloroplast movement in *abi2* is indeed related to the impaired ABA signaling in this mutant. The results obtained for *aba1* and *abi2* are consistent with the idea that ABA acts as a positive regulator of chloroplast responses. ABA appears to sensitize plants to light and to promote a stronger avoidance reaction, which is compatible with the role of avoidance as a stress response on the one hand, and ABA as a stress hormone on the other hand. As already suggested by Rojas-Pierce and coworkers (2014), the PMI1 protein seems a good candidate for an integrator of ABA and light signals to control chloroplast movements, possibly acting via calcium signaling.

Many genes involved in ABA biosynthesis and signaling, including *ABA1* and *ABI4* are regulated by sugars (reviewed in León & Sheen, 2003). On the other hand, we have shown that sugars are involved in the regulation of chloroplast relocations (Banaś & Gabryś, 2007; Eckstein *et al.*, 2012). However, taken together our results suggest that ABA is not an intermediate in sugar signaling to chloroplast movement. Sugars inhibit both chloroplast accumulation and avoidance, whereas ABA is only involved in the avoidance response. The influence of sugars on chloroplast movements in ABA mutants has to be investigated to verify this hypothesis.

The functioning of ABA in the modulation of chloroplast movements should also be considered in the context of cross-talk with other hormones. As can be seen on the example of stomatal movement regulation, the interactions between phototropin and hormone signaling can be very complex and the effects of one hormone may vary depending on the endogenous levels of others (Acharya & Assmann, 2009). Until now, only the role of auxin in the regulation of chloroplast relocations has been studied (Eckstein *et al.*, 2016). Several similarities between the involvements of ABA and auxin on chloroplast movements can be pointed out. Neither hormone affects chloroplast movements when applied directly to leaves. However, the disturbance of auxin and ABA homeostasis (by shoot decapitation and in the *aba1* mutant, respectively) impairs chloroplast movements, but in both cases this can be partially overcome by exogenous hormone application. The analysis of auxin and ABA signaling mutants suggests that the effects of these hormones on the chloroplast movement mechanism are at least partially dependent on long term changes in gene expression. Among the differences between ABA and auxin action in the regulation of chloroplast movements is the involvement in only one (avoidance) or both chloroplast reactions respectively. However, no direct clues to the
cross-talk between different hormones in the control of chloroplast movements have been found yet. The effect of auxin homeostasis disturbance (by shoot decapitation and auxin transport inhibitors) on endogenous hormone levels has been studied, but no major changes have been observed in the content of ABA and several cytokinins (Eckstein et al., 2016). Moreover, the exogenous application of cytokinins to leaves, alone or with auxin, did not affect chloroplast movements (unpublished results).

Differential role of ABA in chloroplast movements in mesophyll vs guard cells

The effect of exogenous ABA on chloroplast movements in mesophyll and guard cells differs significantly. ABA disturbs chloroplast relocations in guard cells, but not in the mesophyll of wild type plants. This may point to differences in signaling pathways and mechanisms of chloroplast relocations in both types of cells.

To date, only two papers have been published which focus on chloroplast movements in guard cells (Königer et al., 2010; Wang et al., 2011). The authors state that chloroplast movements in guard cells are similar to those observed in mesophyll cells. This comparison should be made with great care because both the geometry and functioning of guard cells are different from mesophyll cells. Moreover the chloroplast positioning in guard cells was defined and measured in a different way than the avoidance and accumulation responses in the mesophyll tissue. The former was analyzed along horizontal (epidermal/pore/center position in a guard cell) and vertical (upper/lower/middle part of the cell) axes (Königer et al., 2010). The tissue was fixed with glutaraldehyde which may be a source of artifacts due to the differential thickening of the guard cell walls. Similar to the mesophyll, chloroplast responses differed to some extent in pbot1-1/pbot2 and pbot2 mutants as compared to wild type plants. (Königer et al., 2010). Nevertheless, two important differences between chloroplast responses in guard and mesophyll cells should be pointed out. First, cytochalasin B did not stop the movements completely in guard cells (Wang et al., 2011) which suggests that they are only partly based on the actin cytoskeleton. Secondly, guard cells move themselves while mesophyll cells do not. Consequently, chloroplast relocations in guard cells might be due in part to the movement of the whole cell.

CONCLUSIONS

ABA is not a key player in the control of chloroplast responses in the mesophyll of Arabidopsis, in contrast to guard cells. Exogenous ABA affects the expression of phototropins at the protein level, but this is not reflected in blue light-activated chloroplast relocations. On the other hand, the impairment of chloroplast avoidance in aba1 and aba2 mutants point to the role of ABA in regulating this response. Furthermore, the results of mutant supplementation with exogenous ABA show that not only physiological ABA levels, but also ABA perception via the PYR/PYL/RCAR-P2C-SnRK2 signaling complex, are important for normal chloroplast movement. In physiological conditions, the full toolkit of proteins available in wild type plants makes the influence of ABA invisible.

Overall, ABA appears to enhance plant sensitivity to light and promote the avoidance response. This seems in line with the role of this hormone in drought stress, which is often a consequence of strong light stress in the natural environment. The avoidance reaction is a photoprotective response to excess light. ABA may therefore function as a signal linking plant responses to the two types of stress.

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Conflict of interests

The authors declare that they have no conflict of interests.

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