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A Protein Phosphatase 2A Catalytic Subunit Modulates Blue Light-Induced Chloroplast Avoidance Movements through Regulating Actin Cytoskeleton in Arabidopsis

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Chloroplast avoidance movements mediated by phototropin 2 (phot2) are one of most important physiological events in the response to high-fluence blue light (BL), which reduces damage to the photosynthetic machinery under excess light. Protein phosphatase 2A-2 (PP2A-2) is an isoform of the catalytic subunit of PP2A, which regulates a number of developmental processes. To investigate whether PP2A-2 was involved in high-fluence BL-induced chloroplast avoidance movements, we first analyzed chloroplast migration in the leaves of the *pp2a-2* mutant in response to BL. The data showed that PP2A-2 might act as a positive regulator in phot2-mediated chloroplast avoidance movements, but not in phot1-mediated chloroplast accumulation movements. Then, the effect of okadaic acid (OA) and cantharidin (selective PP2A inhibitors) on high-fluence BL response was further investigated in *Arabidopsis thaliana* mesophyll cells. Within a certain concentration range, exogenously applied OA or cantharidin inhibited the high-fluence BL-induced chloroplast movements in a concentration-dependent manner. Actin depolymerizing factor (ADF)/cofilin phosphorylation assays demonstrated that PP2A-2 can activate/dephosphorylate ADF/cofilin, an actin-binding protein, in Arabidopsis mesophyll cells. Consistent with this observation, the experiments showed that OA could inhibit ADF1 binding to the actin and suppress the reorganization of the actin cytoskeleton after high-fluence BL irradiation. The *adf1* and *adf3* mutants also exhibited reduced high-fluence BL-induced chloroplast avoidance movements. In conclusion, we identified that PP2A-2 regulated the activation of ADF/cofilin, which, in turn, regulated actin cytoskeleton remodeling and was involved in phot2-mediated chloroplast avoidance movements.

Keywords: ADF/cofilin • Blue light • Chloroplast movements • Okadaic acid • Phototropin2 • PP2A-2.

Abbreviations: ADF, actin depolymerizing factor; BL, blue light; CFP, cyan fluorescent protein; ECFP, enhanced cyan fluorescent protein; GFP, green fluorescent protein; LIM, lin11, isl-1, and mec-3; OA, okadaic acid; phot, phototropin; PP1, type 1 protein phosphatases; PP2A, protein phosphatase 2A; PVDF, polyvinylidene difluoride; RL, red light; WT, wild type.

Introduction

As plants are anchored in place by root systems, they have had to develop a series of mechanisms to help them adapt to changing environmental conditions. One of the best-characterized adaptive mechanisms is chloroplast movement, which can be induced by many kinds of external stimuli, including local, light, mechanical or stress effects (Zurzycki 1955, Haupt and Scheuerlein 1990, Suetsugu and Wada 2009). It has been shown that light signaling in chloroplast movement is perceived by phototropins (phot1 and phot2), which were plant-specific serine/threonine protein kinases with two light, oxygen or voltage domains (Huala et al. 1997, Briggs and Huala 1999, Kagawa et al. 2001). According to Sakai et al. (2001), phot1 and phot2 function redundantly in chloroplast accumulation under low blue light (BL) (2–16 $\mu mol\ m^{-2}\ s^{-1}$), but avoidance movements induced by high-fluence BL ($>16\ \mu mol\ m^{-2}\ s^{-1}$) require only phot2.

Previous drug studies proved that most land plant species used actin filaments exclusively (rather than microtubules) for chloroplast movements. Anti-actin drugs (such as latrunculin B), but not anti-microtubule drugs, inhibit chloroplast movements in various green plant species (Suetsugu and Wada 2009). Recent reports show that the mobility of a variety of plant cell organelles, including Golgi bodies, mitochondria, peroxisomes and endoplasmic reticulum, is mediated by class XI myosins, but there is no clear genetic evidence of myosin involvement in chloroplast movement (Avisar et al. 2008, Peremyslov et al. 2008). It is currently held that cp-actin filaments along the chloroplast periphery are associated with chloroplast photorelocation. Reports indicate that the disruption of two genes encoding actin-binding proteins (*chup1* and *thrumin1*) impairs chloroplast photorelocation in response to

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BL (Kadota et al. 2009, Whippo et al. 2011). It has been proven that actin filament stability is conferred by the association of filaments with side-binding and bundling proteins, including fimbrins, formins, LIMs (lin11, isl-1, and mec-3) and villins. Also, several proteins might play a role in these severing and capping activities, including actin depolymerizing factor (ADF)/cofilin, heterodimeric capping protein, villin/gelsolin and Aip1 (Simon and Pon 1996, Maciver 1998, Staiger et al. 2010). One central and presumably biologically relevant property of ADF/cofilins is their ability to sever actin filaments and accelerate actin filament disassembly, as involved in many cellular processes, such as motility at the leading edge of cells, polarized cell growth, endocytosis, phagocytosis, cellular activation, cytokinesis and pathogen intracellular motility (Maciver 1998, Bernstein and Bamburg 2010, Tammana et al. 2010). The *in vivo* activity of ADF/cofilin is regulated through reversible phosphorylation and dephosphorylation at the serine residue; the unphosphorylated form of ADF/cofilin is active (Ambach et al. 2000, Bernstein and Bamburg 2010).

In plants, protein phosphorylation and dephosphorylation, which are catalyzed by protein kinases and phosphatases, respectively, regulate numerous physiological processes and biological development (Smith and Walker 1996, Luan 2003). Protein phosphatase 2A (PP2A) is one of the most abundant types of serine/threonine phosphatase in all eukaryotic cells (Luan 2003). PP2A consists of a catalytic subunit (C), a scaffolding subunit (A) and a regulatory subunit (B) (Mayer-Jaekel and Hemmings 1994). Previous results have proved that protein phosphatases, as protein phosphorylation regulators, positively regulated BL signaling in Arabidopsis. It has been shown that AtPP7 functions as a positive nucleus-localized signaling component that probably acts downstream of cryptochrome in the nucleus in response to BL irradiation (Møller et al. 2003). Previous pharmacological studies showed that the protein phosphatase inhibitors calyculin A, okadaic acid (OA) and tautomycin strongly inhibited BL-dependent stomatal opening in *Vicia faba* (Kinoshita and Shimazaki 1997, Takemiya et al. 2006). Recently, PP2Ac-2, a catalytic subunit of PP2A, was identified to be a specific component of the abscisic acid signaling pathway in Arabidopsis (Pernas et al. 2007). Also, the catalytic subunit of PP2Ac subfamily I acts as a negative regulator in plant defense responses (He et al. 2004). Furthermore, many studies have demonstrated that actin-related protein activation is regulated by protein phosphatase. For example, calyculin A induced the fragmentation of actin filaments and inhibited the formation of randomly oriented long actin filaments induced by abscisic acid or $CaCl_2$ in Arabidopsis guard cells (Hwang and Lee 2001). In human lymphocytes, type 1 protein phosphatases (PP1) and PP2A could associate with cofilin, which was essential for the functional dynamics of the actin cytoskeleton and mediated cofilin activation by dephosphorylating this protein (Ambach et al. 2000).

Although the relationship between the actin cytoskeleton and PP2A has been widely studied, it was still unknown whether PP2A is involved in high-fluence BL-induced chloroplast avoidance movements, which are based on the actin cytoskeleton. In the present study, we used protein phosphatase mutants to investigate the function of protein phosphatases in high-fluence BL-induced chloroplast avoidance movements and demonstrated that PP2A-2 (an isoform of the catalytic subunit of PP2A) modulates high-BL signal transduction. The current investigation revealed that the dephosphorylation/activation of ADF/cofilin by PP2A-2 resulted in changes in the actin cytoskeleton reorganization and participated in BL-induced chloroplast avoidance movements.

Results

High-fluence BL-induced chloroplast avoidance movements are weakened in the absence of PP2A-2

The role of PP2A-2 in BL-induced chloroplast movements was investigated first. It was clear that, after treatment with high-fluence BL ($50\ \mu mol\ m^{-2}\ s^{-1}$, 60 min), almost all chloroplasts in dark-acclimated wild type (WT) Arabidopsis leaves moved to anticlinal walls, parallel to the incident light (**Fig. 1A, B, E, F**). Although chloroplasts also migrated to the anticlinal walls in the leaves of the *pp2a-2* mutant in response to BL, the extent of chloroplast movement was far less than that in the WT (**Fig. 1C, D, G, H**). Quantitative analysis of the chloroplast population revealed that on exposure to BL for 30 min, approximately 70% of chloroplasts were located along the anticlinal walls in the WT (**Fig. 1I**), whereas only approximately 60% of chloroplasts were along the anticlinal walls in the *pp2a-2* mutant (**Fig. 1J**). After extending the exposure time to 60 min, only 15% of chloroplasts were located along the periclinal walls in the WT (**Fig. 1I**), but approximately 35% of chloroplasts could still be observed along the periclinal walls in the *pp2a-2* mutant (**Fig. 1J**).

Next, differences in BL-induced chloroplast movements between WT and *pp2a-2* mutant Arabidopsis were further examined according to the changes in leaf red light (RL) transmittance. The results demonstrated that RL transmittance in both the WT and *pp2a-2* mutant showed a rapid increase after 20 min of treatment with $100\ \mu mol\ m^{-2}\ s^{-1}$ BL (**Fig. 2A**). Importantly, after irradiation for >10 min with $100\ \mu mol\ m^{-2}\ s^{-1}$ BL, the RL transmittance in the WT was higher than in the *pp2a-2* mutant (**Fig. 2A**). However, all F1 progenies of a cross between the *pp2a-2* mutant (female parent) and WT Col-0 (pollen donor) showed similar chloroplast avoidance movements as the WT (**Supplementary Fig. 6**). With using knockout plants of other isoforms of PP2A catalytic subunits, we found that the *pp2a-1* and *pp2a-4* mutants showed the same chloroplast avoidance movement response as compared with the WT (**Supplementary Fig. 2B**). RT–PCR results showed that the expression levels of *PP2A-1* and *PP2A-4* were significant lower in leaves than those of *PP2A-2*, which is consistent with the data from the Arabidopsis eFP Browser, suggesting that the difference

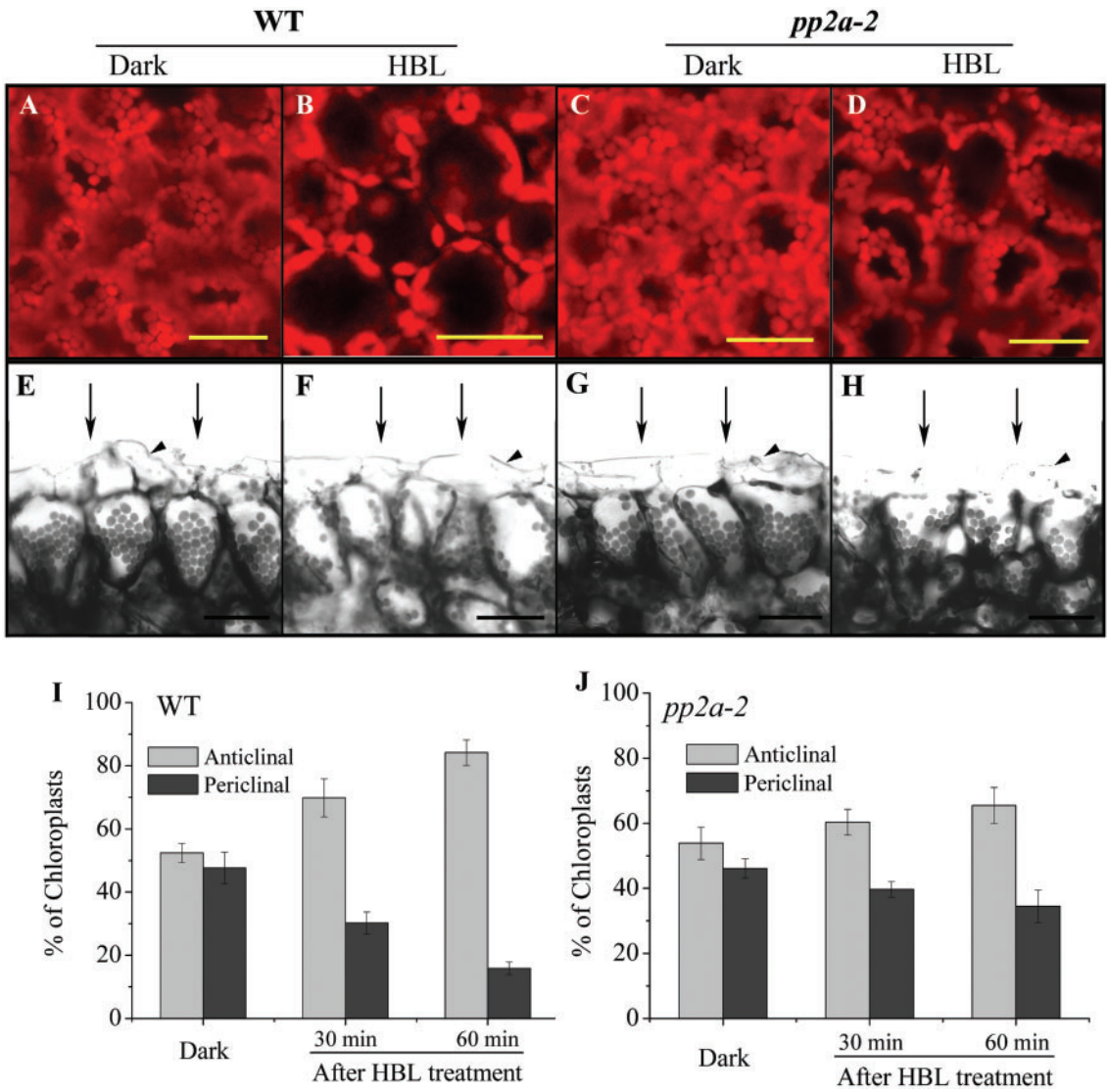

Fig. 1 BL-induced chloroplast movements in WT and *pp2a-2* mutant Arabidopsis leaf cells. Dark-acclimated WT and *pp2a-2* mutant Arabidopsis leaf strips were exposed to sequential 1 h treatments of high-fluence BL (50 μmol m^{-2} s^{-1}). Micrographs from time points 0 min (A and C) and 60 min (B and D) are shown. Cross sections of Arabidopsis leaves exposed to sequential 1 h treatments of high-fluence BL (50 μmol m^{-2} s^{-1}) in WT (E through F) and *pp2a-2* mutant (G through H) Arabidopsis leaf strips show chloroplast positioning in the mesophyll cell layer. Images from time points 0 min (E and G) and 60 min (F and H) are shown. Solid arrowheads indicate the epidermal cell. Arrows indicate the direction of the incident BL. Scale bar = 50 μm. Bar graphs depict the percentage (± SE) of chloroplasts located along the anticlinal cell walls in WT (I) and *pp2a-2* mutant (J) leaf cells, and represent averages for 50–90 cells per high-fluence BL (50 μmol m^{-2} s^{-1}) treatment at 30 and 60 min.

in the gene expression level might be the main reason for the involvement of PP2A-2 in chloroplast avoidance movement among highly similar PP2A catalytic subunits (**Supplementary Fig. 1D**). Further, we also investigated whether RCN1 (an A1 subunit of PP2A), which has been confirmed to play an important role in down-regulating photoactivated phot2 (Tseng and Briggs 2010), was involved in chloroplast movements induced by high-fluence BL. The results showed enhanced chloroplast avoidance movement in the *rcn1* mutant, which corresponded with the previous observation that the phosphorylation level of phot2 was higher in the *rcn1* mutant than in the WT (**Supplementary Fig. 2B**). The difference in the low-fluence BL-induced chloroplast accumulation movements between WT and *pp2a-2* mutant Arabidopsis was also examined. Clearly, the WT and the *pp2a-2* mutant showed a consistent decrease in RL transmittance in response to 0.3 μmol m^{-2} s^{-1} BL irradiation (**Fig. 2B**). However, upon exposure to 5 μmol m^{-2} s^{-1} BL, the short-term increase in RL transmittance disappeared in the *pp2a-2* mutant and, moreover, the magnitude of the decrease was greater than in the WT (**Fig. 2B**). These data indicate that PP2A-2 might act as a positive regulator in phot2-mediated chloroplast avoidance movements (>16 μmol m^{-2} s^{-1}), but is not involved in

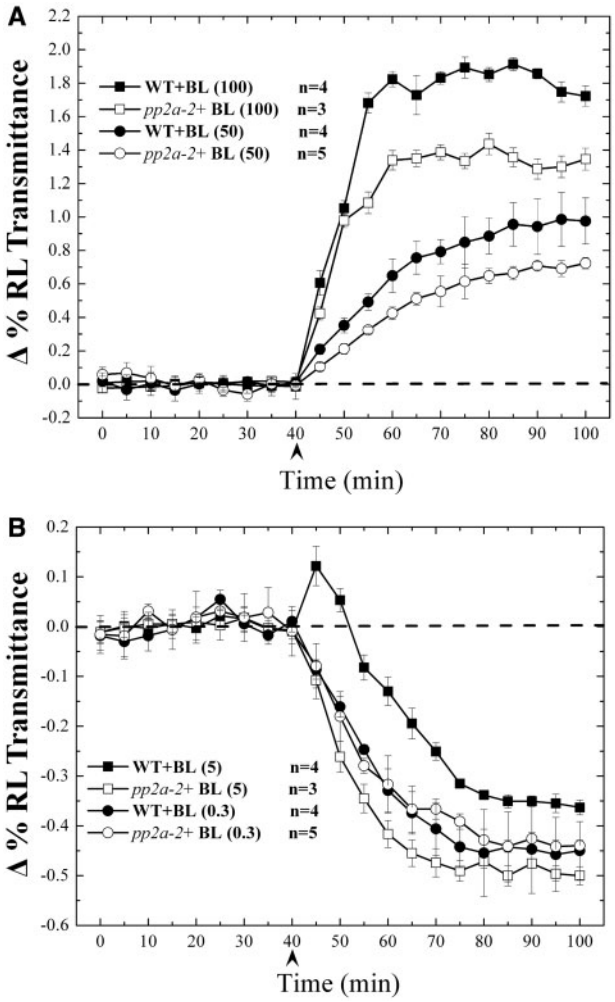

Fig. 2 Chloroplast movement response in *pp2a-2* mutant leaf cells induced by different BL fluence rates. RL transmittances were measured in dark-acclimated leaves for 40 min before exposure to different fluence rates of BL (100, 50, 5 and 0.3 μmol m^{-2} s^{-1}). The start of BL irradiation is indicated by the arrowheads. The results are presented as the average change (±SE) in the percentage RL transmittance of leaves relative to the average value measured before turning on the BL. The number of leaves (*n*) is shown for each treatment.

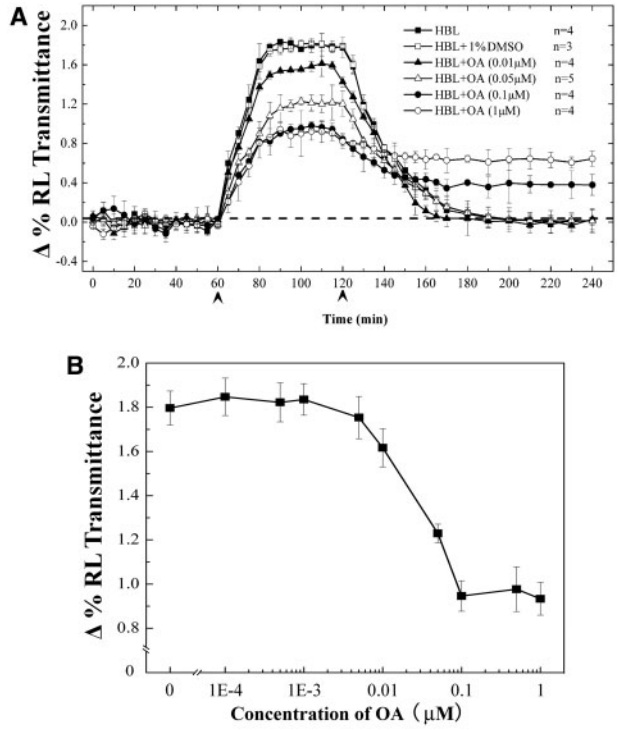

Fig. 3 Effects of OA on high-intensity BL-induced chloroplast movements in WT leaves. (A) Plots show the average change (± SE) in the percentage RL transmittance of leaves relative to the average transmittance measured for the leaves before the BL treatment. RL transmittance was measured in leaves every 5 min, and the treatments were carried out at 0 (adding OA or 1% DSMO), 60 (turning on BL) and 120 min (turning off BL and washout of OA or DSMO by using loading buffer), as indicated by the arrowheads. The individual leaves were incubated in loading buffer containing OA (0.01, 0.05, 0.1 or 1 μM). The number of leaves (*n*) is shown for each treatment. (B) RL transmittances changes were measured after 1 h high-intensity BL (100 μmol m^{-2} s^{-1}) exposure co-treatment with different concentrations of OA. The results are presented as the average change (± SE) in the percentage RL transmittance of leaves relative to the average value measured before turning on the BL. The experiments were repeated at least three times.

phot1-mediated chloroplast accumulation movements (<2 μmol m^{-2} s^{-1}). Taking into account the fact that both phot2 and phot1 were involved in chloroplast movements in response to BL at 2–16 μmol m^{-2} s^{-1}, the enhancement of chloroplast accumulation movements observed in the *pp2a-2* mutant treated with BL of 5 μmol m^{-2} s^{-1} might be ascribed to the attenuation of chloroplast avoidance movements resulting from the absence of PP2A-2 (**Fig. 2B**).

The inhibitory effects of PP2A inhibitor on high-fluence BL-induced chloroplast avoidance movements

To further investigate the role of PP2A-2 in chloroplast avoidance movements induced by high-fluence BL, we examined the change in the percentage RL transmittance of WT leaves in the presence of OA. The results showed that 0.01 μM OA greatly reduced the increase in the RL transmittance of leaves induced by high-fluence BL of 100 μmol m^{-2} s^{-1} and the inhibitory effects reached a maximum when OA was increased to 0.1 or 1 μM (**Fig. 3A**). It should be pointed out that OA did not affect the RL transmittance of leaves in the dark (**Fig. 3A**). A detailed analysis of the dose dependency was also conducted. It seemed that exogenously applied OA inhibited the high-fluence BL-induced chloroplast movements in a concentration-dependent manner within the concentration range of 10^{-3}–10^{-1} μM (**Fig. 3B**). Further study showed that high concentrations of OA (≥0.1 μM) appeared to have toxic effects on normal cell function, because neither changes in the RL transmittance under low BL (**Supplementary Fig. 3**) nor full

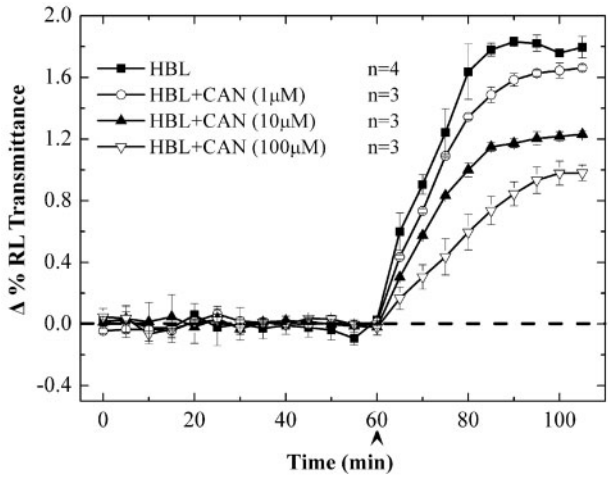

Fig. 4 Effects of cantharidin on high-intensity BL-induced chloroplast movements. RL transmittances were measured in dark-acclimated leaves for 60 min before exposure to 100 μmol m^{-2} s^{-1} BL in the presence of cantharidin (1, 10 and 100 μM). The start of BL irradiation is indicated by the arrowheads. The results are presented as the average change ± SE in the percentage RL transmittance of leaves relative to the average value measured before turning on the BL. The number of leaves (*n*) is shown for each treatment.

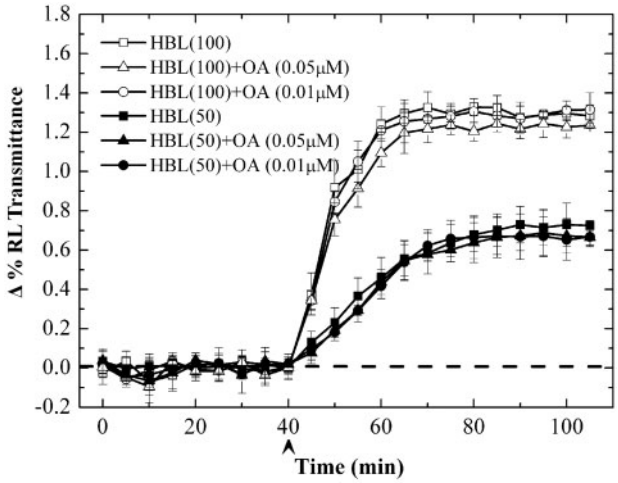

Fig. 5 Effects of OA on high-intensity BL-induced chloroplast movements in *pp2a-2* mutant leaves. RL transmittances were measured in dark-acclimated *pp2a-2* mutant leaves for 40 min before exposure to 50 and 100 μmol m^{-2} s^{-1} BL in the presence of 0.05 and 0.01 μM OA. The start of BL irradiation is indicated by the arrowheads. The results are presented as the average change (±SE) in the percentage RL transmittance of leaves relative to the average value measured before turning on the BL. The experiments were repeated at least three times.

recovery after turning off BL and washout of OA could be found (**Fig. 3A**). Moreover, 1–100 μM cantharidin (another potent and selective PP2A inhibitor) showed a similar inhibitory effect on chloroplast movement in WT leaves (**Fig. 4**).

Further, we examined the chloroplast movements of the *pp2a-2* mutant treated with OA under high-BL exposure. It was clear that regardless of the presence or absence of 0.01–0.05 μM OA, irradiation with 50 or 100 μmol m^{-2} s^{-1} BL for 1 h increased the RL transmittance through the *pp2a-2* mutant leaves by about 0.7 or 1.2% compared with the dark-acclimated phase, respectively (**Fig. 5**). This consistency of increased RL transmittance between OA-treated and -untreated *pp2a-2* mutant leaves implied that the effect of OA on chloroplast avoidance movements was due to the inhibition of PP2A-2. Taken together, these results indicated that the PP2A inhibitors displayed a similar effect on the RL transmittance as the *pp2a-2* mutation.

The difference between the WT and the *pp1* mutant in the chloroplast avoidance movement response induced by high-fluence BL was also examined. Clearly, in response to high-fluence BL, the time course of the increase in the percentage RL transmittance of leaves in the *pp1* and *topp3* mutants was in good accordance with that in the WT (**Supplementary Fig. 2**). By using the selective PP1 inhibitor tautomycin (Takemiya et al. 2006), it was shown that exogenously applied 0.1 and 10 μM tautomycin did not affect the chloroplast movements induced by high-fluence BL (**Supplementary Fig. 2B**). These data suggested that PP1 was perhaps not involved in chloroplast avoidance movements induced by high-fluence BL and that the reduction of these movements by OA might be due to its inhibitory effects on PP2A-2.

In vivo phosphorylation of ADF/cofilin in WT and protein phosphatase mutants

Phototropins were initially found as plasma membrane associated phosphorylated protein under blue light. However, the phosphorylation levels of phot2 were not significantly different in the *pp1* and *pp2a-2* mutants compared with WT Arabidopsis (**Supplementary Fig. 4**). Previous studies have demonstrated that organelles can move along essentially static actin via myosin motor protein or migrate depending upon actin nucleation and polymerization regulated by a couple of actin-binding proteins (Simon and Pon 1996, Maciver 1998). Among these proteins, ADF/cofilin, a small phosphoinositide-sensitive actin-binding protein, has been confirmed to play a vital role in actin dynamics (Staiger et al. 2010, Tholl et al. 2011). Therefore, we investigated whether serine phosphatases were responsible for the activation of ADF/cofilin through dephosphorylation in protein phosphatase-regulated chloroplast movements. First, we detected the phosphorylation level of ADF/cofilin in Arabidopsis leaves acclimated in the dark for 18 h. Using OA to inhibit the serine phosphatases PP1 and PP2A, the results demonstrated that the dephosphorylation of ADF/cofilin could be significantly suppressed by OA (**Fig. 6**). Furthermore, experiments performed in protein phosphatase mutants showed that the phosphorylation level of ADF/cofilin was significantly higher in the *pp2a-2* mutant than in the WT (**Fig. 6A, B**). However, there was no significant difference in

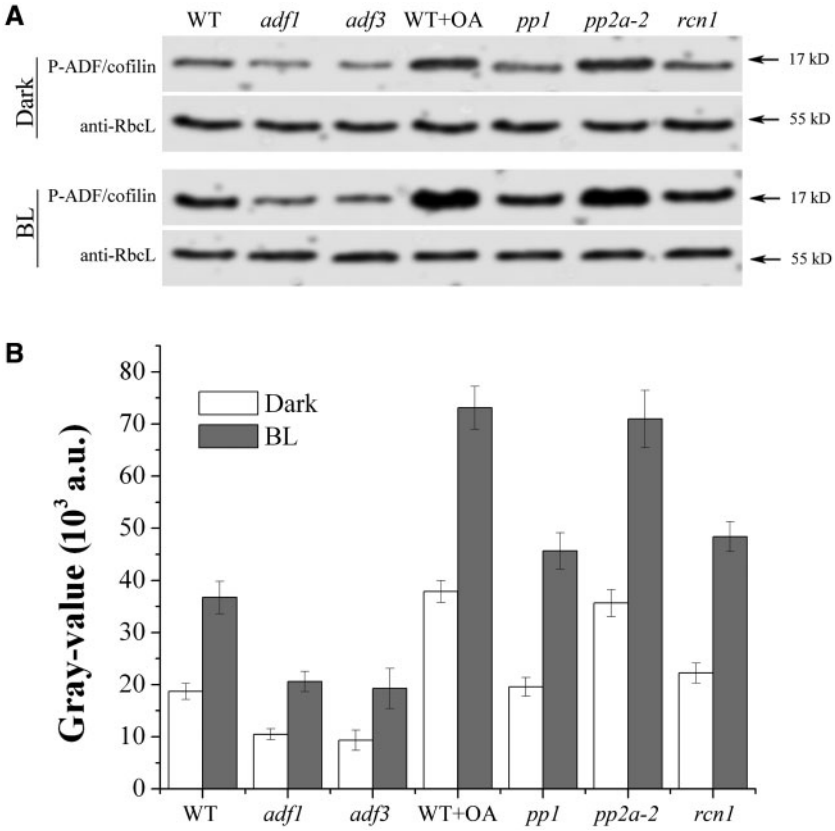

Fig. 6 Phosphorylation levels of ADF/cofilin induced by high-fluence BL in phosphatase mutant leaf cells. (A) The protoplasts were divided into two groups: dark group and BL group (illuminated with 100 μmol m^{-2} s^{-1} BL for 10 min followed by dark recovery for 5 min), and then the total protein (50 μg) extracted from treated protoplasts was subsequently separated by SDS–PAGE (WT, wild type; *adf1*, *adf1* mutant; *adf3*, *adf3* mutant; WT + OA, wild type co-treated with 0.1 μM OA; *pp1*, *pp1* mutant; *pp2a-2*, *pp2a-2* mutant; *rcn1*, *rcn1* mutant). Immunoblots of phospho-ADF/cofilin (P-ADF/cofilin) show the phosphorylation levels of ADF/cofilin. The data were normalized with respect to the form II large subunit of Rubisco (RbcL). (B) Quantitative analysis of the levels of phospho-ADF/cofilin in leaf cells received different treatments. The data represent the mean ±SE.

the phosphorylation level of ADF/cofilin in the *pp1* and *rcn1* mutants compared with the WT. After high-fluence BL irradiation, the phosphorylation level of ADF/cofilin was increased and was in good accordance with that in the dark group, suggesting that ADF/cofilin activity might be modulated by BL signaling, and PP2A-2 dephosphorylated and activated ADF/cofilin constitutively (**Fig. 6A, B**).

Effects of OA on high-fluence BL-induced actin structure reorganization

Western blot analysis demonstrated that OA blocked the dephosphorylation of ADF/cofilin. We thus investigated further the configuration of actin filaments in WT leaf mesophyll cell changes in response to high-fluence BL in the presence of OA. Numerous studies have demonstrated that the actin-based cytoskeleton plays a major role in the positioning and movement of chloroplasts and other organelles (Kandasamy and Meagher 1999). It was shown that chloroplasts were surrounded by basket-like structures of actin filaments that were linked directly or indirectly to larger actin cables (Kandasamy and Meagher 1999, Takagi 2003, Kumatani et al. 2006). As shown in **Fig. 7**, a typical set of F-actin structures formed a meshwork of actin cables around chloroplasts when WT Arabidopsis leaves were kept in the dark for a long time (**Fig. 7A–C**). The green fluorescent protein (GFP)-fABD2 fusion protein-labeled actin cytoskeleton in leaf cells revealed two types of actin filaments: one type of actin filament partially or completely encircled the chloroplasts, forming a basket-like structure, and the other formed a network of fine actin microfilaments, which derived from the bundles and extended randomly into the cytoplasm (**Fig. 7A–C**). The bar graphs show that approximately 85% of mesophyll cells have both basket-like structures and actin filament networks (**Fig. 7M**). After irradiation with high-intensity BL, the chloroplasts relocated their positions to the anticlinal sides of the cell. The configuration of actin filaments on the periclinal side changed, the network of fine actin microfilaments in 80% of mesophyll cells disappeared and the basket-like structure around the chloroplasts was revealed (**Fig. 7D–F, M**). In contrast, after treatment with OA, the

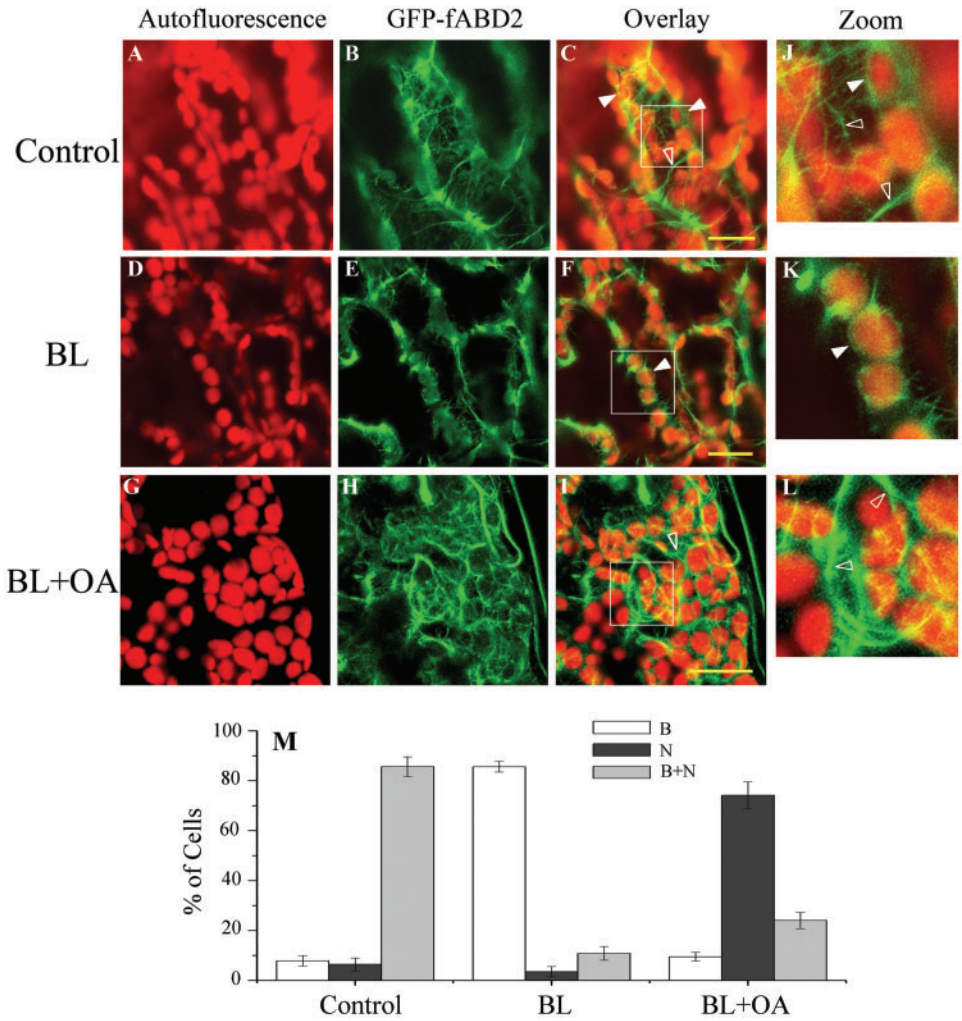

Fig. 7 Effects of OA on organization of the actin cytoskeleton in mesophyll cells of Arabidopsis. (A–L) Confocal images of mesophyll cells expressing GFP-fABD2 with or without 0.1 μM OA treatment after BL irradiation. GFP fluorescence is shown in green and chloroplast autofluorescence in red. The magnified images were partially zoomed out to reveal actin structure details (J–L). Solid arrowheads indicate the basket-like structure and open arrowheads indicate the actin microfilaments network. Scale bar = 25 μm. (M) Bar graphs depict the percentage of cells containing two types of actin structure following treatment of mesophyll cells with or without 0.1 μM OA treatment after BL irradiation. B, cells contained only basket-like structures; N, cells contained only actin filament networks; B + N, cells contained both basket-like structures and actin filament networks. Results represent averages from three independent experiments, each calculated for 50–90 cells.

network of fine actin microfilaments did not disappear and seemed to merge into a meshwork formed by a couple of thick actin cables in each cell in the presence of high-intensity BL irradiation. In addition, the basket-like structure encircling the chloroplasts disappeared (**Fig. 7G–I, M**). Therefore, it was suggested that OA might inhibit the actin cytoskeleton dynamics induced by high-intensity BL.

High-intensity BL-induced chloroplast avoidance movements are weakened in the absence of ADF

Having established that exogenous OA was involved in the phosphorylation level of ADF/cofilin and high-intensity BL-induced actin structure reorganization (**Figs. 6**, **7**), we then examined whether high-intensity BL-induced chloroplast avoidance movements are weakened in the absence of ADF. In this study, the time course of the change in RL transmittance was measured in *adf* mutant leaves in response to BL. The results demonstrated that the RL transmittance through WT, *adf2* and *adf4* mutant leaves increased by approximately 1.8%, but there was less of an increase in RL transmittance (approximately 1.2%) in *adf1* and *adf3* mutant leaves after treatment with high-fluence BL (**Fig. 8A**). When the *adf1* and *adf3* mutants (female parent) were cross-pollinated with the parental WT Col-0 (pollen donor), all F1 progenies showed similar chloroplast avoidance movements as the WT (**Supplementary Fig. 6**). The change in the percentage RL transmittance in response to 0.3 $\mu mol\, m^{-2}\, s^{-1}$ low BL was similar in the WT

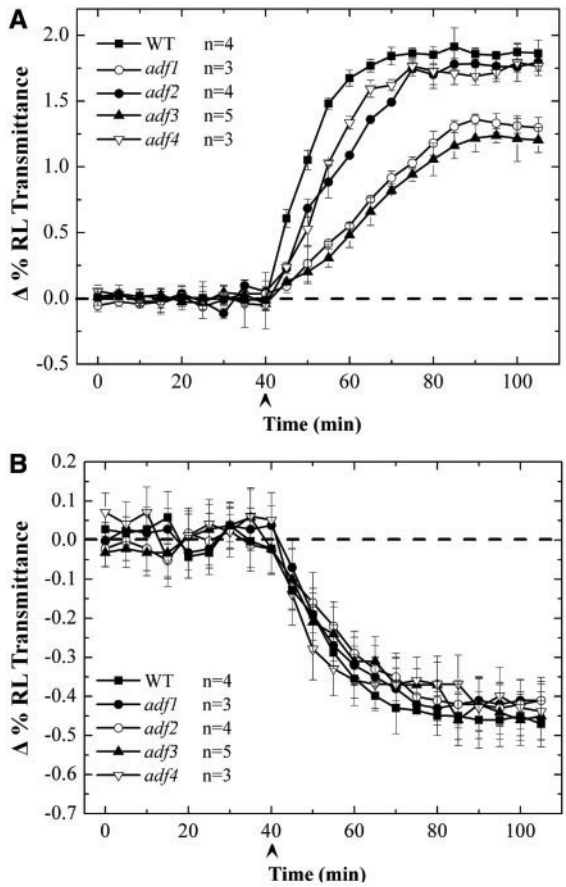

Fig. 8 Chloroplast movement response in *ADF* mutant leaf cells. (A) RL transmittances were measured in dark-acclimated *ADF* mutant leaves for 40 min before exposure to 100 μmol m^{-2} s^{-1} BL. The start of BL irradiation is indicated by the arrowheads. (B) RL transmittances were measured in dark-acclimated *ADF* mutant leaves for 40 min before exposure to 0.3 μmol m^{-2} s^{-1} BL. The start of BL irradiation is indicated by the arrowheads. Results are presented as the average change (±SE) in the percentage RL transmittance of leaves relative to the average value measured before turning on the BL. The number of leaves (*n*) is shown for each treatment.

and *adf* mutants (**Fig. 8B**). Further study showed that the RL transmittances changes in OA-treated *adf1* or *adf3* mutant leaves were similar to OA-untreated group, suggesting that PP2A is involved in the regulation of ADF1 or ADF3 (**Supplementary Fig. 5**). These data indicated that ADF1 and ADF3 may be necessary for high-fluence BL-induced chloroplast avoidance movements. Since the phosphorylation level of ADF/cofilin was significantly higher in the *pp2a-2* mutant than in the WT, it is suggested that PP2A-2 mediated chloroplast avoidance movements through regulating actin cytoskeleton remodeling by dephosphorylation/activation of ADF1 or ADF3.

Effects of PP2A-2 or OA on cellular localization changes in ADF1 in response to high-fluence BL

In order to verify that PP2A-2 could regulate ADF1 cellular localization in response to high-fluence BL, the ADF1 coding sequence was fused to the GFP reporter gene and transiently expressed in Arabidopsis cells. The ADF1-GFP fusion protein predominantly localized to a network of thick and long cytoplasmic bundles in WT and *pp2a-2* mutant mesophyll protoplasts (**Fig. 9A**). Interestingly, after high-fluence BL irradiation, the ADF1-GFP fusion protein was randomly distributed in the cytoplasm of *pp2a-2* mutant mesophyll protoplasts instead of a network, which was inconsistent with the ADF1 cellular localization in WT cell in response to high-fluence BL (**Fig. 9A**). Further, following transient transfection of GFP-fABD2 Arabidopsis mesophyll protoplasts with the cyan fluorescent protein (CFP)-ADF1 construct, we found that the distribution of the CFP-ADF1 fusion protein was consistent with that of the GFP-fABD2 fusion protein-labeled actin cytoskeleton with or without high-fluence BL exposure. However, after treatment with OA, the CFP-ADF1 fusion protein was approximately localized randomly in the cytoplasm of mesophyll protoplasts, which was practically distributed inconsistently with actin cytoskeleton in the presence of high-intensity BL irradiation (**Fig. 9B**). Therefore, these results suggested that OA or reduced PP2A-2 activity might inhibit ADF1 binding to the actin cytoskeleton after high-intensity BL.

Discussion

BL-induced chloroplast migration may be ubiquitous in higher plants (Zurzycki 1955, Haupt and Scheuerlein 1990, Suetsugu and Wada 2009). It has been proposed that the induction of chloroplast movements is dependent on phototropins, which have been shown to be autophosphorylated in response to BL (Kagawa et al. 2001, Sakai et al. 2001). Although correlations between the phosphorylation of phototropins and chloroplast movements are well established, unfortunately, the mechanisms of the signaling cascades of phosphorylation of downstream molecules are still not clear. Extensive progress has been made in our understanding of the role of phosphatases in protein dephosphorylation in animal cellular signaling, but functional analyses of plant phosphatases are limited (Smith and Walker 1996, Luan 2003). In this work, a loss-of-function mutant in PP2A-2 (an isoform of the catalytic subunit of PP2A) with approximately 30% reduced PP2A activity (**Supplementary Fig. 1C**) was used to investigate the role of PP2A in the regulation of chloroplast movements induced by high-fluence BL. By using confocal scanning microscopy and ultraviolet-visible spectrometry, we investigated the differences between *pp2a-2* mutant and WT mesophyll cells after exposure to high-intensity BL irradiation, and these results contributed to the general idea that PP2A-2 might be involved in chloroplast avoidance movements.

The first question we addressed is what is the role of PP2A-2 in chloroplast avoidance movements induced by high-intensity BL? In plants, the physiological roles of protein phosphatases in photoreceptor-mediated light signaling transduction were identified by using protein phosphatase inhibitors and

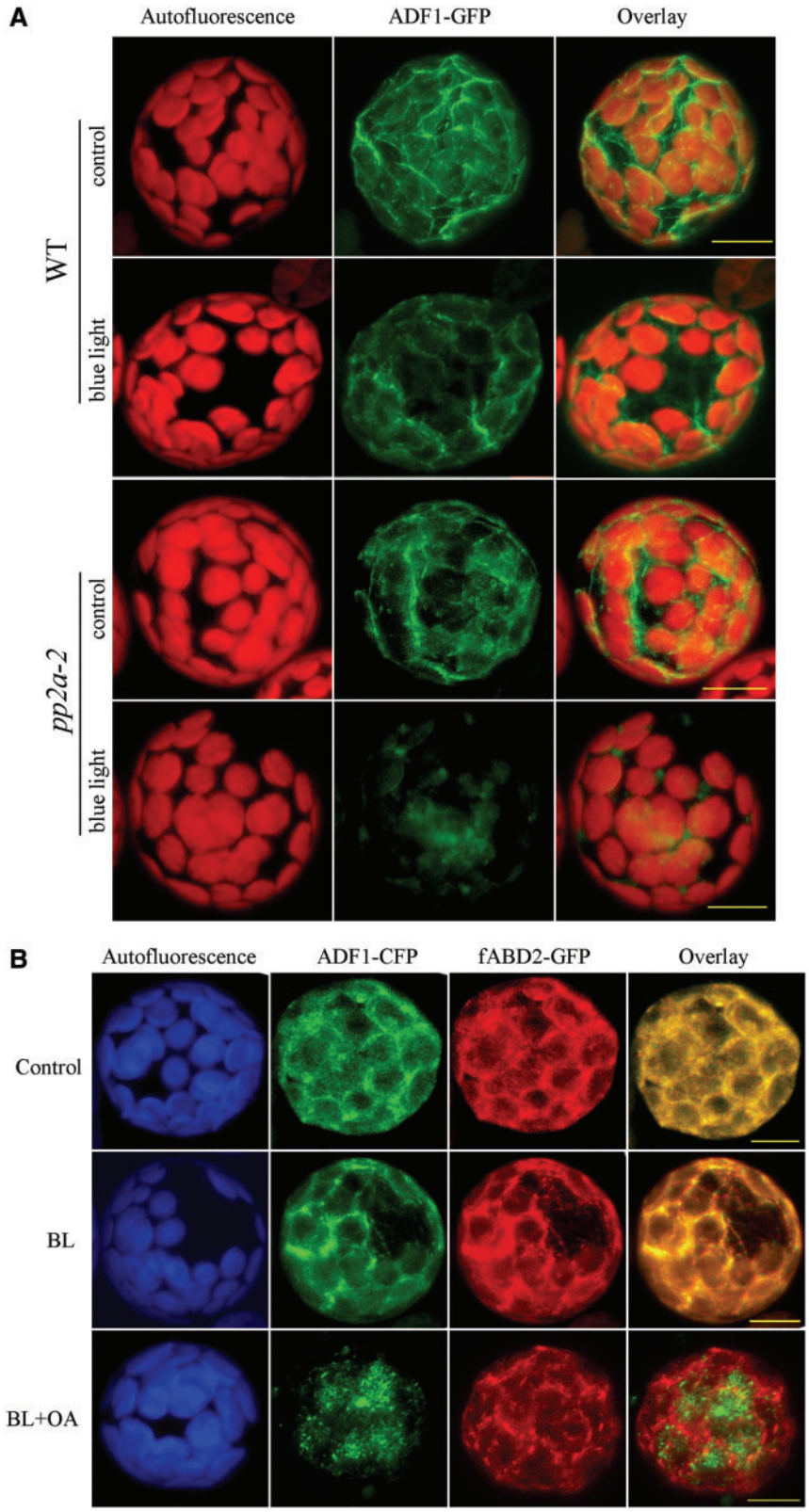

Fig. 9 Effects of PP2A-2 or OA on cellular localization changes in ADF1 in response to high-fluence BL. (A) Subcellular localization of ADF1-GFP in WT or *pp2a-2* mutant mesophyll cells after BL irradiation. GFP fluorescence is shown in green and chloroplast autofluorescence in red. Scale bar = 10 μm. (B) Co-localization of ADF1-CFP- and GFP-fABD2-labeled actin cytoskeleton in WT mesophyll cells with or without 0.1 μM OA treatment after BL irradiation. GFP fluorescence is shown in red, CFP fluorescence is shown in green and chloroplast autofluorescence in blue. The yellow color shows the co-localization of GFP and CFP. Scale bar = 10 μm.

Arabidopsis transgenic mutants. For example, a catalytic subunit of PP2A designated FyPP has been demonstrated to modulate phytochrome-mediated light signals in flowering time control in Arabidopsis; phytochrome-specific PP5 was found to control light signal flux by enhancing phytochrome stability and affinity for a signal transducer (Kim et al. 2002, Ryu et al. 2005). Here, we used the *pp2a-2* mutant and PP2A inhibitors to explore the function of PP2A-2 in high-intensity BL-induced chloroplast avoidance movements. In our study, the time courses of the change in RL transmittance and confocal microscopy revealed that the *pp2a-2* mutation impaired the phot2-mediated chloroplast avoidance movements in response to high-fluence BL, but it was not involved in phot1-mediated chloroplast accumulation movements (**Figs. 1**, **2**). In parallel, within a certain concentration range, exogenously applied OA or cantharidin inhibited the high-fluence BL-induced chloroplast movements in a concentration-dependent manner (**Figs. 3**, **4**). Furthermore, exogenously applied tautomycin or *pp1* mutants displayed a consistent change in RL transmittance in comparison with the WT in response to high-fluence BL (**Supplementary Fig. 2**). This indicated that PP2A-2 might act as a positive regulator in phot2-mediated chloroplast avoidance movements and PP1 might have no relation with high-intensity BL-induced chloroplast avoidance movements (**Figs. 1–5**, **Supplementary Figs. 2**, **3**).

Another question we addressed is how PP2A-2 regulates phot2-mediated chloroplast avoidance movements. First, phototropins have been shown to be autophosphorylated in response to BL signaling, so we suspected that PP2A-2 might function as an intermediate to modulate phot2-mediated chloroplast avoidance movements, possibly by transferring the phosphorylated state of phototropins to downstream signal molecules. However, this hypothesis was not supported by the experimental data from autoradiographic analysis, which showed that the phosphorylation levels of phot2 were not significantly different between the WT and the *pp2a-2* mutant after 5 min of high-intensity BL treatment (**Supplementary Fig. 4**). Next, we found that the phosphorylation level of ADF/cofilin in the *pp2a-2* mutant was significantly higher than in the WT and the *pp1* mutant. In addition, OA prevented the dephosphorylation of ADF/cofilin in Arabidopsis leaf cells (**Fig. 6**), which was consistent with the results obtained by Ambach *et al*., proving that PP2A not only associated with cofilin but also directly dephosphorylated this protein and thereby activated cofilin (Ambach et al. 2000). It seemed that PP2A-2 might act as a positive regulator of ADF/cofilin, since it could constitutively dephosphorylate and activate ADF/cofilin. In plants, phosphatases are traditionally classified into four subgroups (PP1, PP2A, PP2B and PP2C) based on biochemical and pharmacological properties. Each subgroup of protein phosphatases has different members, which execute many aspects of biological function. Here, we found a member of the PP2A subgroup, PP2A-2, that might not affect the phot2 phosphorylation status in the same way as RCN1, which has been confirmed to play an important role in down-regulating photoactivated phot2 (Tseng and Briggs 2010). The results showed that phosphorylation levels of ADF/cofilin increased after high-intensity BL irradiation, while there was no significant difference in the phosphorylation level of ADF/cofilin in the *rcn1* mutant (which had a high phot2 phosphorylation status after high-fluence BL irradiation) compared with the WT (**Fig. 6**). These data implied that ADF might be modulated by BL signaling but not through the phot2-mediated pathway (**Fig. 6**). Also, our experiments show that phot2 phosphorylation status in the *rcn1* mutant was consistent with that in earlier studies and the phosphorylation level of phot2 also increased after treatment with OA (**Supplementary Fig. 4**). This suggests that RCN1 was involved in chloroplast avoidance movements through affecting the phot2 phosphorylation status, while PP2A-2 might be involved in chloroplast avoidance movements by dephosphorylating/activating ADF/cofilin. Taking into account the fact that the regulation of the actin cytoskeleton was downstream of phot2 in the high-intensity BL-induced chloroplast avoidance movement process, OA might inhibit the chloroplast avoidance movement by inhibiting PP2A-2 activation and affecting the ADF/cofilin phosphorylation status, although it could also inhibit RCN1-mediated phot2 dephosphorylation.

Numerous studies have demonstrated that chloroplast photorelocation movement depends on the actin cytoskeleton (Kandasamy and Meagher 1999, Takagi 2003, Kumatani et al. 2006). It is well known that dynamic actin cytoskeleton rearrangements are modulated by numerous actin-binding proteins, which sense multiple environmental stimuli and regulate the F-actin network through a series of biochemical mechanisms (Simon and Pon 1996, Maciver 1998, Staiger et al. 2010). Here, our results showed that an actin-binding protein, ADF/cofilin, might be involved in chloroplast avoidance movements induced by high-intensity BL (**Fig. 8**). In plant and animal cells, ADF/cofilin is an abundantly expressed, evolutionarily conserved actin-binding protein that participates in regulating the dynamics of the actin cytoskeleton (Staiger et al. 2010, Tholl et al. 2011). Both its interaction with actin and its subcellular localization are determined by the reversible phosphorylation of the serine residue. The dephosphorylated forms of ADF/cofilin are active in binding actin and can promote actin filament dynamics. Plant ADF/cofilins play important roles in pollen tube growth (Chen et al. 2003), root formation (Thomas and Schiefelbein 2002) and cold acclimation (Ouellet et al. 2001). The Arabidopsis ADF/cofilin gene family consists of 11 expressed members that group phylogenetically into four ancient subclasses. Recently, Ruzicka et al. (2007) reported that subclass I ADFs (*ADF1*, *ADF2*, *ADF3* and *ADF4*) were expressed much more in leaves than the other three subclasses (and even recorded no expression). In our study, since we were investigating chloroplast movements in leaves, we examined the high-intensity BL-induced chloroplast avoidance movement response in subclass I ADF mutants. The results showed that high-intensity BL-induced chloroplast avoidance movements are weakened in the absence of ADF1 and ADF3, indicating that ADF1 and ADF3

might be necessary for high-fluence BL-induced chloroplast avoidance movements (Fig. 8). Also, the RT–PCR results showed that the expression level of ADF2 was significantly lower in leaves, whereas ADF4 showed a similarly high expression level as ADF1 and ADF3 in leaves (Supplementary Fig. 1E). Previous studies reported that ADF4 showed a marked preference for the ADP-G-actin compared with ADF1 because of different amino acid sequences, although the expression patterns of ADF4 were found to be similar to those observed for ADF1 and ADF3 (Tian et al. 2009). By analyzing the sequence alignment of ADFs, the C-terminal-specific F-actin binding sequences showed a difference between ADF4 and ADF1 (or ADF3) (Supplementary Fig. 1F). Thus, we presumed that the difference in the expression level in leaves and in the C-terminal-specific F-actin binding sequences perhaps led to the results showing that ADF1 and ADF3 were involved in chloroplast avoidance movements, but not ADF2 and ADF4 (Supplementary Fig. 1). The consistency of the increased RL transmittance between OA-treated and -untreated *adf1* or *adf3* mutant leaves suggested that PP2A is involved in the regulation of ADF1 or ADF3 (Supplementary Fig. 5). Moreover, it was obvious that the degree of increase in the RL transmittance in *adf1* or *adf3* mutant leaves was consistent with that in the *pp2a-2* mutant under the same high-intensity BL condition. Collectively, these findings demonstrate that PP2A-2 could activate/dephosphorylate ADF/cofilin and then regulate high-intensity BL-induced chloroplast avoidance movements. Further, we wanted to determine whether PP2A-2 was involved in high-fluence BL-induced changes of the actin cytoskeleton structure. Recent detailed microscopic analyses revealed that short actin filaments around the periphery of chloroplasts (cp-actin filaments) were involved in chloroplast movement (Kadota et al. 2009, Tsuboi and Wada 2011, Yamashita et al. 2011). These actin filaments showed rapid BL-induced dynamic changes during chloroplast photorelocation. In response to high-fluence BL irradiation, the cp-actin filaments rapidly disappeared within 1–2 min on the irradiated side of chloroplasts, the newly formed cp-actin filaments began to appear at the other side of moving chloroplasts and then induced a biased localization (Kadota et al. 2009, Kong and Wada 2011). Using GFP-fABD2 fusion protein to label the actin cytoskeleton (Sheahan et al. 2004), we found that OA could suppress the disintegration of randomly distributed actin filaments and the formation of cp-actin filaments around the chloroplasts periphery, and then caused the formation of long thick bundles of actin filaments after high-fluence BL irradiation (Fig. 7). Observation of the cellular localization of ADF1-GFP or ADF1-CFP fusion proteins revealed that in the cytoplasm of WT mesophyll protoplasts, ADF1 was binding with actin with or without high-fluence BL exposure; however, after OA treatment or with reduced PP2A-2 activity, ADF1 showed an inconsistent localization comparing with actin cytoskeleton in the presence of high-fluence BL irradiation (Fig. 9). Since the phosphorylation of ADF/cofilin was kept a high level in *pp2a-2* mutant or OA-treated WT cells (Fig. 6), it was suggested that OA or reduced PP2A-2 activity might keep ADF/cofilin at a high phosphorylation level and then inhibit ADF/cofilin binding to the actin cytoskeleton after high-intensity BL treatment. Thus, we concluded that PP2A-2 regulated the actin cytoskeleton reorganization through activating/dephosphorylating ADF/cofilin.

For the first time, we demonstrate that PP2A-2 functions as an intermediate to modulate phot2-mediated high-intensity BL signal transduction in chloroplast avoidance movements. Also, we found that PP2A-2 modulates high-fluence BL-induced chloroplast avoidance movements through regulating actin cytoskeleton remodeling by activation of ADF/cofilin, providing the first clues that the interaction between protein phosphatase and the actin cytoskeleton is implicated in BL-induced chloroplast avoidance movements. Future studies will be required to elucidate the mechanism of ADF/cofilin involvement in PP2A-2-modulated chloroplast avoidance movements.

Materials and Methods

Chemicals

OA (BioVision, Mountain View, CA, USA) was dissolved in DMSO to produce a 0.6 mM stock solution, which was aliquoted. [^{32}P]orthophosphate was purchased from Beijing Fu-Rui Bio-Engineering Company (Beijing, China). Antibodies against phosphor-cofilin and cantharidin were purchased from Sigma–Aldrich, China (Shanghai, China). Unless stated otherwise, the remaining chemicals were of analytical grade from Chinese companies.

Plant material

Seeds of the Arabidopsis GFP-fABD2 line were kindly provided by Prof. M. Sheahan (University of Newcastle, Australia). The Arabidopsis mutants *pp1* (At2g29400; SALK_057537), *pp2a-2* (At1g10430; SALK_150673), *adf1* (AT3G46010; GK-312C03.01), *adf2* (AT3G46000; SALK_022613), *adf3* (AT5G59880; SAIL_501_F01) and *adf4* (AT5G59890; SALK_063457) were purchased from NASC (http://arabidopsis.info). The mutants are all in the Col-0 background. Seeds were sown in water-soaked Scott's Plug or Metro Mix and incubated at 4°C in darkness for 3–4 d. Seedlings were grown in soil in a plant growth chamber (Conviron, model E7/2, Winnipeg, Canada) under a 12-h light/12-h dark cycle, a photon fluence rate of 100 μmol m^{-2} s^{-1} and a temperature of 22.5/21.5°C (light/dark).

Laser confocal scanning microscopy

Microscopic observations were performed using a Zeiss LSM 510 laser confocal scanning microscope (LSM510/ConfoCor2, Carl-Zeiss, Jena, Germany) implemented on an inverted microscope (Axiovert 100). For excitation, the 458/488-nm line of an Ar-ion laser was used. CFP signals were visualized with excitation at 458 nm and emission at 470–500 nm using a band-pass filter. GFP signals were visualized with excitation at 488 nm and emission at 500–550 nm using a band-pass filter. Chloroplast

autofluorescence (488-nm excitation) was visualized at 650 nm using a long-pass filter.

To analyze the chloroplast movements in living mesophyll cells, fresh leaves were gently cut into 1-mm sections and then mounted under a coverslip on a microscope slide with loading buffer according to a modified procedure, as described previously (Wen et al. 2008). Micrographs of cross sections from each treatment were captured using a × 40 oil-immersion objective lens on the Zeiss LSM510 and analyzed with Zeiss 'LSM Image Browser' Rel 3.2 image processing software (Zeiss, Germany).

For calculating the numbers of chloroplasts located along anticlinal and periclinal walls, a thin temporary section was prepared from the leaves after treatment with BL. Then, leaf sections were mounted under a coverslip on a microscope slide with loading buffer. Optical images of temporary sections with a 0.5 -μm pinhole were taken at × 20 magnification by using the same confocal system described above, and then the numbers of chloroplasts along the anticlinal and periclinal walls were calculated.

Transmittance measurements

In plants, the change in leaf RL transmittance has been proven to be a reliable indicator of chloroplast movements by our group and others (DeBlasio et al. 2005, Wen et al. 2008). RL transmittances were measured using the ultraviolet-visible spectrometer (Lambda 35, Perkin-Elmer, UK), according to the previously described protocol (Wen et al. 2008). Leaves from 3- to 4-week-old Arabidopsis plants were excised and dark acclimated for 9–15 h in 50 mM KCl/10 mM Tris-MES (pH 6.1, loading buffer) before being treated with BL and/or phosphatase inhibitors. Different photon flux densities of BL (0.3, 5, 50 or 100 μmol m^{-2} s^{-1}) were obtained by changing the height between the leaf and a set of light-emitting diodes (450 ± 25 nm), which were mounted perpendicular to the leaf surface. The photon flux density was measured using a quantum meter (LI-COR 250; LI-COR, Lincoln, NE, USA) equipped with a light sensor (LI-COR 190SA; LI-COR). After BL treatment and/or co-incubation with phosphatase inhibitors for the indicated time, the leaves gently sandwiched between two glass slides were immediately inserted into the solid sample holder of the ultraviolet-visible spectrometer for measuring RL transmittance. To characterize the recovery dynamics of chloroplast movements, the changes in the RL transmittance were determined at the indicated time after the BL source was turned off and OA was removed by washing five times with the loading buffer. For each leaf, the change in the percentage RL transmittance was calculated as: Δ% RL transmittance = $(T_t - T_0)/T_0$, where T_t and T_0 were the average percentage RL transmittance values after and before the BL and/or inhibitor treatments, respectively. The results were presented as the average change in the percentage RL transmittance for the indicated number of leaves.

Western blotting

Protoplasts were isolated from Arabidopsis leaves (14–21 d old) according to a procedure described previously with slight modification (Gao et al. 2008). For the OA treatment group, the protoplasts were firstly incubated with OA for 1 h, which was then removed by washing out five times with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glucose and 1.5 mM MES-KOH, pH 5.6). After BL treatment (100 μmol m^{-2} s^{-1}, 10 min) followed by dark recovery for 5 min, the protoplasts suspension were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 15 mM NaCl, 1% Triton X-100 and 100 mg ml^{-1} phenylmethylsulfonyl fluoride) and incubated on ice with gentle shaking on a level shaker for 30 min. The samples were centrifuged at 12,000 × *g* for 5 min at 4°C and the supernatants were transferred to new 1.5-ml tubes. A 100-times diluted protein sample supernatant was used to determine the protein concentrations by the method of Bradford (1976). The samples were separated by 12% SDS–PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and stained using Ponceau S. The resulting membrane was blocked with 5% skim milk and incubated with antibodies against phosphor-cofilin (Sigma) and polyclonal antibodies against the form II large subunit of Rubisco (RbcL) followed by IRDye 800 secondary antibody (Rockland Immunochemicals). Detection and quantitative analysis were performed using the LI-COR Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, Inc., Lincoln, NE, USA), according to a previously described method (Huang et al. 2011).

Plasmid constructs and transient transfection

The pNTL-AtADF1-GFP (a derivative of pBluescript II KS, Stratagene) was kindly provided by Clement Thomas (Plant Molecular Biology, CRP-Sante, Luxembourg). Enhanced cyan fluorescent protein (ECFP) was amplified by PCR under the templates of pECFP (Clontech Laboratories), using the following primers: forward, 5′-CGGGATCCATGGTGAGCAAGGGCGAGG AGCT-3′; reverse, 5′- GGACTAGTTTACTTGTACAGCTCGTCC ATGCCG-3′. The PCR products amplified from ECFP were digested with *Bam*HI and *Spe*I, and then gel purified and ligated into the sites of the cloning vector pNTL-AtADF1-GFP to yield the pNTL-AtADF1-ECFP plasmid. The complete sequence integrity of all constructs was confirmed by DNA sequencing.

Protoplasts were isolated from Arabidopsis leaves (14–21 d old) according to a previously described procedure with slight modification (Gao et al. 2008). Before transfection, the protoplasts in W5 solution were bathed on ice for 30 min and then as much supernatant as possible was removed without touching the protoplast pellet. The protoplasts were resuspended in mannitol-$MgCl_2$ solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES pH 5.7) at room temperature and the concentration was adjusted to about 2×10^5 protoplasts ml^{-1} with the mannitol-$MgCl_2$ solution. For transfection, 100 μL of protoplasts in mannitol-$MgCl_2$ solution was incubated with 10–20 μg of plasmid DNA and 120 μL of polyethylene glycol (PEG) solution containing 40% (w/v) PEG-4000, 0.2 M mannitol and 0.1 M $CaCl_2$. After incubation for 5–30 min at room temperature, 0.5 ml of W5 solution was added to stop the transfection. After

centrifugation to remove the PEG solution, the protoplasts were collected and resuspended in 250 μL of W5 solution (24-well plates). Transfected protoplasts were left in the dark at room temperature for 8–20 h to allow the expression of the AtADF1-GFP or AtADF1-ECFP fusion protein.

Supplementary data

Supplementary data are available at PCP online.

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