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RESEARCH PAPER

Hydrogen peroxide is involved in high blue light-induced chloroplast avoidance movements in *Arabidopsis*

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Abstract

One of the most important functions of blue light (BL) is to induce chloroplast movements in order to reduce the damage to the photosynthetic machinery under excess light. Hydrogen peroxide (H_2O_2), which is commonly generated under various environmental stimuli, can act as a signalling molecule that regulates a number of developmental processes and stress responses. To investigate whether H_2O_2 is involved in high-fluence BL-induced chloroplast avoidance movements, a laser scanning confocal microscope and a luminescence spectrometer were used to observe H_2O_2 generation *in situ* with the assistance of the fluorescence probe dichlorofluorescein diacetate (H_2DCF-DA). After treatment with high-fluence BL, an enhanced accumulation of H_2O_2, indicated by the fluorescence intensity of DCF, can be observed in leaf cells of *Arabidopsis thaliana*. Exogenously applied H_2O_2 promotes the high-fluence BL-induced chloroplast movements in a concentration-dependent manner within the range of 0–10^{-4} M, not only increasing the degree of movements but also accelerating the start of migrations. Moreover, the high-fluence BL-induced H_2O_2 generation and the subsequent chloroplast movements can be largely abolished by the administration of the H_2O_2-specific scavenger catalase and other antioxidants. In addition, in-depth subcellular experiments indicated that high-fluence BL-induced H_2O_2 generation can be partly abolished by the addition of diphenyleneiodonium (DPI), which is an NADPH oxidase inhibitor, and the blocker of electron transport chain dichlorophenyl dimethylurea (DCMU), respectively. The results presented here suggest that high-fluence BL can induce H_2O_2 generation at both the plasma membrane and the chloroplast, and that the production of H_2O_2 is involved in high-fluence BL-induced chloroplast avoidance movements.

Key words: Blue light, chloroplast avoidance movements, hydrogen peroxide, RL transmittance.

Introduction

Plants have developed a series of highly sophisticated short- and long-term mechanisms that help them to adapt to changing environmental conditions. For example, when exposed to low-fluence blue light (BL), the chloroplasts accumulate perpendicular to the incident light, along the periclinal cell walls. In response to high-fluence BL, the chloroplasts move parallel to the incident light, along the anticlinal cell walls (Zurzycki, 1955). This BL-induced relocation of the chloroplasts is regarded as an adaptive mechanism to improve the efficiency of photosynthesis and to avoid any damage by strong light (Seitz, 1972).

It is known that phototropin1 (phot1) and phot2, as plant-specific BL receptors, can mediate BL-induced chloroplast movements (Briggs *et al*., 2001). Phot1 was first identified as a BL receptor responsible for phototropism by using an *Arabidopsis* mutant that showed impaired phototropic bending in response to BL (Christie *et al*., 1998). Later, a homologue of phot1 was also isolated from an *Arabidopsis* mutant defective in the avoidance response and was renamed phot2 (Jarillo *et al*., 1998; Briggs and Christie, 2002). In general, phot2 functions under a relatively high intensity of BL. For example, it can, as a photoreceptor mediating the

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Abbreviations: aa, amino acid; AsA, ascorbic acid; BL, blue light; CAT, catalase; DCMU, dichlorophenyl dimethylurea; DPI, diphenyleneiodonium; GL, green light; H_2DCF-DA, dichlorofluorescein diacetate; H_2O_2, hydrogen peroxide; LOV domains, light, oxygen, and voltage domains; phot, phototropin; RL, red light; ROS, reactive oxygen species.

photoavoidance response of chloroplasts, prevent strong light from damaging the photosynthetic apparatus (Jarillo *et al*., 2001; Kagawa *et al*., 2001; Sakai *et al*., 2001). Recent molecular characterization has shown that both phototropins contain a Ser/Thr protein kinase domain located within their C terminus. Furthermore, the N-terminal region of phot1 and phot2 both contain a repeated motif of 110 amino acids (aa), designated LOV1 and LOV2 (Christie *et al*., 1999; Christie and Briggs, 2001). LOV1 and LOV2 belong to the Per–Arnt–Sim (PAS) domain superfamily, which are found in a variety of proteins. They have been reported to mediate protein–protein interactions and to function as internal sensors of oxygen, light, and redox changes in the electron transport system or overall cellular redox status (Taylor and Zhulin, 1999; Briggs and Christie, 2002).

An increasing body of evidence has shown that redox seems to intervene in the regulation of multiple processes functional at virtually every stage of plant development (Buchanan and Balmer, 2005). Therefore, tight control of redox homeostasis is fundamental in cell metabolism. Although plant cells possess an array of antioxidant mechanisms to cope with high rates of reactive oxygen species (ROS) production, most forms of biotic or abiotic stimulus could result in the enhanced production of ROS, which inevitably disrupts the balance of the cellular redox status (Mou *et al*., 2003). It has been widely reported that hydrogen peroxide (H_2O_2), as a major member of ROS, acts at subtle levels as a second messenger molecule in biological processes such as development and stress perception (Levine *et al*., 1994; Orozco-Cárdenas *et al*., 2001). In higher plants, H_2O_2 can be generated by several different pathways, including a plasma membrane localized NADPH oxidase, glycollate oxidase, and the Mehler reaction in the chloroplasts. The H_2O_2 signal can be transmitted through alterations in Ca^{2+} fluxes and the cellular redox state, both of which are considered to be very early events that follow the rise in H_2O_2 levels (Rentel and Knight, 2004).

Although it has been reported that strong light could change the cellular redox state and trigger the xanthophyll cycle, which in turn plays a role in modulating the BL-dependent chloroplast movements (Tlalka *et al*., 1999), it is still not clear whether the H_2O_2 signal is directly involved in BL-induced chloroplast movements, and what is the source of the H_2O_2 generation. To address these questions, the changes in chloroplast movement in response to H_2O_2 were therefore examined using *Arabidopsis*. In this work, evidence is provided that H_2O_2, which was generated at both the cell membrane and the chloroplast by prolonged exposure to high-fluence BL, may be involved in the high-fluence BL-induced chloroplast avoidance movements. The mechanism whereby H_2O_2 was perceived to be underlying the BL-induced chloroplast movements is also discussed.

Materials and methods

Chemicals

Dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR) was dissolved in DMSO to produce a 100 mM stock solution, which was aliquoted. Catalase (CAT, bovine liver), diphenyleneiodonium (DPI), ascorbic acid (AsA), and dichlorophenyl dimethylurea (DCMU) were from Sigma (St Louis). Unless stated otherwise, the remaining chemicals were of analytical grade from Chinese companies.

Plant material

Seeds of *Arabidopsis* 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 12/12 h light/dark cycle, a photon fluence rate of 100 μmol m^{-2} s^{-1}, and a day/night temperature cycle of 22±0.5 °C. All the experiments were performed on the leaves from 4–5-week-old seedlings and repeated at least three times.

Transmittance measurements

In plants, chlorophyll is the main absorber of red light. Due to the migration of the chloroplasts to the anticlinal walls the chlorophyll distributed at the cell surface will be reduced, leading to an increase in red light (RL) transmittance. Upon migration of the chloroplasts to the periclinal cell walls, the majority of chlorophyll will be distributed at the cell surface, leading to a decrease in RL transmittance (DeBlasio *et al*., 2003, 2005). Therefore the increase in RL transmittance is used to indicate the avoidance movement and the decrease in RL transmittance to indicate the accumulation movement. In our experiments, RL transmittance was measured using the ultraviolet-visible spectrometer (Lambda 35, Perkin-Elmer, UK) according to the protocol described by DeBlasio *et al*. with some modifications (DeBlasio *et al*., 2003). Leaves from 4–5-week-old *Arabidopsis* plants were excised and dark-acclimated for 9–15 h in the 50 mM KCl/10 mM TRIS-MES (pH 6.1, loading buffer). The dark-adapted leaves were incubated with different concentrations of H_2O_2 (0–10^{-1} M) for 1 h in the dark, and were then directly exposed to different photon flux density of BL for different times. The different photon flux density of BL (0.3, 5, 15, 30, or 60 μmol m^{-2} s^{-1}) was obtained by changing the height between the leaf and a set of light-emitting diodes (LED) (450±25 nm), which were mounted perpendicular to the leaf surface. 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 or/and H_2O_2 treatments for the indicated times, the leaves that had been 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 RL transmittance was determined at the indicated time after the BL source was turned off and H_2O_2 was removed by washing five times with the loading buffer. For each leaf, change in percentage RL transmittance was calculated as:

$$\triangle\%\,\mathrm{RL\,transmittance} = (T_t - T_0)/T_0$$

where T_t and T_0 are the average percentage RL transmittance value after and before the BL or/and H_2O_2 treatments, respectively. The results are presented as the average change in percentage RL transmittance for the indicated number of leaves.

Dye loading

Freshly prepared abaxial leaf strips were first incubated for 30 min in 10 ml loading buffer to eliminate any background ROS that may

be produced during the preparation of the leaf strips. The leaf strips were then placed in a small Petri dish containing 2.97 ml loading buffer and incubated with 30 μl of H_2DCF-DA from a 100 mM stock solution in DMSO for 10–15 min in the dark before the different treatments.

Luminescence spectrometer

The dye-loaded leaf strips were transferred to a dish containing fresh buffer to wash off excess dye and subsequently irradiated with BL, RL, or green light (GL) of 30 μmol m^{-2} s^{-1} for different times.

At the indicated times, a leaf strip was placed flat onto a plastic holder and fixed at both ends with silicon grease and the DCF fluorescence emitted from the leaf strip was recorded with a luminescence spectrometer (LS55, Perkin-Elmer, UK). The luminescence spectrometer was set to an excitation of 488 nm and an emission of 525 nm, with a slit width of 2.5 nm. To determine the effects of antioxidants on ROS production, the final concentration of 100 units ml^{-1} catalase, 10 μM DCMU, 200 μM AsA, 50 μM DPI or 50 mM mannitol was added into the dish just before light irradiation.

Laser scanning confocal microscope

In situ detection of ROS production was performed using a commercial laser scanning microscope (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, a blue argon-ion laser (488 nm) set on 3% power was used; the emission fluorescence was collected by the 500–550 nm bandpass for DCF fluorescence. The viability of the cells within the mesophyll tissue under these media conditions was >80%, as tested by fluorescein diacetate staining (data not shown).

To analyse the effects of exogenous H_2O_2 on 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. Micrographs of cross-sections from each treatment were captured at ×20 magnification using the commercial laser scanning confocal microscope.

For calculating the numbers of chloroplasts located along the anticlinal and periclinal walls, a thin temporary section was prepared from the leaves after treatment with BL or H_2O_2. Then, leaf sections were mounted under a coverslip on a microscope slide with loading buffer. The 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 the numbers of chloroplasts in the anticlinal and periclinal walls were calculated.

Results

The effects of exogenous H_2O_2 on BL-induced chloroplast movements

The effects of the exogenously applied H_2O_2 on high-fluence BL-induced chloroplast avoidance movements were investigated first. It is clear that the chloroplasts of mesophyll cells of dark-acclimated *Arabidopsis* leaves after a 60 min treatment with BL at 30 μmol m^{-2} s^{-1} move to the anticlinal walls, parallel to the incident light (Fig. 1A, B). Moreover, the chloroplast movements induced by high-fluence BL are more marked in the presence of 100 μM H_2O_2 than in the absence of H_2O_2 (Fig. 1C, D). Analysis of chloroplast position showed that, in leaves exposed to high-fluence BL for 30 min, approximately 60% of the chloroplasts were located along the anticlinal walls (Fig. 1I), whereas under the same light conditions, approximately 85% of the chloroplasts were along the anticlinal walls in the presence of exogenous H_2O_2 (Fig. 1J). With the extension of the exposure time to 60 min, only 8% of the chloroplasts were located at the periclinal walls in the presence of exogenous H_2O_2, although 20% of the chloroplasts could still be observed in the periclinal walls without H_2O_2 treatment.

Effects of different concentrations of H_2O_2 on BL-induced changes in RL transmittance

It has been proved that the changes in light transmittance and absorption through intact leaves could be used as a reliable indicator of chloroplast movements (DeBlasio *et al*., 2005; Luesse *et al*., 2006). Chloroplast migration to the periclinal cell walls in response to low-fluence rates of light results in a decrease in RL transmittance through leaves due to greater surface area coverage by chloroplasts, whereas under high-light conditions, chloroplasts migrate to the anticlinal walls, and RL transmittance increases as the amount of surface area covered by chloroplasts decreases. To confirm the enhanced effects of H_2O_2 on BL-induced chloroplast movements further, the change in percentage RL transmittance of leaves was examined in the presence of H_2O_2. Exogenous application of H_2O_2 increased the RL transmittance of leaves in a dose-dependent manner at a concentration of H_2O_2 $\leqslant 10^{-4}$ M in the presence of 30 μmol m^{-2} s^{-1} BL, although it has no effects on the RL transmittance of leaves in the darkness (Fig. 2). Moreover, when BL was removed, the RL transmittance of leaves could recover to the original level. Within the concentration range of $0–10^{-4}$ M, the maximum promotion of the RL transmittance was observed after BL for 30 min. For example, the RL transmittance of leaves was increased to 196% of the original level by H_2O_2 of 10^{-4} M. However, high concentrations of H_2O_2 (10^{-1}–10^{-3} M) appeared to have a harmful effect on the normal function of the cell because neither changes nor full recovery in the RL transmittance could be found (see insert of Fig. 2).

Different intensity of BL-induced changes in RL transmittance in the presence of H_2O_2

Figures 3 and 4 show a time-course of the change in RL transmittance in *Arabidopsis* leaves in response to sequential treatments of low-, intermediate-, and high-intensity BL (0.3, 5, 15, 30, and 60 μmol m^{-2} s^{-1}, respectively). Upon exposure to 15, 30, and 60 μmol m^{-2} s^{-1} BL, RL transmittance through *Arabidopsis* leaves increased by approximately 0.6%, 0.77%, and 1.0%, respectively. However, a more prominent increase in the RL transmittance (0.8%, 1.5%, and 1.8%, respectively) could be observed in leaves after being treated

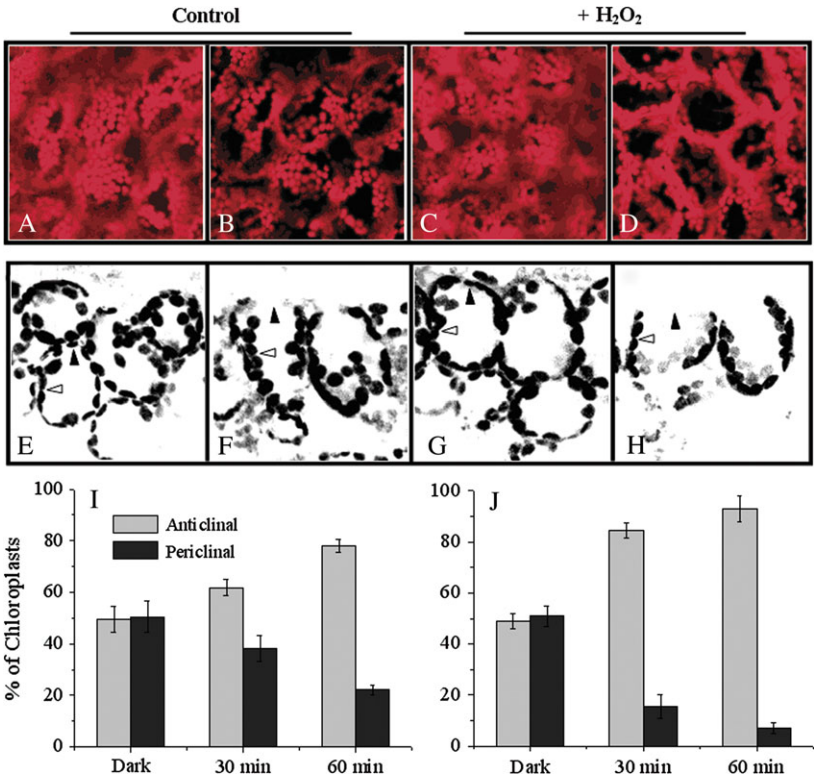

Fig. 1. The effects of exogenous H_2O_2 on BL-induced chloroplast movements. Dark-acclimated *Arabidopsis* leaf strips were exposed to sequential 1 h treatments of high-fluence BL (30 μmol m^{-2} s^{-1}) in the absence (A, B) or presence (C, D) of 100 μM H_2O_2. Micrographs from time points 0 min (A, C), and 60 min (B, D) are shown. Cross-sections of *Arabidopsis* leaves exposed to sequential 1 h treatments of high-fluence BL (30 μmol m^{-2} s^{-1}) in the absence (E, F) or presence (G, H) of 100 μM H_2O_2 show chloroplast positioning in the mesophyll cell layer. Images from time points 0 min (E, G) and 60 min (F, H) are shown. Solid arrowheads show the periclinal cells walls. Open arrowheads show the anticlinal cells walls. Bar graphs depict the percentage (±SE) of chloroplasts located along the anticlinal and periclinal cells walls in the absence (I) or presence (J) of H_2O_2, and represent averages from 60–100 cells per high-fluence BL (30 μmol m^{-2} s^{-1}) treatment.

simultaneously with high-fluence BL and 10^{-4} M H_2O_2 (Fig. 3). In addition, regardless of the presence or absence of H_2O_2, irradiation with 0.3 or 5 μmol m^{-2} s^{-1} BL for 1 h decreased RL transmittance through *Arabidopsis* leaves by about 0.4% compared with the dark-acclimated phase (Fig. 4). Specifically, upon exposure to 5 μmol m^{-2} s^{-1} continuous BL, transmittance increased for about 15 min before decreasing to a value lower than that measured before the BL treatment. However, the time of increasing transmittance was extended from 15 min to 40 min in the presence of H_2O_2 (Fig. 4). These data indicated that exogenous H_2O_2 could not only increase the degree of high fluence BL-induced chloroplast movements but also accelerate the trigger of the chloroplast migration induced by both low and high BL.

High-fluence BL induces H_2O_2 production in mesophyll cells

Having established that exogenous H_2O_2 was involved in the promotion of chloroplast avoidance movements induced by high-fluence BL in the above leaf bioassay and RL transmittance analysis experiments (Figs 1–4), it was then examined whether high-fluence BL might increase the level of H_2O_2 in mesophyll cells. In this study, the change in intracellular H_2O_2 level was directly measured by a sensitive fluorophore dichlorofluorescein (H_2DCF). The non-polar diacetate ester (H_2DCF-DA) of H_2DCF enters the cell and is hydrolysed to the more polar, non-fluorescent compound H_2DCF, which is therefore trapped. Subsequent oxidation of H_2DCF by H_2O_2, catalysed by peroxidases, yields the highly fluorescent DCF (Allan and Fluhr, 1997; Cathcart *et al.*, 1983).

Figure 5 shows the dynamics of H_2O_2 generation indicated by the relative fluorescence intensity of DCF in the abaxial leaf strip during exposure to 0.3 μmol m^{-2} s^{-1} BL or 30 μmol m^{-2} s^{-1} light (BL, RL, and GL). It is clear that the intensity of DCF fluorescence in leaf strips after treatment with high-fluence BL increased more significantly than with RL. In addition, only very low fluorescence intensity could be detected after exposure to 0.3 μmol m^{-2} s^{-1} BL, whereas no fluorescence occurred after GL treatment compared with the control (Fig. 5A). The experiments performed on leaf strips using phototropin mutants show that the DCF fluorescence caused by intracellular H_2O_2 increased more significantly in wild type and the *phot1* single mutant than in the *phot2* single

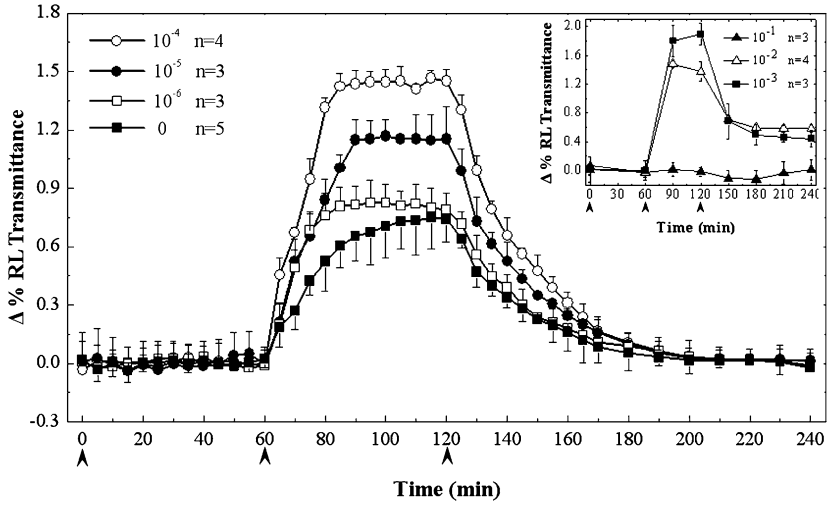

Fig. 2. Effects of different concentrations of H_2O_2 on BL-induced chloroplast movements. The plots show the average change (±SE) in the percentage of RL transmittance of leaves relative to the average transmittance measured before the BL treatment. RL transmittance was measured in leaves every 5 min, and the treatments were carried out at 0 min (adding H_2O_2), 60 min (turning on BL), and 120 min (turning off BL and washing out H_2O_2 by using loading buffer) indicated by the arrowheads. The individual leaves were incubated in loading buffer containing H_2O_2 (10^{-4} M, 10^{-5} M, 10^{-6} M, and 0 M). The RL transmittance in the insert was measured under high concentrations of H_2O_2 (10^{-3}–10^{-1} M). Number of leaves (*n*) is shown for each treatment.

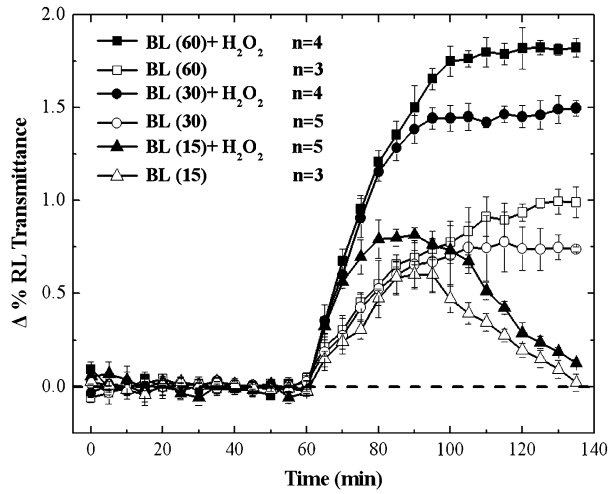

Fig. 3. Chloroplast movement response in *Arabidopsis* leaves induced by high-fluence BL. RL transmittances were measured in dark-acclimated leaves for 60 min before exposure to BL (15, 30, and 60 μmol m^{-2} s^{-1}) co-treatment with 10^{-4} M H_2O_2 application (solid symbols) and without H_2O_2 (open symbols). Results are presented as the average change ±SE in the percentage of RL transmittance of leaves relative to the average value measured before turning on the BL. Number of leaves (*n*) is shown for each treatment.

mutant and the *phot1phot2* double mutant after treatment with high-fluence BL (Fig. 5B). This suggested that the phot2 photoreceptor is related to the generation of H_2O_2 induced by high-fluence BL. A single cell assay performed using laser scanning confocal microscopy also illustrated that 30 μmol m^{-2} s^{-1} BL induced a significant increase in DCF fluorescence intensity in mesophyll cells, and the accumulated H_2O_2 was observable at both the chloroplast and the plasma membrane of mesophyll cells after treatment with high-fluence BL for 30 min (Fig. 6A–F). The result clearly showed that a large quantity of H_2O_2 had been generated in the presence of phot2 during the process of high-fluence BL-induced chloroplast avoidance movements.

The effects of scavengers on H_2O_2 generation and chloroplast avoidance movements

To investigate the role of intracellular H_2O_2 in high-fluence BL-induced chloroplast avoidance movements, scavengers of ROS were used to abolish the intracellular H_2O_2. As shown in Fig. 7, the increases in DCF fluorescence intensity in abaxial leaf strips induced by

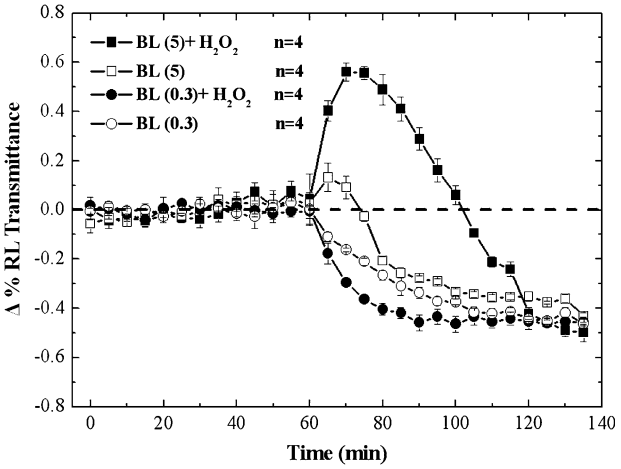

Fig. 4. Chloroplast movement response in *Arabidopsis* leaves induced by low- and intermediate-fluence BL. RL transmittances were measured in dark-acclimated leaves for 60 min before exposure to BL (0.3 and 5 μmol m^{-2} s^{-1}) co-treatment with 10^{-4} M H_2O_2 application (solid symbols) and without H_2O_2 (open symbols). Results are presented as the average change ±SE in the percentage of RL transmittance of leaves relative to the average value measured before turning on the BL. Number of leaves (*n*) is shown for each treatment.

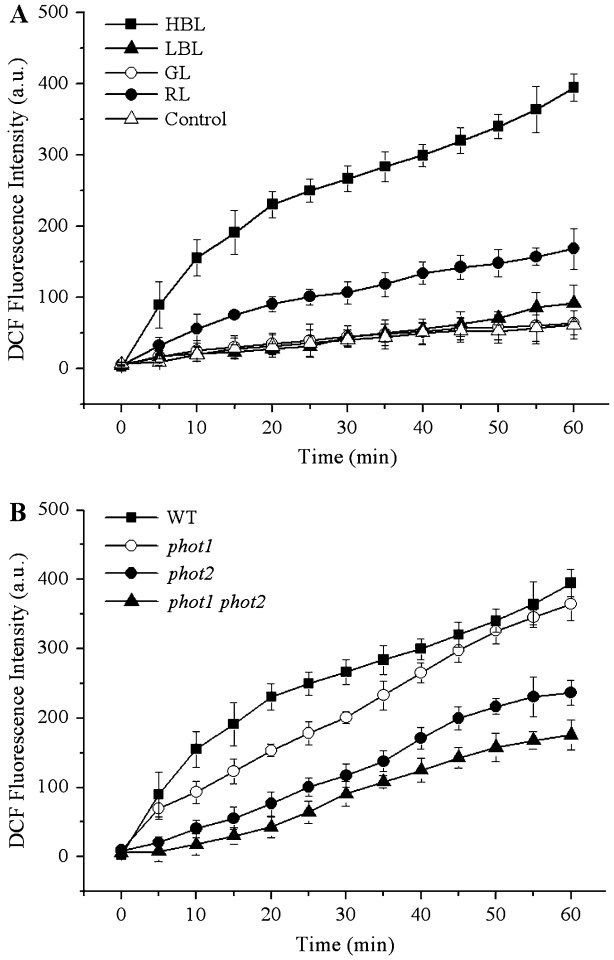

Fig. 5. Determination of H_2O_2 generation under different light conditions in *Arabidopsis* leaf strips by the DCF fluorescence assay. In (A), DCF fluorescence was immediately measured in leaf strips of wild-type (WT) *Arabidopsis* after treatment with 30 μmol m^{-2} s^{-1} BL (HBL), GL, RL, and 0.3 μmol m^{-2} s^{-1} BL (LBL) for the indicated times. In (B), DCF fluorescence was immediately measured in leaves strips of *phot1*, *phot2*, and *phot1phot2* mutants after treatment with 30 μmol m^{-2} s^{-1} BL for the indicated times. The values are the mean ±SE of three replicates.

both 30 min and 60 min exposure to high-fluence BL were abolished by 100 units ml^{-1} CAT, a specific scavenger of H_2O_2. A single cell assay also demonstrated that, after treatment with CAT, H_2O_2 generation was remarkably scavenged in high BL-treated mesophyll cells of *Arabidopsis* leaves (Fig. 8A–H). Other scavengers of ROS (such as AsA and mannitol) have a similar effect on H_2O_2 generation induced by high-fluence BL (data not shown).

The effects of scavengers of ROS on high-fluence BL-induced chloroplast avoidance movements, indicated by a change in the percentage RL transmittance of the leaves, are shown in Fig. 9. Apparently these scavengers could, to a different extent, inhibit the increase in RL transmittance of leaves induced by high-fluence BL. This indicated that intracellular H_2O_2 may be necessary for high-fluence BL-induced chloroplast avoidance movements.

The effects of DCMU and DPI on H_2O_2 generation

It was noted that H_2O_2 induced by high-fluence BL occurred at both the chloroplast and plasma membrane of mesophyll cells (Fig. 6A–F). In order to determine how H_2O_2 is generated at these two subcellular sources, the changes in DCF fluorescence intensity of leaves induced by high-fluence BL were further examined in the presence of DCMU, an inhibitor of photosynthetic electron transport or DPI, a specific inhibitor of NADPH oxidase (Gray and Cresswell, 1984; Schneider *et al*., 2003). As shown in Fig. 10, addition of 10 μM DCMU before high-fluence BL treatment partly abolished the increases in DCF fluorescence intensity in *Arabidopsis* leaves. This suggested that the photosynthetic process is responsible for the generation of H_2O_2 induced by high-fluence BL at the chloroplast. In a parallel experiment, 50 μM DPI could also partly abolish high-fluence BL-induced DCF fluorescence caused by intracellular H_2O_2. This implied that the reduction of fluorescence intensity is due to the blocking of H_2O_2 generation through NADPH oxidase by DPI.

Discussion

Under various environmental and developmental stimuli, ROS are commonly generated in many biological systems (Potikha *et al*., 1999). It has been widely confirmed that ROS appear to play a crucial role in the physiological and pathological processes of plants. ROS generated by light could serve as factors to control the redox state, functioning

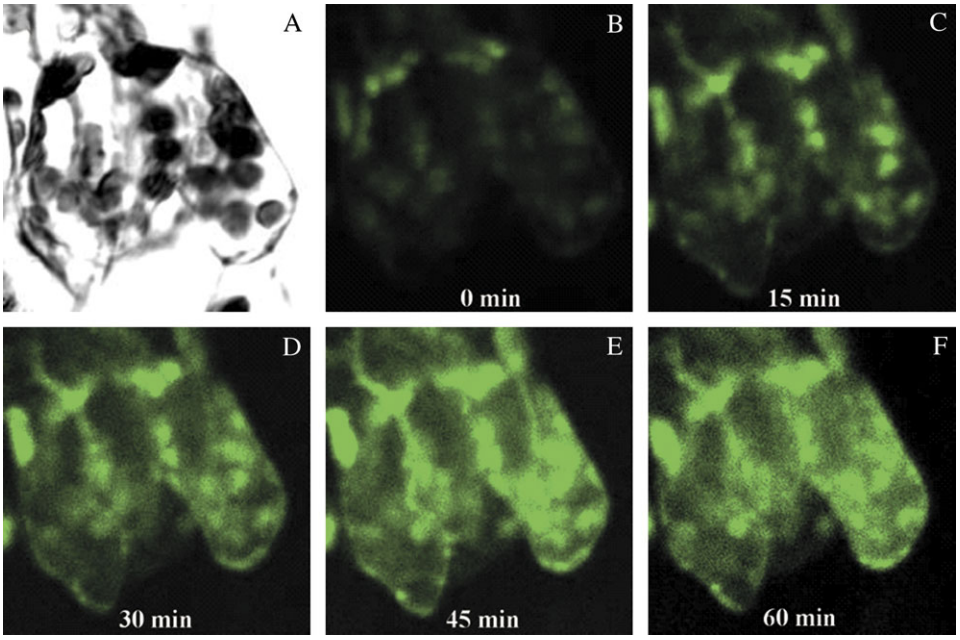

Fig. 6. *In situ* inspection of high-fluence BL-induced production of H_2O_2 in *Arabidopsis* mesophyll cells. The dye-loaded leaf strip was mounted under a cover slip on a microscope slide with loading buffer. During the 1 h treatment period with 30 μmol m^{-2} s^{-1} high-fluence BL, micrographs of DCF fluorescence in the mesophyll cells were taken at the indicated times by a laser scanning confocal microscope (B–F) as described in the Materials and methods. Bright field of mesophyll cells was also shown (A).

as controlling factors for redundant pathways of light signal transduction. For example, H_2O_2, a form of ROS, is implicated as an intermediary messenger in the BL-induced stomatal movement through mediating the activity of an H^+ pump in the plasma membrane (Zhang *et al*., 2004).

Here, new evidences is provided that H_2O_2 is involved in high fluence BL-induced chloroplast avoidance movements in *Arabidopsis* mesophyll cells. The following results support this conclusion: (i) exogenously applied H_2O_2 might accelerate the trigger for chloroplast migration and enhance high-fluence BL-induced chloroplast avoidance movements, (ii) high-fluence BL could induce H_2O_2 production at both the chloroplast and the cell membrane, (iii) scavenging of endogenous H_2O_2 blocked the high-fluence BL-induced chloroplast avoidance movements, (iv) the *phot2* mutant with impaired chloroplast avoidance movements showed much less H_2O_2 generation relative to the *phot1* mutant, (v) the changes in H_2O_2 level were consistent with chloroplast avoidance movements.

It has been established that low and intermediate light at fluence rates less than 16 μmol m^{-2} s^{-1} cause chloroplast accumulation, while high light at fluence rates above 16 μmol m^{-2} s^{-1} induces the chloroplast avoidance movements (Jarillo *et al*., 2001; Kagawa *et al*., 2001; Sakai *et al*., 2001). In an experiment using a variety of BL fluence rates, we also found two similar chloroplasts movements without H_2O_2 (Figs 3, 4). Moreover, microscope imaging and statistical analysis demonstrated that exogenous application of 100 μM H_2O_2 could promi-

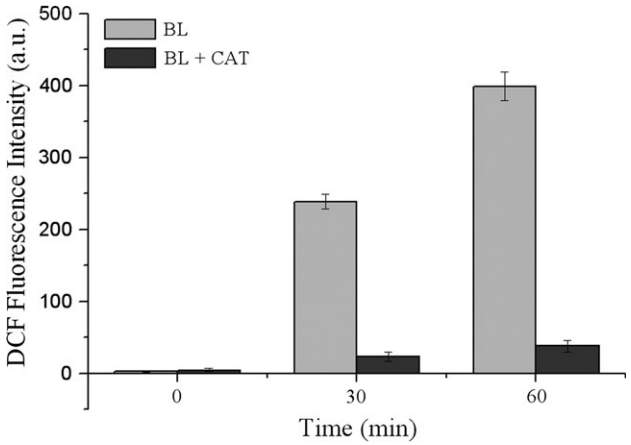

Fig. 7. The effects of CAT on high-fluence BL-induced production of H_2O_2 in *Arabidopsis* leaf strips. DCF fluorescence was immediately measured using a luminescence spectrometer in leaf strips after treatment with 30 μmol m^{-2} s^{-1} BL for the indicated times in the presence or absence of 100 units ml^{-1} CAT.

nently promote high-fluence BL-induced chloroplast avoidance movements (Fig. 1). Further RL transmittance assays confirmed that, within the range of 5–60 μmol m^{-2} s^{-1} BL, the extent of the chloroplast avoidance movements was greater in the presence of 100 μM H_2O_2 than in the absence of it (Fig. 3). This is compatible with the fact that H_2O_2 is a versatile signal molecule in a variety of biological responses (Zhang *et al*., 2001; Yoshida and Hasunuma, 2004). Although H_2O_2 could accelerate the

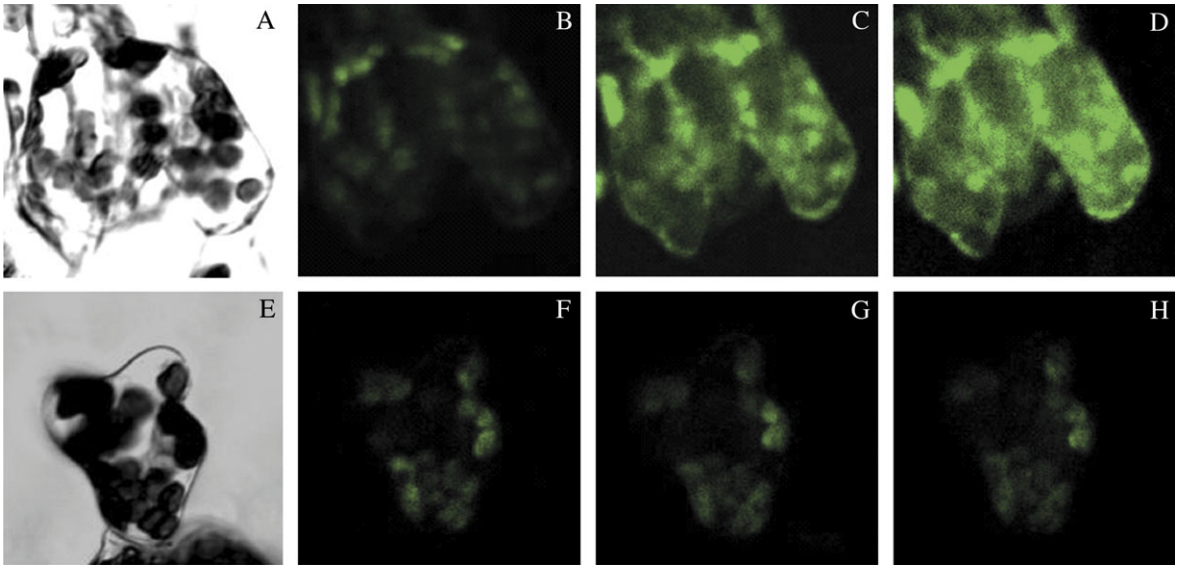

Fig. 8. The effects of CAT on high-fluence BL-induced production of H_2O_2 in mesophyll cells of *Arabidopsis*. Mesophyll cells were exposed to sequential 1 h treatments of 30 μmol m^{-2} s^{-1} BL in the absence (B–D) or presence (F–H) of 100 units ml^{-1} CAT. Micrographs from time points 0 min (B, F), 30 min (C, G), and 60 min (D, H) are shown. Bright field of mesophyll cells was also shown (A, E).

trigger of the low BL-induced chloroplast migration as well as in the high BL-induced chloroplast avoidance movements, it had no effect on the degree of low BL-induced chloroplast accumulation movements (Fig. 4), indicating that the H_2O_2 effect may be specific to high-fluence BL-induced chloroplast avoidance movements but not the low light accumulation response. This is consistent with the finding that low BL could not cause the generation of endogenous H_2O_2 as compared to the control (Fig. 5A), implying that H_2O_2 is not necessary for the low BL-induced chloroplast accumulation movements. As for the accelerated effects of H_2O_2 on the initiation of the low BL-induced chloroplast migration, it is possible that exogenously applied H_2O_2 provoked the change in this chloroplast movement with no physiological significance. However, the underlying mechanism by which H_2O_2 accelerated the low BL-induced accumulation movement may be interesting and needs to be explored further.

Using H_2DCF-DA as a probe of H_2O_2, it was demonstrated that a large quantity of H_2O_2 was induced by high-fluence BL at the levels of the cell and leaf (Figs 5A, 6), which is consistent with the result obtained by Chandrakuntal *et al*. (2004). Analysis of the time-courses of H_2O_2 generation and chloroplast avoidance movements revealed that the migration of chloroplasts induced by high-fluence BL exhibited dynamics similar to the production of H_2O_2 within 60 min (Figs 1, 3–5). Furthermore, high-fluence BL-induced chloroplast avoidance movements could be largely reduced by using CAT to scavenge the intracellular H_2O_2 (Figs 7–9). AsA and mannitol also have a similar inhibitory effect on high-fluence BL-induced chloroplast avoidance movements

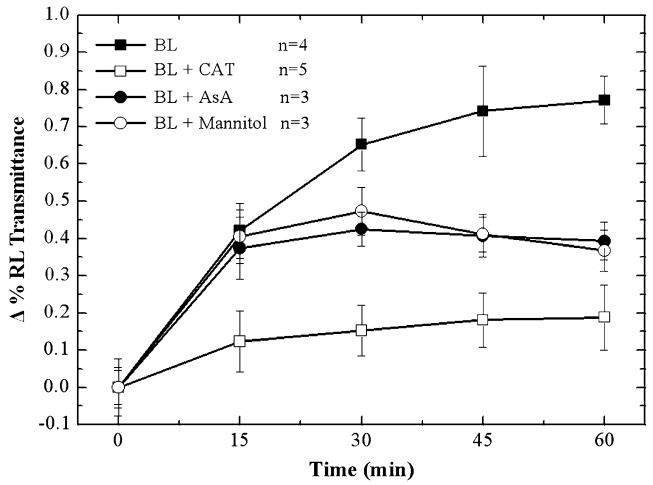

Fig. 9. Effect of scavengers of ROS on high-fluence BL-induced chloroplast avoidance movements in *Arabidopsis* leaf strips. The plots show the average change (±SE) in the percentage of RL transmittance of leaves relative to the average transmittance measured for the leaves before the BL treatment in the presence of 100 units ml^{-1} CAT, 200 μM AsA, or 50 mM mannitol. Number of leaves (*n*) is shown for each treatment.

(Fig. 9). This result clearly demonstrated that H_2O_2 might function as an intermediate in high-fluence BL signalling in chloroplast avoidance movements.

How does H_2O_2 mediate the high-fluence BL-induced chloroplast avoidance movements? This question might be answered by applying the fact that ROS can alter the cellular redox state. In *Arabidopsis* plants, phototropin contained two LOV domains, which are relatively conserved in a variety of proteins and are thought to function

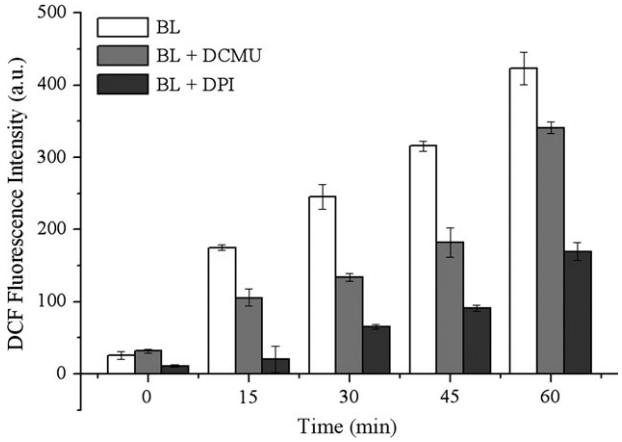

Fig. 10. The effects of DCMU and DPI on high-fluence BL-induced production of H_2O_2 in *Arabidopsis* leaf strips. DCF fluorescence in the leaf strips was measured immediately using a luminescence spectrometer after treatment with 30 μmol m^{-2} s^{-1} BL for the indicated times in the presence of 10 μM DCMU or 50 μM DPI.

as internal sensors of oxygen, redox potential, and light (Huala *et al*., 1997; Taylor and Zhulin, 1999). For example, ROS may control the reversibility of the adduct formation between the chromophore and cysteine residue, which could be modified by ROS, within the LOV domain in phototropin (Crosson and Moffat, 2001). It has also been reported that intracellular ROS may change the redox state of the LOV domain in WC-1 protein and thus regulate the protein signal transduction in photomorphogenesis (Yoshida and Hasunuma, 2004). In our experiment, H_2O_2 was produced in the presence of high-fluence BL (Figs 5, 6). Hence, the H_2O_2 generated might act directly on phot2 by changing the redox state of the LOV domain of phot2 and mediating BL signalling transduction in chloroplast avoidance movements.

In the washout experiments, at concentrations lower than 10^{-4} M, the effects of H_2O_2 on chloroplast migration were reversible, whereas at concentrations higher than 10^{-4} M, the effects were irreversible (Fig. 2). This suggested that at low concentrations the effects of H_2O_2 could be attributed to the activation of a signalling cascade in governing chloroplast movements, whereas at high concentrations H_2O_2 might damage cellular function. In fact, a similar dual role of H_2O_2 in regulating stomatal movements depending on its concentration has been described in previous work (Zhang *et al*., 2001). The evidence that BL-induced H_2O_2 production enhanced chloroplast migration in *Arabidopsis* leaves exposed to BL, and that the BL-induced chloroplast movements was reversible by washout and could be decreased by H_2O_2 removal, indicate that BL-induced H_2O_2 does not damage plant cells, and it can act as a modulator of BL-induced chloroplast movements.

In higher plants, ROS can be generated by several different pathways in a diverse range of physiological response (Allan and Fluhr, 1997; Bolwell *et al*., 1998). These pathways may include a cell wall localized peroxidase (Bolwell *et al*., 1995), amine oxidases (Allan and Fluhr, 1997), NADPH oxidases (Van Gestelen *et al*., 1997; Xing *et al*., 1997), and nuclei (Ashtamker *et al*., 2007). Furthermore, the highly energetic reactions of photosynthesis and an abundant oxygen supply make the chloroplast a particularly rich potential source of ROS. It has been suggested that when NADPH availability is limited, O_2 competing for electrons from photosystem I leads to the generation of ROS through the Mehler reaction (Munekage *et al*., 2004).

The subcellular source and possible molecular events involved in H_2O_2 generation in mesophyll cells and leaf strips treated with high-fluence BL have been investigated. A single cell assay (Fig. 6) showed that the plasma membrane and the chloroplasts might be the main regions of H_2O_2 production. Results from experiments applying DPI and DCMU suggested that the plasma membrane localized NADPH oxidases and photosynthetic electron transport are responsible for the H_2O_2 generation at the plasma membrane and chloroplasts, respectively (Fig. 10). It has been reported that the activity of NADPH oxidases could be activated by the increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) to catalyse NADPH and O_2 to generate ROS in plant development and environmental defence (Lamb and Dixon, 1997; Sagi and Fluhr, 2001). Moreover, a number of studies showed that the increase in $[Ca^{2+}]_{cyt}$ induced by BL could be commonly observed in many experimental systems (Sato *et al*., 1999; Tlalka and Fricker, 1999). Mutants in *phot2* show a lower H_2O_2 production under the same BL conditions (Fig. 5B), indicating that phototropin2 is required for the possible generation of H_2O_2 induced by high-fluence BL. From the DPI and *phot2* mutant experimental results, we can thus speculate that the high-fluence BL-induced increase in $[Ca^{2+}]_{cyt}$ via phototropin2 may activate the activity of NADPH oxidase to generate H_2O_2 at the plasma membrane, and in turn the H_2O_2 generated may act on phototropin2 to promote the chloroplast movements. This speculation is consistent with the concept that phototropin2 is responsible for avoidance movements (Sakai *et al*., 2001). However, it should also be noted that DPI has been demonstrated to inhibit blue light-induced phosphorylation of phototropin in *Vicia faba* (Kinoshita *et al*., 2003). In future studies, it might be intriguing to determine whether the inhibition of phototropin phosphorylation could lead to the decrease in H_2O_2 production. On the other hand, BL, like RL, as photosynthetically active radiation energy, could also be absorbed by photosystems to produce H_2O_2 through the Mehler reaction (Fig. 5A). But H_2O_2 generated from photosynthesis seems not to be associated with chloroplast movements in our present study. Further studies are needed to clarify how the photoreceptors responsible for chloroplast movements

perceive H_2O_2 from different sources and whether H_2O_2 from the plasma membrane and the chloroplasts are both required for high-fluence BL-induced chloroplast avoidance movements.

In summary, the accumulated evidence suggests that H_2O_2 is generated parallel to chloroplast avoidance movements induced by high-fluence BL, and that H_2O_2 itself cannot alter chloroplast position but it can promote chloroplast avoidance movements in the presence of BL, providing a new insight into the ROS and BL signal network. These results not only suggest a new role of H_2O_2 in high-fluence BL-induced chloroplast avoidance movements, but also provides a possible explanation of the interaction between ROS and LOV domains. At present, the relationship between H_2O_2 and other intracellular signal molecules (Ca^{2+}, phot1, and phot2) is unknown and needs further study.

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