Heterotrimeric G-protein is involved in phytochrome A-mediated cell death of *Arabidopsis* hypocotyls

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The heterotrimeric guanine nucleotide-binding protein (G-protein) has been demonstrated to mediate various signaling pathways in plants. However, its role in phytochrome A (phyA) signaling remains elusive. In this study, we discover a new phyA-mediated phenotype designated far-red irradiation (FR) preconditioned cell death, which occurs only in the hypocotyls of FR-grown seedlings following exposure to white light (WL). The cell death is mitigated in the $G\alpha$ mutant *gpa1* but aggravated in the $G\beta$ mutant *agb1* in comparison with the wild type (WT), indicative of antagonistic roles of GPA1 and AGB1 in the phyA-mediated cell-death pathway. Further investigation indicates that FR-induced accumulation of nonphotoconvertible protochlorophyllide (Pchl⁶³³), which generates reactive oxygen species (ROS) on exposure to WL, is required for FR-preconditioned cell death. Moreover, ROS is mainly detected in chloroplasts using the fluorescent probe. Interestingly, the application of H₂O₂ to dark-grown seedlings results in a phenotype similar to FR-preconditioned cell death. This reveals that ROS is a critical mediator for the cell death. In addition, we observe that *agb1* is more sensitive to H₂O₂ than WT seedlings, indicating that the G-protein may also modify the sensitivity of the seedlings to ROS stress. Taking these results together, we infer that the G-protein may be involved in the phyA signaling pathway to regulate FR-preconditioned cell death of *Arabidopsis* hypocotyls. A possible mechanism underlying the involvement of the G-protein in phyA signaling is discussed in this study.

Keywords: heterotrimeric G protein, phytochrome A, ROS, cell death, Pchl⁶³³

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Introduction

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) are signaling switches that are highly conserved in eukaryotes. These proteins precisely transduce extracellular signals from the ligand-activated G-protein-coupled receptors (GPCR) to the downstream effectors. In vertebrates, G-proteins are involved in the recognition and transduction of diverse signals such as light, calcium, odorants, small molecules, and proteins [1, 2]. In contrast to mammalian cells that contain 23 $G\alpha$, 6 $G\beta$, and 12 $G\gamma$ genes, plant cells have a smaller number of genes encoding G-protein subunits. For example, the *Arabidopsis* genome encodes single canonical α (GPA1) and β (AGB1) subunits and

two γ (AGG1 and AGG2) subunits [3–6]. The other components central to G-protein signaling, such as GPCR and regulators of G-protein signaling (RGS), are also sparsely found in plants [7]. Nevertheless, the G-protein signaling pathway has been demonstrated to mediate a wide range of plant responses to light, phytohormones, sugar, and various stresses [7, 8]. Further, there are an increasing number of physiological processes that are mediated by the G-protein signaling pathway [9, 10].

Light is an important signal that dramatically influences plant growth and development as well as photosynthesis [11]. Plants possess several photoreceptors including the blue/ultraviolet A (UV-A)-absorbing cryptochromes and the red/far-red irradiation (FR)-absorbing photochromes to monitor the light quality, intensity, and directionality [12]. Since the visual transduction cascade in rod photoreceptor cells represents a classical model system for GPCR signaling [13], this well-understood signaling pathway has promoted researchers to investigate a similar role of G-proteins in the light sensing of plants. The early experiments to test this hypothesis were conducted with two adenosine diphos-

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phate (ADP)-ribosylating drugs, namely, the cholera toxin that constitutively activates the G-protein pathway and the pertussis toxin that switches off the G-protein signaling pathway [14-17]. Similarly, two chemical agents, GTP γ s (a G α -subunit activator) and GDP β s (a G α -subunit inhibitor), have also been used in the study of the G-protein signaling pathway [18, 19]. The results derived from these experiments supported the hypothesis that the G-protein on the plasma membrane is involved in light signaling pathways. The most interesting results reflecting G-protein mediation of light signaling were derived from the study on a tomato phytochrome A (phyA)-deficient mutant known as *aurea*, in which chloroplast development is severely suppressed [18, 19]. Microinjection of exogenous GTP γ s and cholera toxin into *aurea* hypocotyl cells restored chloroplast development and anthocyanin biosynthesis, suggesting that G-protein activation triggers almost a complete cellular response initiated by phyA.

Recently, two papers have published conflicting data on G-protein involvement in phyA-mediated signal transduction using genetic and molecular approaches [20, 21]. Okamoto *et al.* [20] showed that the overexpression of either a native or a constitutively active form of GPA1 driven by a glucocorticoid-inducible promoter resulted in hypersensitivity to light, including red, FR, and blue light that affected hypocotyl elongation. It was found that phyA was essential for the enhanced response of the G α overexpressors after exposure to FR; this result supported the involvement of the G-protein in the phyA signaling pathway. In contrast, using the null mutants and various overexpressors of G-protein subunits, Jones' group [21] eliminated the possibility of a direct role for the G-protein in red- and FR-light signaling with regard to hypocotyl growth in *Arabidopsis*. These conflicting results raise questions regarding the involvement of the G-protein in phyA signaling.

In this study, we discovered a new phyA-dependent phenotype designated FR-preconditioned cell death. Recent reports have revealed that the G-protein is involved in cell death induced by oxidative stress (such as ozone) and pathogenic invasion [22-24]. Thus, we took advantage of the phyA-dependent cell death system to examine whether the G-protein is involved in this process. Our results demonstrate that *gpa1* and *agb1* mutants had opposite effects on phyA-dependent cell death, and support the hypothesis that the G-protein is involved in the phyA signaling pathway.

Results

FR-preconditioned cell death is phyA-dependent

When wild-type (WT) seedlings were continuously grown in FR (fluence rate 5 $\mu\text{mol}/\text{m}^2/\text{s}$) for 3 d followed by continuous exposure to white light (WL) (fluence rate

70 $\mu\text{mol}/\text{m}^2/\text{s}$) for an additional 3 d in half-strength MS medium containing 1% sucrose, we observed that most hypocotyls were dehydrated, and they then wilted. This occurred despite the fact that their cotyledons were able to partially turn green (Figure 1A). A careful examination showed that cell death initiated from the middle section of the hypocotyls 1 d after the seedlings were transferred from FR to WL. However, the seedlings that were grown in blue or red light for 3 d and then transferred to WL did not exhibit this phenotype (data not shown). Thus, we designated this phenotype as FR-preconditioned cell death. As far as we know, such cell death in hypocotyls has not been reported to date. Cell death was not visible if FR-grown seedlings were transferred to the dark; this

Figure 1 FR-preconditioned cell death of hypocotyls is phyA-dependent. **(A)** Morphology of WT, *phyA*, and *phyB* seedlings. Seedlings were grown in darkness for 1.5 d, then FR for 3 d followed by a 3-d exposure to WL. Arrows indicate light-induced wilting of the middle section of the hypocotyls. Scale bar, 1 mm. **(B)** Percentage of WT, *phyA*, and *phyB* seedlings exhibiting hypocotyl cell death. FR-WL indicates that seedlings grown in darkness for 1.5 d followed by FR for 3 d were transferred to WL for 3 d. Dark-WL indicates that seedlings grown in the dark for 5 d were transferred to WL for 3 d. The bars indicate the standard deviations from 3 independent experiments. Asterisks above the bar indicate a significant difference in cell death ($P < 0.01$) between *phyA* and WT or *phyB* seedlings.

indicated that light was required for cell death. We also observed that the longer the seedlings were incubated in FR, the more the hypocotyls wilted (data not shown). In addition, unlike FR-blocked cotyledon greening that can be rescued by addition of sugar to the growth medium [25], FR-preconditioned cell death of hypocotyls occurred in the presence of sugar. It was noteworthy that seedlings with wilted hypocotyls could develop new green leaves in a plate (Figure 1A); this indicated that the shoot meristem was insensitive to FR.

Because only the photoreceptor *phyA* senses FR, we questioned whether cell death was specifically mediated by *phyA*. Therefore, we assayed the hypocotyl response of the *phyA* and *phyB* mutants together with the WT under similar conditions. In order to minimize the difference in the rapidity of seed germination, seeds stratified at 4 °C for 4 d were germinated for 1.5 d in the dark. The etiolated seedlings were then incubated in FR for 3 d and subsequently in WL for 3 d. As expected, wilted hypocotyls were

not visible in the *phyA* mutant but were clearly observed in the *phyB* mutant (Figure 1A). In order to quantitatively determine the degree of cell death, the seedlings were stained with Evans blue solution to specifically stain the dead cells [26]. The results showed that 72.6% and 71.2% of *phyB* and WT seedlings, respectively, were stained by Evans blue, whereas only 7.8% of the *phyA* plants were stained (Figure 1B). The seedlings grown in the dark for 5 d and subsequently exposed to WL continuously for 3 d were used as a control; these seedlings were barely stained by Evans blue (Figure 1B). Thus, FR-preconditioned cell death was specifically mediated by *phyA*.

FR-preconditioned cell death is affected by the G-protein

Based on increasing evidence indicating that the G-protein signaling pathway is involved in the process of cell death induced by various stresses [22–24], we explored whether the G-protein also modulated FR-preconditioned cell death. As shown in Figure 2A, among the hypocotyls

Figure 2 The G-protein is involved in FR-preconditioned cell death of hypocotyls. FR-WL indicates that seedlings grown in the dark for 1.5 d followed by FR for 3 d were transferred to WL for 3 d. Dark-WL indicates that seedlings grown in the dark for 5 d were transferred to WL for 3 d. The bars indicate the standard deviations from 3 independent experiments. Cell death in the mutant hypocotyls indicated by 1 and 2 asterisk(s) above the bar is significantly different from that in WT at the levels of $P < 0.05$ and $P < 0.01$, respectively. **(A)** The cell-death rate in the hypocotyls of the seedlings with the *phyA*, WT, *gpa1*, *agb1*, and *gpa1 agb1* genetic backgrounds. **(B)** Cell death was quantified by Evans blue staining. **(C)** Chlorophyll content in cotyledons of seedlings grown in FR-WL or dark-WL. FR-blocked cotyledon greening was not affected by G-protein mutations. **(D)** Percentage of seedlings with cell death in the single *phyA* and double *phyA gpa1*, *phyA agb1* mutants was measured under FR-WL or dark-WL conditions.

evaluated by Evans blue staining, the $G\alpha$ null mutant *gpa1* exhibited a lower cell-death rate (45.2%) while the $G\beta$ null mutant *agb1* exhibited a higher rate (90.1%) compared to the WT. This suggested that GPA1 was a positive player while AGB1 was a negative player in FR-preconditioned cell death. The antagonistic action of the GPA1 and AGB1 subunits was also demonstrated during cell death caused by pathogens and ozone stress [22-24]. In addition, the *gpa1 agb1* double mutant exhibited a cell-death rate similar to that of the *agb1* mutant, indicating that AGB1 was the main transducer of the G-protein signaling pathway.

To quantify FR-preconditioned cell death, the Evans blue stain was extracted from the dyed hypocotyls and measured by spectrometry. Consistent with the other results, the *gpa1* mutant exhibited lower intensity Evans blue staining while the *agb1* and *gpa1 agb1* mutants exhibited higher intensity staining as compared to the WT (Figure 2B). There was no difference observed in cell death between the WT and the mutant seedlings that were grown in the dark for 5 d and then exposed to WL (Figure 2B). In addition, we examined the effect of the G-protein on FR-blocked cotyledon greening, which is a typical phenotype mediated by the *phyA* signaling pathway. Our results showed that the chlorophyll contents in the cotyledons of WT, *gpa1*, and *agb1* seedlings grown either in FR or in the dark, was very similar (Figure 2C); however, the chlorophyll content of these seedlings grown in FR was significantly lower than that of *phyA* (Figure 2C). This indicated that the G-protein was not involved in the process of FR-suppressed cotyledon greening. Taken together, the results suggested that the G-protein-mediated *phyA* signaling pathway was tissue specific.

To obtain genetic evidence, we constructed *phyA gpa1* and *phyA agb1* double mutants in order to examine whether *phyA* and G-protein were localized in the same pathway during FR-preconditioned cell death. Our results showed that both *phyA gpa1* and *phyA agb1* double mutants exhibited the same rate of hypocotyl cell death as that of the

Figure 3 THF1 is involved in FR-preconditioned cell death. Results from WT, single (*thf1* and *gpa1*) and double (*gpa1 thf1*) mutant seedlings are presented. The values are the mean $\pm$ SD ($n = 3$). Asterisks above the bar indicate $p < 0.01$ vs WT.

Figure 4 FR-preconditioned cell death is not associated with POR expression. **(A)** Levels of *PORA* and *PORB* transcripts were analyzed by RT-PCR after 28 cycles in hypocotyls of seedlings. The actin transcript was used as a quantitative control. **(B)** The total level of POR protein in hypocotyls of seedlings was analyzed by western blotting. The band marked with a star represents the POR protein. Other bands are the degraded forms of POR.

phyA mutant (Figure 2D); this suggested that the sole FR receptor *phyA* is epistatical to the G-protein.

Thylakoid formation 1 (THF1) protein is involved in FR-preconditioned cell death

THF1 is identified as a GPA1 interacting protein that is highly conserved in oxygenic photosynthetic organisms

[27]. Although it has been shown that THF1 functions in the sugar signaling pathway mediated by the G-protein, it is not clear whether THF1 is also involved in FR-preconditioned cell death. We adopted a genetic approach to answer this question using the *thf1* null mutant. The result showed that the death rate of the *thf1* seedlings was 18.7% (Figure 3), which was lower than that of WT and *gpa1* seedlings; this suggested that THF1, like GPA1, positively regulates FR-preconditioned cell death. The genetic relationship between *THF1* and *GPA1* was further examined using the *thf1 gpa1* double mutant. The death rate of *thf1 gpa1* hypocotyls was lower than that of *gpa1* but higher than that of *thf1*; this implied that a complex genetic relationship exists between *GPA1* and *THF1* in the regulation of FR-preconditioned cell death.

Expression of protochlorophyllide oxidoreductase (POR)

is not associated with FR-preconditioned cell death of hypocotyls

Previous studies on the FR-blocked cotyledon greening have revealed that POR is a critical determiner of seedling greening and survival [25, 28]. The *phyA* mutant may have escaped cell death because the expression of *PORA* is not suppressed under FR, and *PORA*-bound protochlorophyllide (Pchl_{ide}) is converted to non-toxic chlorophyllide (Chl_{ide}) in the presence of light [28]. We therefore studied the role of POR in FR-preconditioned cell death. Our RT-PCR analysis data with gene-specific primers showed that both *PORA* and *PORB* were expressed in the dark (Figure 4A). After FR exposure, *PORA* was only detected in the hypocotyls of *phyA* (Figure 4A), whereas *PORB* was expressed in all genetic backgrounds (Figure 4A). A comparison of the *PORB* expression levels in FR-grown hypocotyls demonstrated that the expression in *agb1* was

Figure 5 Nonphotoconvertible Pchl_{ide} (Pchl_{ide}⁶³³) is required for FR-preconditioned cell death. Pchl_{ide} was extracted from 50 hypocotyls of seedlings grown in the dark for 5 d (Dark-WL) or in the dark for 1.5 d followed by FR for 3 d (FR-WL) and then exposure to WL for 30 min. **(A)** Room temperature fluorescence spectra of Pchl_{ide} extracted from hypocotyls of WT, *phyA*, *gpa1*, and *agb1* seedlings. **(B)** The level of Pchl_{ide}⁶³³ quantified from (A). The bars indicate standard deviations from three independent experiments. Significant differences ($P < 0.01$) in Pchl_{ide}⁶³³ levels between mutants and WT are indicated by two asterisks above the bar.

slightly higher than that in the WT, whereas *gpa1* exhibited a similar expression level as those of the WT. The POR protein level was examined by western blot analysis. In accordance with the RT-PCR results, after FR exposure POR accumulation was found to be higher in *phyA* and *agb1* than that in WT, but *gpa1* exhibited similar accumulation levels as that of the WT (Figure 4B). Thus, our results showed that there was a low probability of POR being the primary factor responsible for the difference in FR-preconditioned cell death between the G-protein mutants and the WT.

Nonphotoconvertible Pchl⁶³³ is required for FR-preconditioned cell death

It has been known that seedlings grown in the dark or in FR for prolonged periods of time will be photobleached upon exposure to WL due to over-accumulated Pchl⁶³³ [25, 29–31]. There are two types of Pchl⁶³³, namely, photoconvertible (Pchl⁶⁵⁵) and nonphotoconvertible (Pchl⁶³³). When exposed to light, Pchl⁶⁵⁵ is converted to Chl⁶⁷¹ within a short time for chlorophyll biosynthesis, while Pchl⁶³³ acts as a sensitizer causing photooxidative damage in cellular and subcellular structures [32]. We reasoned that the observed difference in the rate of cell death between mutant and WT hypocotyls might be due to the Pchl⁶³³ accumulated during FR treatment. To test this hypothesis, we used a fluorescence spectrometer to measure the level of Pchl⁶³³ in the hypocotyls of the seedlings grown in FR for 3 d and then exposed to WL for 30 min. Our results showed that Pchl⁶³³ production was clearly induced in WT, *gpa1*, and *agb1* hypocotyls when they were grown in FR compared to when they were grown in the dark (Figure 5A and 5B); further, *agb1* accumulated a higher level of Pchl⁶³³ than WT and *gpa1*. It is important to note that the *phyA* mutant did not accumulate Pchl⁶³³ in FR. This finding indicated that the survival of the *phyA* hypocotyls was probably associated with minimal accumulation of

Pchl⁶³³ *in vivo*. Similarly, *agb1* had a high cell-death rate and also accumulated a high level of Pchl⁶³³ in its hypocotyls compared with that accumulated in the WT hypocotyls. However, the level of Pchl⁶³³ accumulated in the *gpa1* mutant was not proportional to its cell-death rate (Figure 5A and 5B); this suggested that other factors are involved in suppression of cell death in *gpa1*.

If the Pchl⁶³³ accumulated in hypocotyls during FR treatment is required for cell death, we predicted that the level of Pchl⁶³³ in the hypocotyls of *thf1* seedlings should be low. In fact, the fluorescent assay demonstrated that Pchl⁶³³ production was suppressed as compared to the WT as a result of the THF1 mutation (Figure 6A). Quantitative analysis revealed that the amount of Pchl⁶³³ produced by the *thf1* mutant was as low as that produced by the *phyA* mutant, which was approximately 20% of the Pchl⁶³³ level produced by the WT (Figure 6B). However, there was no difference observed in the Pchl⁶³³ levels among WT, *phyA*, and *thf1* seedlings when they were grown in the dark (Figure 6B). Taking these results together, we infer that FR-induced Pchl⁶³³ is required for cell death.

Reactive oxygen species (ROS) is localized in chloroplasts

Based on the presence of Pchl⁶³³ in hypocotyls, we hypothesized that ROS might be released from Pchl⁶³³ upon WL exposure. To confirm this hypothesis, we mapped the subcellular localization of the ROS (mainly H₂O₂) produced in hypocotyls using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). As expected, no fluorescence could be detected in the hypocotyls of WT seedlings that were grown in the dark and subsequently exposed to WL (Figure 7A); this indicated that there was no ROS accumulation. Moreover, the strong chloroplast autofluorescence (red) observed in these seedlings indicated that the chloroplast development was normal (Figure 7A).

Figure 6 The *thf1* mutant does not accumulate Pchl⁶³³. **(A)** Room temperature fluorescence spectra of the extracted Pchl⁶³³ from hypocotyls of WT, *phyA*, and *thf1* seedlings. **(B)** The level of Pchl⁶³³ was quantified from (A). The bars indicate standard deviations from three independent experiments. Significant differences ($P < 0.01$) in the level of Pchl⁶³³ between mutants and WT are indicated by two asterisks above the bar.

Figure 7 Fluorescence microscopic image of ROS localization in the cells of WT hypocotyls. **(A)** H₂DCFDA fluorescence was not detectable in the hypocotyls grown in the dark for 5 d and then transferred to WL for an additional day. Scale bar, 1 mm. **(B)** Strong H₂DCFDA fluorescence was observed in the cells of the hypocotyls that were grown in FR for 3 d followed by exposure to WL for an additional day. Scale bar, 1 mm. **(C)** The H₂DCFDA fluorescence shown in **(B)** was mainly emitted from the plastids/chloroplasts. Insets show H₂DCFDA fluorescence in the chloroplasts of guard cells (white square) and dermal cells (red square). Scale bar, 200 μ m.

Figure 8 H₂O₂ treatment mimics FR-preconditioned cell death of hypocotyls. Seedlings grown in the dark for 5 d were treated with 1 mM H₂O₂ for 2 h, washed with deionized water, and put in WL for 1 d. **(A)** Typical morphology of a seedling with hypocotyl cell death 6 h after H₂O₂ treatment. Scale bar, 2 mm. **(B)** The effect of exogenous H₂O₂ on cell death of WT, *phyA*, *gpa1*, *agb1*, and *thf1* seedlings. Cell death was measured with Evans blue staining. Error bars indicate SD ($n = 3$). Significant differences ($P < 0.01$) in cell death between mutants and WT are indicated by two asterisks above the bar.

In the FR-grown hypocotyls, however, the H₂DCFDA-stained fluorescent signal (green) was strong in the middle section of hypocotyls (Figure 7B); this implied that ROS

was generated in these cells. Interestingly, chloroplast auto-fluorescence was emitted mainly from the upper section of the hypocotyls (Figure 7B). A detailed inspection revealed

that the green fluorescent region coincided with the position of the plastids/chloroplasts (Figure 7C). Thus, this result was consistent with the idea that ROS was derived from plastid-localized Pchl^{ide}⁶³³.

H₂O₂ treatment mimics FR-preconditioned cell death

Based on the above data, we questioned whether exogenous oxidative stress could mimic FR-preconditioned cell death. Therefore, we treated 5-d-old dark-grown seedlings with 1 mM of H₂O₂ under WL for 2 h; H₂O₂ was then removed from the plates. As expected, the H₂O₂ exposure induced a similar phenotype as that observed in FR-preconditioned cell death approximately 6 h after treatment (Figure 8A). We then quantified cell death by Evans blue staining. Our result showed that there was no difference in the cell-death rate among the *gpa1*, *phyA*, and WT seedlings. However, the cell death rate was more severe in the *agb1* and *thf1* mutants than that in the WT (Figure 8B); this suggested that *agb1* and *thf1* are more sensitive to oxidative stress than *gpa1*, *phyA*, and WT. These results provide further evidence supporting the notion that oxidative stress mediates FR-preconditioned cell death.

Discussion

FR-preconditioned cell death of hypocotyls is regulated by phyA signaling

PhyA is the sole photoreceptor responsible for photomorphogenesis of seedlings grown in continuous FR light. Functional analysis of phyA via multiple approaches has revealed its global effects on various physiological responses such as shade avoidance, floral induction, entrainment of the circadian clock, and chloroplast biogenesis [33, 34]. New phenotypes with defective phyA signaling pathway are continuously being examined. In the present study, we discovered that FR-preconditioned cell death, which occurs only in the hypocotyls of FR-grown seedlings after WL treatment, is also mediated by the photoreceptor phyA. It is well documented that when etiolated seedlings are grown in the absence of sugar in FR for a prolonged time period (>1 d), cotyledon greening is impeded [25, 28]. This block in cotyledon greening due to FR can be rescued by the addition of sugar to the growth media [25, 28]. In contrast, FR-preconditioned hypocotyl cell death takes place even on media containing sugar, while other parts of the seedlings, such as the cotyledons and shoot meristems, can grow; this suggests that the cell death phenotype is unique to the hypocotyls under our growth conditions.

The hypocotyl of *Arabidopsis* is a model system for studying the molecular mechanism underlying the cell elongation and signal transduction networks because of its anatomical simplicity and its growth behavior [35]. Genetic

screenings for the altered nature of hypocotyl growth under the given conditions have aided in the identification of many important genes related to light response, hormones, and metabolism. However, little attention has been paid to the event of cell death known as photobleaching that normally occurs after seedlings are transferred from FR or darkness to WL. Screening of mutants resistant to FR-induced killing in the absence of sucrose led to the isolation of a novel phyA signal-transduction gene (*PAT1*), which acts as an early signaling component in the cytoplasm [36]. In addition, the phyA signaling pathway has been demonstrated to function in the SA-induced hypersensitive response [37]. We therefore speculate that hypocotyls could also be used as a potential model system for the identification of important components in the phyA-mediated cell-death pathway when sugar is present in the growth media.

Effect of G-protein on phyA-dependent cell death

Generally, ROS is suggested to function in two ways, namely, as an oxidant and as a signal. As an oxidant, ROS directly reacts with various biomolecules such as proteins, lipids, and DNA, and causes irreversible cellular damage [38, 39]. ROS also serves as an important signal regulating global changes in gene expression [40, 41]. These two functions may be linked *in vivo* and can barely be distinguished in response to environmental stimuli. However, recent progress achieved as a result of studies on the *flu* seedlings, which rapidly accumulate Pchl^{ide} in the dark and generate singlet oxygen upon exposure to light, have shown that the primary effect of ROS is the activation of a broad range of signaling pathways without direct damage to chloroplast biogenesis [29, 42].

The results derived from this study indicate that the G-protein may affect FR-induced cell death via both ROS generation and its signaling pathway. As reported previously [43], we also demonstrated that FR can up-regulate the level of Pchl^{ide} in the hypocotyls of the WT seedlings. It is interesting that the *AGB1* mutation leads to the production of more Pchl^{ide}⁶³³ than that produced by the WT. Thus far, excess accumulation of Pchl^{ide}⁶³³ in the WT under FR is thought to result from the suppression of PORA expression [28]. However, our results failed to establish a good correlation between POR expression and FR-preconditioned cell death in hypocotyls. For example, the *agb1* mutant expressing a higher level of PORB, which is the main isoform expressed in FR [44], shows more severe FR-preconditioned cell death than WT. One explanation is that the *AGB1* mutation also generates more Pchl^{ide} than that generated by the WT. Another possibility is that the *AGB1* mutation affects the binding of POR to Pchl^{ide} in a cell.

We observed that FR-preconditioned cell death always

occurred in the middle section of the hypocotyls. Erdei *et al.* [31] observed a similar light response in the epicotyls of dark-germinated pea seedlings. In their model, the photosensitivity of the epicotyls was explained by the balance between POR and Pchl_{ide} that is crucial for survival of dark-grown epicotyls, along which the Pchl_{ide} gradient can be detected [45]. Pchl_{ide}⁶³³ was thought to bind to unknown proteins [46]. The AGB1 mutation appears to enhance the ratio of Pchl_{ide}⁶³³ to photoactive Pchl_{ide}⁶⁵⁵. Until date, little information is available regarding the regulation of this ratio in plants. Our data (unpublished) indicated that hypocotyls of dark-germinated seedlings of the *agb1* mutant were more resistant to light-induced wilting than those of the WT. This result can be explained by the higher level of POR expression in *agb1* than that in the WT. AGB1 mutation may influence the binding of POR to Pchl_{ide} and lead to the production of nontransformable Pchl_{ide} in FR. Moreover, AGB1 might also have an impact on the sensitivity of hypocotyls to ROS because *agb1* suffers more severe cell death than that incurred by the WT when treated with the same concentration of H₂O₂. A similar role of Gβ in oxidative stress has also been demonstrated in mammalian cells, where H₂O₂ directly activates the G-protein signaling pathway and the Gβγ subunit is the main signal transducer involved in the protection of the cells against oxidative stress via downstream activation of the mitogen-activated protein kinase cascade. In the case of *gpa1*, although the mechanism underlying reduction of FR-preconditioned cell death as a result of the mutation is unclear because there is no difference observed in the accumulation of Pchl_{ide} and sensitivity to H₂O₂ between the *gpa1* and WT seedlings, our data are in agreement with previous reports on GPA1-mutation-induced reduction of cell damage caused by ROS stress [23]. As Joo *et al.* [23] reported, in FR light the pattern of ROS generation in *gpa1* might be altered as compared to that in the WT. We speculate that since the double *gpa1 agb1* mutant exhibits the same level of cell death as that of the single *agb1* mutant, it is possible that the GPA1 mutation resulted in a lower level of FR-preconditioned cell death than that of WT due to the presence of free AGB1 in the *gpa1* seedlings. This hypothesis regarding G-protein-regulated FR-preconditioned cell death is similar to that of lateral root formation, where AGB1 may act downstream of GPA1 to negatively regulate lateral root formation.

G-protein may finely regulate the phyA signaling pathway

The early studies via a pharmacological approach showed that the G-protein was an important transducer in the phyA signaling pathway for chloroplast development in the hypocotyls of *aurea*. Recently, the gene controlling the pale

green phenotype of the *aurea* mutant was isolated and found to be the phytochromobilin synthase that catalyzes the conversion of biliverdin IX α into 3Z-phytochromobilin during phytochrome chromophore biosynthesis [47]. The chlorophyll-deficient phenotype of the *aurea* mutant is due to the suppression of 5-aminolevulinic acid (ALA) synthesis caused by a high level of heme and subsequent reduction in Pchl_{ide} synthesis [48]. Thus, we reasoned that the GPA1-promoted chloroplast development observed in *aurea* might partially release the inhibition of ALA synthesis caused by heme. It would be interesting to examine whether ALA accumulation is altered in the *gpa1* mutant. Besides ALA, chlorophyll biosynthesis can be modulated by the G-protein via another key factor, namely, POR, whose expression in FR is always slightly higher in the *agb1* mutant than that in the WT. Thus, the G-protein may finely tune the regulation of tetrapyrrole biosynthesis.

Further investigation is required to determine the mechanism underlying the mediation of the phyA signaling pathway by the G-protein. However, the involvement of the G-protein in FR-preconditioned cell death in hypocotyls provides a new opportunity to unravel the link between phyA and the G-protein at a molecular level. FR appears to trigger a process that is extremely harmful for plant growth because it suppresses POR expression and simultaneously promotes Pchl_{ide} accumulation. The genetic dissection of the molecular mechanism underlying the plant's response to FR has revealed the involvement of many positive regulators, such as PAT1, FHY1, FHY3, FIN2, and FAR1 [36, 49-51], as well as negative regulators, such as PSI2 and SPA1 [52, 53]. They are involved in the phyA signaling pathway or phytochrome chromophore biosynthesis, suggesting that the phyA signaling pathway is tightly regulated and integrated in the whole cellular signaling network. We propose that the G-protein is one of the switches involved in finely tuning the regulation of the phyA signaling pathway. Okamoto *et al.* [20] demonstrated that FHY1 played a role in GPA1-promoted hypocotyl elongation under FR. FHY1 is also involved in FR-suppressed chlorophyll biosynthesis since the expression of *HEMA1* markedly decreased in the mutant [54]. Thus, FHY1 might be an appropriate candidate linking the G-protein and one branch of the phyA signaling pathway. Recent research has determined that the interaction of activated phyA with FHY1, FHL1, NADPK2, and PSK1 is an early event in cytoplasmic phyA signaling. Among them, FHY1 and FHL1 were required for phyA translocation from the cytoplasm to the nucleus [55-57], and NADPK2 was suggested to potentially activate the G-protein by increasing the level of GTP [20]. We suggest two possibilities for crosstalk between G-protein and phyA signaling in regulation of chlorophyll biosynthesis. One possibility is that the G-protein affects the process of

phyA nuclear translocation via FHY1 or FHL1. Another is that the G-protein signaling pathway activated by phyA in the cytoplasm regulates nuclear gene expression. However, in phyA-mediated FR responses, only the inhibition of gravitropism was found to be regulated by the cytoplasmic phyA signaling pathway [58]. Thus, the latter possibility of G-protein involvement in the phyA signaling pathway may be excluded. It would be interesting to investigate whether the G-protein has an impact on the rapidity of the phyA movement from the cytoplasm to the nucleus using a combination of genetic and molecular approaches.

Materials and Methods

Plant materials, growth conditions, and light sources

Arabidopsis thaliana WT (ecotype Columbia, Col-0) plants were used in this study. All the mutants used were in the Col-0 background. The *phyA* (*phyA-211*) and *phyB* (*phyB-9*) mutants were obtained from the Arabidopsis Biological Resources Center (Columbus, OH). The other mutants are *gpa1* (*gpa1-4*), *agb1* (*agb1-2*) and *thf1* (*thf1-1*) [27]. The double mutants used in the experiment were created by using the abovementioned single mutants and confirmed by plant genotyping in the F2 generation.

Surface-sterilized seeds stratified at 4 °C for 4 d were germinated on half-strength Murashige and Skoog (MS) medium with 1% of sucrose for 1.5 d in darkness before FR irradiation at 22 °C. FR light was provided by the far-red diodes (model E-30, Percival Scientific, Boone, IA) at the peak wavelength of 730 nm with a plexiglas filter (FRF 700, Westlake Plastics, Lenni, PA). The light strength was determined using a spectroradiometer (ASD, Boulder, CO). After FR irradiation, the seedlings were subsequently transferred to WL (TL-D, 30W, Philip, Shanghai). WL fluence rates were measured using a ZDS-10 Luxmeter (Xuelian Instrument Factory, Shanghai). At least 50 seedlings from each genotype were sampled for further analysis in the same experiment.

Qualitative and quantitative determinations of cell death in hypocotyls

More than 50 seedlings were examined for each qualitative or quantitative assay of cell death in hypocotyls. For the qualitative assay of cell death, hypocotyls showing a visible wilted phenotype or Evans blue staining under the FR-induced cell death condition were recorded. For quantitative measurement of cell death, seedlings were completely submerged in 2.5% (w/v) solution of Evans blue dye for 10 min, then washed three times with distilled water. The dye bound to the dead cells in hypocotyls was extracted using a solution containing 50% (v/v) methanol and 1% (w/v) sodium dodecyl sulfate (SDS) at 60 °C for 30 min and then quantified by measuring the absorbance at 600 nm [26].

Chlorophyll and Pchl_a measurement

Chlorophyll was extracted using 80% acetone at 4 °C for 24 h in the dark. The chlorophyll content was determined using a spectrophotometer (UNIC UV-2102 PCS, Shanghai) [59]. Protochlorophyllide was extracted and measured by a previously described method [60]. In each replicate experiment, 50 hypocotyls were sampled during the analysis.

Fluorescence microscopic imaging of ROS and H₂O₂ treatment

Subcellular localization of ROS was examined by fluorescence microscopic imaging [23]. Fluorescence was observed with a LSM 510 META laser scanning confocal microscope (ZEISS, Germany), with excitation at 488 nm and emission at 530 nm. For H₂O₂ treatment, seedlings grown for 5 d in the dark were shifted to WL and immediately submerged in 1 mM H₂O₂ solution for 2 h. They were then washed with deionized water and placed in WL for 1 d; thereafter, the cell death was quantitatively measured.

Semiquantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using the RNAGents total RNA isolation system (Promega). Reverse transcription was performed according to the manufacturer's instructions (Reverse transcription system, Promega). Gene expression was determined using gene-specific primers by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) or quantitative real-time RT-PCR. Primers for the POR genes were as follows: PORA-F: CAT TAC ACT CTT TAA GTC TTA AAC G; PORA-R: AAC CAC GTT TCC TTT TCT AAG T; PORB-F: CTC TCT CTT TGT TCG AGT ACC AAC; and PORB-R: GTA TTC GTG TTC CCG GTA ATG G. The amplified DNA fragments were separated by agarose gel electrophoresis and analyzed using GIS-2010 (Tanon, Shanghai).

Protein extraction and western blot analysis

Total protein was extracted and quantified by a previously described method [25]. Protein expression was detected by immunoblot analysis. Total protein (40 µg) was loaded in each well. Signals were detected using a medical film processor (SRX-101A, Konica, Minolta, Tokyo). Anti-POR antibody was obtained from Agrisera, and used at a dilution of 1:2 000.

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