ACTA ABP BIOCHIMICA POLONICA

Vol. 63, No 3/2016
449–458
http://dx.doi.org/10.18388/abp.2016_1382

Regular paper

Abscisic acid and blue light signaling pathways in chloroplast movements in *Arabidopsis* mesophyll*

Aleksandra Eckstein[1]✉, Weronika Krzeszowiec[1], Agnieszka Katarzyna Banaś[1], Franciszek Janowiak[2] and Halina Gabryś[1]✉

[1]Jagiellonian University, Faculty of Biophysics, Biochemistry and Biotechnology, Department of Plant Biotechnology, Kraków, Poland;
[2]The Franciszek Górski Institute of Plant Physiology of Polish Academy of Sciences, Kraków, Poland

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

Received: 20 March, 2016; revised: 26 April, 2016; accepted: 29 June, 2016; available on-line: 02 August, 2016

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 (Jarillo *et al.,* 2001; Kagawa *et al.*, 2001; Sakai *et al.,* 2001). Apart from chloroplast redistribution they also control other acclimation responses: phototropism (Sakai *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; Oikawa *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; Anielska-Mazur *et al.,* 2009; Augustynowicz *et al.,* 2009; Eckstein *et al.,* 2016).

Absicisic 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 reponses 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 zexanthin 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

✉e-mail: Halina Gabryś e-mail: halina.gabrys@uj.edu.pl; Aleksandra Eckstein e-mail: aleksandra.eckstein@uj.edu.pl

*A preliminary report on the same subject was presented at the 43rd Winter School of the Faculty of Biochemistry, Biophysics and Biotechnology, 16–20 February 2016, Zakopane, Poland.

Abbreviations: ABA, abscisic acid; BL, blue light; DW, dry weight; phot1, phot2, phototropin1 and 2

xanthophyll cycle. The *aba1* mutant shows reduced growth and significantly lower endogenous ABA content (Rock & Zeevaart, 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 *ABI1* and *ABI2* (*ABA INSENSITIVE*) genes (Leung *et al.,* 1994; Meyer *et al.,* 1994; Leung *et al.,* 1997; Rodriguez *et al.,* 1998). The *abi1* and *abi2* mutants exhibit constitutive phosphatase activity and in consequence SnRK2 inactivation, resulting in plant insensitivity to ABA. *abi1* and *abi2* show reduced sensitivity to exogenous ABA and incresed ABA accumulation under low water potential, but normal stomatal behaviour (Korneef *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 *abi4* 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 apparatus 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.,* 2013). 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 membrane 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 stomatal 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 phytotoxin fusicoccin (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 *PMI1* (*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-photochemical quenching2-1*) mutant, also called *aba1-6* (*abscisic acid1-6*), 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 *abi4-1* (*abscisic acid insensitive4-1*, CS8104), *abi2-1* (*abscisic acid insensitive2-1*, CS23) and *aba1-6* (*abscisic acid1-6*, 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 23°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). *UBQ10* 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 $16\,000 \times g$, 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 Clarity 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: 36 μmol m^{-2} s^{-1} and 72 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

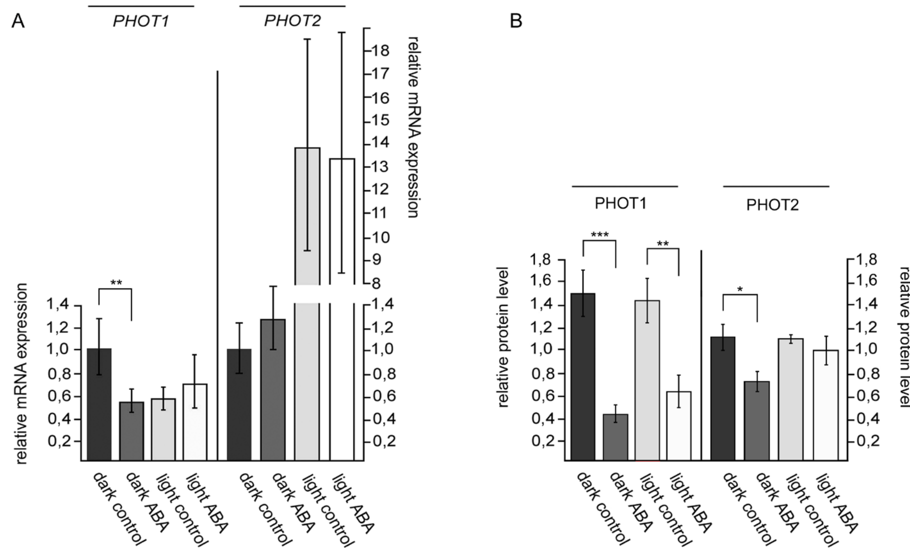

Figure 1. The expression of phototropin1 and phototropin2 in leaves of WT *A. thaliana* at mRNA (A) and protein (B) levels.
5-week-old plants were pretreated with control buffer or 100 μM ABA, dark-adapted overnight and then either illuminated with 100 μmol m^{-2} s^{-1} white fluorescent light in the 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, ***$p<0.001$).

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.

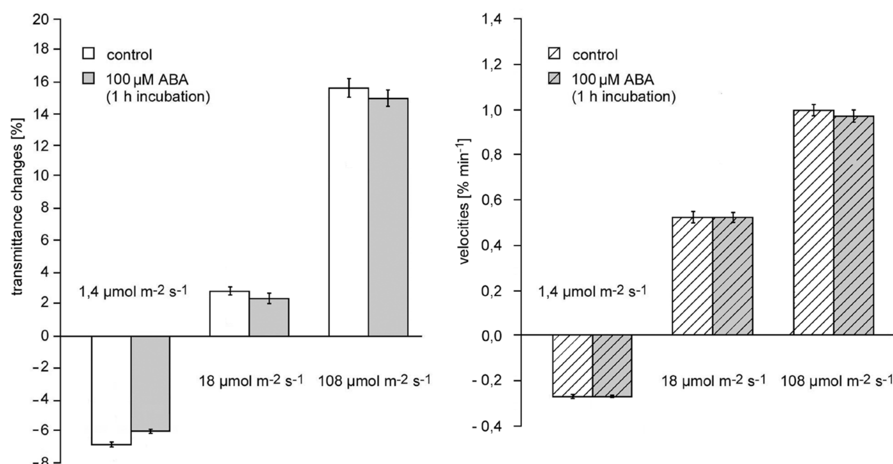

Figure 2. Amplitudes (left) and velocities (right) of transmittance changes reflecting chloroplast relocations induced by continuous blue light of increasing fluence rates (indicated on graph, each step 45 min) in WT *A. thaliana* leaves pretreated with 100 μM ABA for 1 h in the dark.
Results are means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

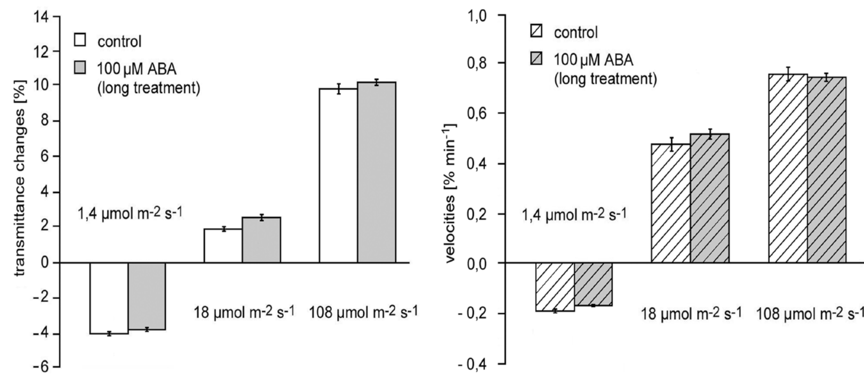

Figure 3. Amplitudes (left) and velocities (right) of transmittance changes reflecting chloroplast relocations induced by continuous blue light of increasing fluence rates (indicated on graph, each step 45 min) in WT *A. thaliana* leaves pretreated with 100 µM ABA for 48 h.

Results are means of 12 (control samples) or 15 (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).

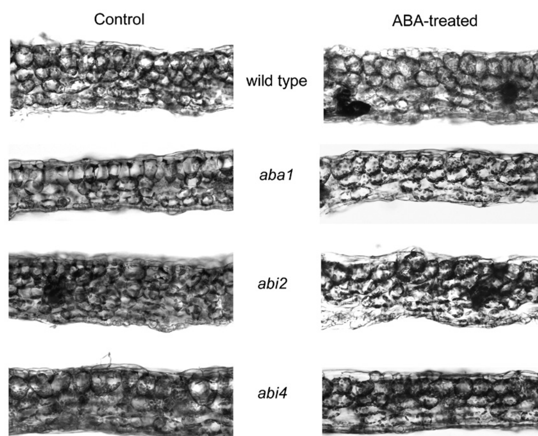

Figure 4. Cross-sections of fresh leaves of *A. thaliana* WT and *aba1*, *abi2*, *abi4* mutant plants, untreated or subjected to long-term ABA treatment (100 µM, 48 h).

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: *abi4-1*, *abi2-1* (*aba 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 *abi4* 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 *abi2* and p=0.0491, p= 0.0245, p=0.0104 for *aba1*. At 18 µmol m^{-2} s^{-1} *abi2* exibited 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

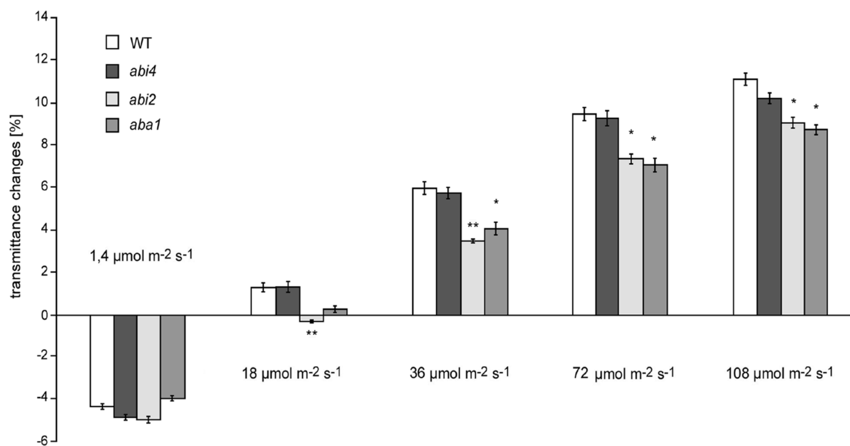

Figure 5. Amplitudes of transmittance changes reflecting chloroplast relocations induced by continuous blue light of increasing fluence rates (indicated on graph, each step 45 min) in leaves of *A. thaliana*: WT, *abi4*, *abi2* and *aba1*.
Results are means of 6 to 8 independent experiments, error bars represent S.E. Asterisks indicate the statistical significance of differences between wild type and mutant plants (*p*-values calculated using Student's *t*-test: **p*=0.01–0.05, ***p*=0.001–0.01).

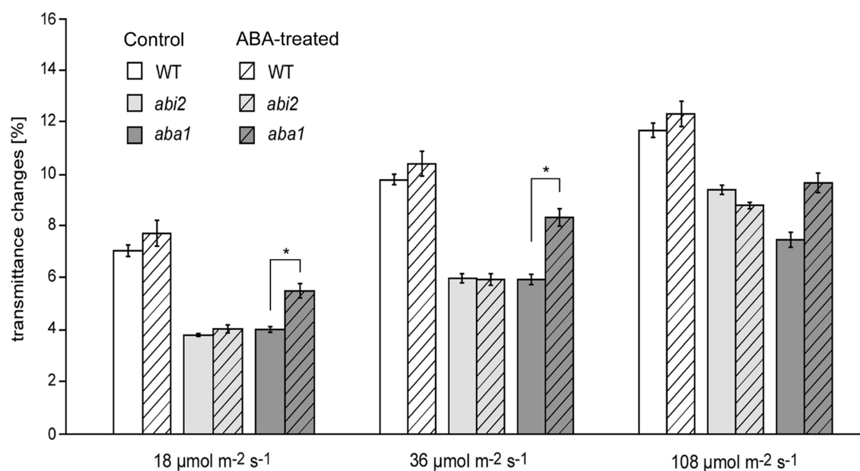

Figure 6. Amplitudes of transmittance changes reflecting chloroplast relocations induced by continuous blue light of increasing fluence rates (indicated on graph, each step 45 min) in leaves of *A. thaliana* WT, *abi2* and *aba1*, pretreated with 100 μM ABA for 16 h.
Results are means of 6 independent experiments , error bars represent S.E. Asterisks indicate the statistical significance of differences between the respective control and ABA-treated plants (p-values calculated using Student's *t*-test: **p*=0.01–0.05).

mutants. The responses of WT and *abi2* chloroplasts to all three BL intensities were unaffected by ABA treatment. By contrast, exogenous ABA treatment of *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 *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 *aba1* mutant can be partially rescued by exogenous ABA while changes introduced by the *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 *et al.,* 1998; Jarillo *et al.,* 2001; Kagawa *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 *et al.,* 2002; Sztatelman *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 *PHOT1* and *PHOT2* was strongly regulated by light, which is consistent with the results of Łabuz and coworkers (2012), but almost unaffected by ABA (Fig. 1A). Only *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 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 (Grabalska & 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 (Suetsugu *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 ABI4 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.a. trehalose), nitrogen, ascorbate and ROS signaling (reviewed in León *et al.,* 2013). The ABI4 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 ABI4 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 *aba1* 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 responses 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 *phot1-5/phot2* and *phot2* 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 *abi2* 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-PP2C-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.

Acknowledgements

The authors wish to thank Dr. Olga Sztatelman for critical reading of the manuscript.

Financial support: Financial support from the Polish National Science Centre, grant no. 2011/01/B/NZ3/02160 "Phototropin signaling in higher plants" is gratefully acknowledged. The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a partner of the Leading National Research Center (KNOW) supported by the Ministry of Science and Higher Education and benefits from the structural funds from the European Union – grant no: POIG.02.01.00-12-064/08.

Conflict of interests

The authors declare that they have no conflict of interests.

REFERENCES

Acharya BR, Assmann SM (2009) Hormone interactions in stomatal function. *Plant Mol Biol* **69**: 451–462. https://doi.org/10.1007/s11103-008-9427-0..

Aggarwal C, Łabuz J, Gabryś H (2013) Phosphoinositides play differential roles in regulating phototropin1-and phototropin2-mediated chloroplast movements in *Arabidopsis*. *PLoS One* **8**: e55393. https://doi.org/journal.pone.0055393 .

Anielska-Mazur A, Bernaś T, Gabryś H (2009) In vivo reorganization of the actin cytoskeleton in leaves of *Nicotiana tabacum* L. transformed with plastin-GFP: Correlation with light-activated chloroplast responses. *BMC-Plant Biol* **64**: 1–14. https://doi.org/10.1186/1471-2229-9-64.

Assmann SM, Schwartz A (1992) Synergistic effect of light and fusicoccin on stomatal opening. *Plant Physiol* **98**: 1349-1355. https://doi.org/10.1104/pp.98.4.1349 .

Assmann SM, Snyder JA, Lee Y-RJ (2000) ABA-deficient (*aba1*) and ABA-insensitive (*abi1-1*, *abi2-1*) mutants of *Arabidopsis* have a wild-type stomatal response to humidity. *Plant Cell Environ* **23**: 387–395. https://doi.org/10.1046/j.1365-3040.2000.00551.x.

Augustynowicz J, Krzeszowiec W, Gabrys H (2009) Acquisition of plastid movement responsiveness to light during mesophyll cell differentiation. *Int J Dev Biol* **53**: 121–127. https://doi.org/10.1387/ijdb.062140ja.

Banaś AK, Aggarwal C, Łabuz J, Sztatelman O, Gabryś H (2012) Blue light signalling in chloroplast movements. *J Exp Bot* **63**: 1559–1574. https://doi.org/10.1093/jxb/err429.

Banaś AK, Gabryś H (2007) Influence of sugars on blue light-induced chloroplast relocations. *Plant Signal Behav* **2**: 221–230. https://doi.org/ 10.4161/psb.2.4.4392.

Barrero JM, Piqueras P, González-Guzmán M, Serrano R, Rodríguez PL, Ponce MR, Micol JL (2005) A mutational analysis of the ABA1 gene of *Arabidopsis thaliana* highlights the involvement of ABA in vegetative development. *J Exp Bot* **56**: 2071–2083. https://doi.org/10.1093/jxb/eri206.

Blackman PG, Davies WJ (1983) The effects of cytokinins and ABA on stomatal behaviour of maize and *Commelina*. *J Exp Bot* 34: 1619–1626. https://doi.org/10.1093/jxb/34.12.1619.

Brady SM, McCourt P (2003) Hormone cross-talk in seed dormancy. *J Plant Growth Regul* **22**: 25–31. https://doi.org/10.1007/s00344-003-0018-7.

Cao FY, Yoshioka K, Desveaux D (2011) The roles of ABA in plant–pathogen interactions. *Journal Plant Res* **124**: 489–499. https://doi.org/10.1007/s10265-011-0409-y.

Christie JM, Reymond P, Powell GK, Bernasconi P, Raibekas AA, Liscum E, Briggs WR (1998). *Arabidopsis* NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. *Science* **282**: 1698–1701. https://doi.org/ 10.1126/science.282.5394.1698.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol* **139**: 5–17. https://doi.org/10.1104/pp.105.063743.

DeBlasio SL, Luesse DL, Hangarter RP (2005) A plant-specific protein essential for blue-light-induced chloroplast movements. *Plant Physiol* **139**: 101–114. https://doi.org/10.1104/pp.105.061887 .

Desikan R, Last K, Harrett-Williams R, Tagliavia C, Harter K, Hooley R, Hancock JT, Neill, SJ (2006) Ethylene-induced stomatal closure in Arabidopsis occurs via AtrbohF-mediated hydrogen peroxide synthesis. *The Plant Journal* **47**: 907–916. https://doi.org/10.1111/j.1365-313X.2006.02842.x.

Dubas E, Janowiak F, Krzewska M, Hura T, Żur I (2013) Endogenous ABA concentration and cytoplasmic membrane fluidity in microspores of oilseed rape (*Brassica napus* L.) genotypes differing in responsiveness to androgenesis induction. *Plant Cell Rep* **32**: 1465–1475. https://doi.org/10.1007/s00299-013-1458-6.

Eckstein A, Krzeszowiec W, Waligórski P, Gabryś H (2016) Auxin and chloroplast movements. *Physiol Plantarum* **156**: 351–366. https://doi.org/10.1111/ppl.12396.

Eckstein A, Zięba P, Gabryś H (2012) Sugar and light effects on the condition of the photosynthetic apparatus of *Arabidopsis thaliana* cultured in vitro. *J Plant Growth Regul* **31**: 90–101 https://doi.org/10.1007/s00344-011-9222-z.

Finkelstein RR (1994) Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. *Plant J* **5**: 765–771. https://doi.org/10.1046/j.1365-313X.1994.5060765.x.

Finkelstein RR, Wang ML, Lynch TJ, Rao S, Goodman HM (1998) The Arabidopsis abscisic acid response locus ABI4 encodes an APETALA2 domain protein. *Plant Cell* **10**: 1043–1054. https://doi.org/10.1105/tpc.10.6.1043.

Gabryś H (2004) Blue light-induced orientation movements of chloroplasts in higher plants: Recent progress in the study of their mechanisms. *Acta Physiol Plant* **26**: 473–478. https://doi.org/10.1007/s11738-004-0038-3.

Gabryś H (2012) Blue-Light-Activated Chloroplast Movements: Progress in the Last Decade. In *Progress in Botany 73*. Lüttge U, Beyschlag W, Büdel B, Francis D eds, pp 189–205. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-22746-2_7.

Gabryś H, Krzeszowiec W (2012) Chloroplast movements induced by light: diversity of mechanisms in various taxa. In *Biological diversity*. Łaska G ed, pp 9–24. Bialystok: Polish Botanical Society. .

Grabalska M, Malec P (2004) Blue Light-induced chloroplast reorientations in *Lemna trisulca* L. (Duckweed) are controlled by two separable cellular mechanisms as suggested by different sensitivity to Wortmannin. *Photochem Photobiol* **79**: 343–348. https://doi.org/10.1111/j.1751-1097.2004.tb00019.x.

Haubrick LL, Torsethaugen G, Assmann SM (2006) Effect of brassinolide, alone and in concert with abscisic acid, on control of stomatal aperture and potassium currents of *Vicia faba* guard cell protoplasts. *Physiol Plantarum* **128**: 134–143. https://doi.org/10.1111/j.1399-3054.2006.00708.x.

Haupt W (1999) Chloroplast movement: from phenomenology to molecular biology. In *Progress in Botany*. Esser K, Kadereit JW, Runge M eds, pp 3–36. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-59940-8_1.

Haupt W, Scheuerlein R (1990) Chloroplast movement. *Plant Cell Environ* **13**: 595–614. https://doi.org/10.1111/j.1365-3040.1990.tb01078.x.

Hubbard KE, Nishimur N, Hitomi K, Getzoff ED, Schroeder JI (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes Dev* **24**: 1695–1708. https://doi.org/10.1101/gad.1953910.

Inoue SI, Kinoshita T, Takemiya A, Doi M, Shimazaki KI (2008) Leaf positioning of Arabidopsis in response to blue light. *Mol Plant* **1**: 15–26. https://doi.org/10.1093/mp/ssm001.

Iwabuchi K, Sakai T, Takagi S (2007) Blue light-dependent nuclear positioning in *Arabidopsis thaliana* leaf cells. *Plant Cell Physiol* **48**: 1291–1298. https://doi.org/10.1093/pcp/pcm095.

Jarillo JA, Gabrys H, Capel J, Alonso JM, Ecker JR, Cashmore AR (2001) Phototropin-related NPL1 controls chloroplast relocation induced by blue light. *Nature* **410**: 952–954. https://doi.org/10.1038/35073622.

Kadota A, Yamada N, Suetsugu N, Hirose M, Saito C, Shoda K, Ichikawa S, Kagawa T, Nakanod A, Wada M (2009) Short actin-based mechanism for light-directed chloroplast movement in Arabidopsis. *Proc Natl Acad Sci USA* **106**: 13106–13111. https://doi.org/10.1073/pnas.0906250106.

Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M (2001) *Arabidopsis* NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. *Science* **291**: 2138–2141. https://doi.org/10.1126/science.291.5511.2138.

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002) Chloroplast avoidance movement reduces photodamage in plants. *Nature* **420**: 829–832. https://doi.org/10.1038/nature01213.

Kinoshita T, Shimazaki KI (2001) Analysis of the phosphorylation level in guard-cell plasma membrane H^+-ATPase in response to fusicoccin. *Plant Cell Physiol* **42**: 424–432. https://doi.org/10.1093/pcp/pce055.

Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki KI (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414**: 656–660. https://doi.org/10.1038/414656a.

Kodama Y, Suetsugu N, Kong SG, Wada M (2010). Two interacting coiled-coil proteins, WEB1 and PMI2, maintain the chloroplast photorelocation movement velocity in Arabidopsis. *Proc Natl Acad Sci USA* **107**: 19591–19596. https://doi.org/10.1073/pnas.1007836107.

Koornneef M, Reuling G, Karssen CM (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol Plant* **61**: 377–383. https://doi.org/10.1111/j.1399-3054.1984.tb06343.x.

Königer M, Jessen B, Yang R, Sittler D, Harris GC (2010) Light, genotype, and abscisic acid affect chloroplast positioning in guard cells of *Arabidopsis thaliana* leaves in distinct ways. *Photosynth Res* **105**: 213–227. https://doi.org/10.1007/s11120-010-9580-6.

Koussevitzky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, Surpin M, Lim J, Mittler R, Chory J (2007) Signals from chloroplasts converge to regulate nuclear gene expression. *Science* **316**: 715–719. https://doi.org/10.1126/science. 1140516.

Krzeszowiec W, Rajwa B, Dobrucki J, Gabryś H (2007) Actin cytoskeleton in *Arabidopsis thaliana* under blue and red light. *Biol Cell* **99**: 251–260. https://doi.org/10.1042/BC20060077.

Krzeszowiec W, Gabrys H (2007) Phototropin mediated relocation of myosins in *Arabidopsis thaliana*. *Plant Signal Behav* **2**: 333–336. https://doi.org/10.4161/psb.2.5.4509.

León P, Sheen J (2003) Sugar and hormone connections. *Trends Plant Sci* **8**: 110–116. https://doi.org/10.1016/S1360-1385(03)00011-6.

León P, Gregorio J, Cordoba E (2013) ABI4 and its role in chloroplast retrograde communication. *Front Plant Sci* **3**: 304. https://doi.org/10.3389/fpls.2012.00304.

Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J (1994) *Arabidopsis* ABA response gene ABI1: features of a calcium-modulated protein phosphatase. *Science* **264**: 1448–1451. https://doi.org/10.1126/science.7910981.

Leung J, Merlot S, Giraudat J (1997) The *Arabidopsis* ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* **9**: 759–771. https://doi.org/10.1105/tpc.9.5.759.

Lohse G, Hedrich R (1992) Characterization of the plasma-membrane H^+-ATPase from *Vicia faba* guard cells. *Planta* **188**: 206–214. https://doi.org/10.1007/BF00216815.

Luesse DR, DeBlasio SL, Hangarter RP. (2006) Plastid movement impaired 2, a new gene involved in normal blue-light-induced chloroplast movements in *Arabidopsis*. *Plant Physiol* **141**: 1328–1337. https://doi.org/10.1104/pp.106.080333.

Łabuz J, Sztatelman O, Banaś AK, Gabryś H (2012) The expression of phototropins in *Arabidopsis* leaves: developmental and light regulation. *J Exp Bot* **63**: 763–771. https://doi.org/10.1093/jxb/ers061.

Łabuz J, Hermanowicz P, Gabryś H (2015) The impact of temperature on blue light induced chloroplast movements in *Arabidopsis thaliana*. *Plant Science* **239**: 238–249. https://doi.org/10.1016/j.plantsci.2015.07.013.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**: 1064–1068. https://doi.org/10.1126/science.1172408.

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, Frey A, Marion-Poll, A (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *EMBO J* **15**: 2331..

Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J* **25**: 295–303. https://doi.org/10.1046/j.1365-313x.2001.00965.x.

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* **264**: 1452–1455. https://doi.org/10.1126/science.8197457.

Mori IC, Pinontoan R, Kawano T, Muto S (2001) Involvement of superoxide generation in salicylic acid-induced stomatal closure in *Vicia faba*. *Plant Cell Physiol* **42**: 1383–1388..

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. *Annu Rev Plant Biol* **56**: 165–185. https://doi.org/10.1146/annurev.arplant.56.032604.144046.

Nauš J, Rolencová M, Hlaváčkova V (2008) Is chloroplast movement in tobacco plants influenced systemically after local illumination or burning stress? *Journal Integr Plant Biol* **50**: 1292–1299. https://doi.org/10.1111/j.1744-7909.2008.00743.x.

Oikawa K, Kasahara M, Kiyosue T, Kagawa T, Suetsugu N, Takahashi F, Kanegae T, Niwa Y, Kadota A, Wada M (2003) Chloroplast unusual positioning1 is essential for proper chloroplast positioning. *Plant Cell* **15**: 2805–2815. https://doi.org/10.1105/tpc.016428.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071. https://doi.org/10.1126/science.1173041.

Pilot G, Lacombe B, Gaymard F, Cherel I, Boucherez J, Thibaud J-B, Sentenac H (2001) Guard cell inward K+ channel activity in Arabidopsis involves expression of the twin channel subunits KAT1 and KAT2. *J Biol Chem* **276**: 3215–3221. https://doi.org/10.1074/jbc.M007303200.

Rock CD, Zeevaart JA (1991) The aba mutant of Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. *Proc Natl Acad Sci USA* **88**: 7496–7499. https://doi.org/10.1073/pnas.88.17.7496 .

Rodriguez PL, Benning G, Grill E (1998) ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in Arabidopsis. *FEBS Lett*, **421**: 185–190. https://doi.org/ 10.1016/S0014-5793(97)01558-5.

Rojas-Pierce M, Whippo CW, Davis PA, Hangarter RP, and Springer PS (2014) PLASTID MOVEMENT IMPAIRED1 mediates ABA sensitivity during germination and implicates ABA in light-mediated chloroplast movements. *Plant Physiol Biochem* **83**: 185–193. https://doi.org/10.1016/j.plaphy.2014.07.014.

Rook F, Hadingham SA, Li Y, Bevan MW (2006) Sugar and ABA response pathways and the control of gene expression. *Plant Cell Environ* **29**: 426–434. https://doi.org/10.1111/j.1365-3040.2005.01477.x.

Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Okada K (2001) Arabidopsis nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. *Proc Natl Acad Sci USA* **98**: 6969–6974. https://doi.org/10.1073/pnas.101137598.

Sakamoto K, Briggs WR (2002) Cellular and subcellular localization of phototropin 1. *Plant Cell* **14**: 1723–1735. https://doi.org/10.1105/tpc.003293 .

Senn G (1908) Die Gestalts- und Lageveranderung der Pflanzenchromatophoren. Leipzig Stuttgart: W. Engelmann (in German).

Shu K, Zhang H, Wang S, Chen M, Wu Y, Tang S, Xie Q (2013) ABI4 regulates primary seed dormancy by regulating the biogenesis of abscisic acid and gibberellins in *Arabidopsis*. *PLoS Gen* **9**: e1003577. https://doi.org/10.1371/journal.pgen.1003577.

Sirichandra C, Wasilewska A, Vlad F, Valon C, Leung J (2009) The guard cell as a single-cell model towards understanding drought tolerance and abscisic acid action. *J Exp Bot* **60**: 1439–1463. https://doi.org/10.1093/jxb/ern340.

Suetsugu N, Kagawa T, Wada M (2005) An auxilin-like J-domain protein, JAC1, regulates phototropin-mediated chloroplast movement in Arabidopsis. *Plant Physiol* **139**: 151–162. https://doi.org/10.1104/pp.105.067371.

Suhita D, Kolla VA, Vavasseur A, Raghavendra AS (2003) Different signaling pathways involved during the suppression of stomatal opening by methyl jasmonate or abscisic acid. *Plant Sci* 164: 481–488. https://doi.org/10.1016/S0168-9452(02)00432-6.

Sztatelman O, Waloszek A, Banaś AK, Gabryś H (2010) Photoprotective function of chloroplast avoidance movement: In vivo chlorophyll fluorescence study. *J Plant Physiol* **167**: 709–716. https://doi.org/10.1016/j.jplph.2009.12.015.

Sztatelman O, Łabuz J, Hermanowicz P, Banaś AK, Bażant A, Zgłobicki P, Aggarwal C, Nadzieja M, Krzeszowiec W, Strzalka W, Gabryś H (2016) Fine tuning chloroplast movements through physical interactions between phototropins. *J Exp Bot*. https://doi.org/10.1093/jxb/erw265.

Takagi S (2003) Actin-based photo-orientation movement of chloroplasts in plant cells. *J Exp Biol* **206**: 1963–1969. https://doi.org/10.1242/jeb.00215.

Takemiya A, Shimazaki K (2010) Phosphatidic acid inhibits blue light-induced stomatal opening via inhibition of protein phosphatase. *Plant Physiol* **153**: 1555–1562. https://doi.org/ 10.1104/pp.110.155689 .

Takemiya A, Sugiyama N, Fujimoto H, Tsutsumi T, Yamauchi S, Hiyama A, Tada Y, Christie JM, Shimazaki KI (2013). Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. *Nature Commun* **4**: 2094. https://doi.org/ 10.1038/ncomms3094.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S (2005) Ethylene inhibits abscisic acid-induced stomatal closure in *Arabidopsis*. *Plant Physiol* **138**: 2337–2343. https://doi.org/10.1104/pp.105.063503 .

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S (2006) Cytokinin and auxin inhibit abscisic acid-induced stomatal closure by enhancing ethylene production in *Arabidopsis*. *J Exp Bot* **57**: 2259–2266. https://doi.org/10.1093/jxb/erj193.

Tlałka M, Runquist M, Fricker M (1999) Light perception and the role of the xanthophyll cycle in blue-light-dependent chloroplast movements in *Lemna trisulca* L. *Plant J* **20**: 447–459. https://doi.org/10.1046/j.1365-313x.1999.00614.x.

Trojan A, Gabryś H (1996) Chloroplast distribution in *Arabidopsis thaliana* (L.) depends on light conditions during growth. *Plant Physiol* **111**: 419–425. https://doi.org/10.1104/pp.111.2.419.

Tseng TS, Craig Whippo C, Hangarter RP, Briggsa WR (2012) The Role of a 14-3-3 Protein in stomatal opening mediated by PHOT2 in *Arabidopsis*. *Plant Cell* **24**: 1114–1126. https://doi.org/10.1105/tpc.111.092130 .

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc Natl Acad Sci USA* **106**: 17588–17593. https://doi.org/10.1073/pnas.0907095106.

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant Cell Physiol* **51**: 1821–1839. https://doi.org/10.1093/pcp/pcq156.

Verslues PE, Bray EA (2006) Role of abscisic acid (ABA) and *Arabidopsis thaliana* ABA-insensitive loci in low water potential-induced ABA and proline accumulation. *J Exp Bot* **57**: 201–212. https://doi.org/10.1093/jxb/erj026.

Wada M, Kagawa T, Sato Y (2003) Chloroplast movement. *Annu Rev Plant Biol* **54**: 455–468. https://doi.org/10.1146/annurev.arplant.54.031902.135023.

Wada M, Suetsugu N (2004) Plant organelle positioning. *Curr Opin Plant Biol* **7**: 626–631. https://doi.org/10.1016/j.pbi.2004.09.005.

Walczak T, Gabryś H (1980) New type of photometer for measurements of transmission changes corresponding to chloroplast movements in leaves. *Photosynth* **14**: 65–72. .

Walker-Simmons MK, Abrams SR (1991) Use of ABA immunoassays. In *Abscisic acid physiology and biochemistry*. Davies WJ, Jones HG eds, pp 53–63. Oxford: Bios Scientific Publishers. .

Wang XL, Gao XQ, Wang XC (2011) Stochastic dynamics of actin filaments in guard cells regulating chloroplast localization during stomatal movement. *Plant Cell Environ* **34**: 1248–1257. https://doi.org/10.1111/j.1365-3040.2011.02325.x.

Wind JJ, Peviani A, Snel B, Hanson J, Smeekens SC (2013) ABI4: versatile activator and repressor. *Trends Plant Sci* **18**: 125–132. https://doi.org/10.1016/j.tplants.2012.10.004.

Yamburenko MV, Zubo YO, Börner T (2015) Abscisic acid affects transcription of chloroplast genes via protein phosphatase 2C-dependent activation of nuclear genes: repression by guanosine-3'-5'-bisdiphosphate and activation by sigma factor 5. *Plant J* **82**: 1030–1041. https://doi.org/10.1111/tpj.12876.

Zhang Z, Feng L-Y, Cheng J, Tang H, Xu F, Zhu F, Zhao ZY, Yuan M, Chen Y-E, Wang J-H, Yuan S, Lin H-H (2013) The roles of two transcription factors, ABI4 and CBFA, in ABA and plastid signalling and stress response. *Plant Mol Biol* **83**: 445–458. https://doi.org/10.1007/s11103-013-0102-8 .

Zhang X, Wang H, Takemiya A, Song C, Kinoshita T, Shimazaki K (2004) Inhibition of blue light-dependent H+ pumping by abscisic acid through hydrogen peroxide-induced dephosphorylation of the plasma membrane H+-ATPase in guard cell protoplasts. *Plant Physiology* **136**: 4150–4158. https://doi.org/10.1104/pp.104.046573 .

Zurzycki J (1955) Chloroplast arrangement as a factor in photosynthesis. *Acta Soc Bot Pol* **24**: 27–63.