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Light Primes the Thermally Induced Detoxification of Reactive Oxygen Species During Development of Thermotolerance in *Arabidopsis*

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Reactive oxygen species (ROS) serve as critical signaling mediators in plant adaptation responses to environmental stimuli. ROS biosynthesis and metabolism should be tightly regulated, because they often impose oxidative damage on biological molecules, such as DNA and proteins, and on cellular structures. It is known that at high temperatures, ROS rapidly accumulate in plant tissues. Thus, a quick activation of ROS-scavenging systems is necessary for thermal adaptation. However, it is largely unknown how the thermo-induced ROS-detoxifying capacity is enhanced by environmental factors at the molecular level. Here, we demonstrated that environmental light primes the thermally induced ROS detoxification process for development of thermotolerance in Arabidopsis. While the ROS detoxification capacity was markedly enhanced in light-pre-treated plants at high temperatures, its enhancement was not as evident in dark-pre-treated plants. ASCORBATE PEROXIDASE 2 (APX2) is a representative ROS-scavenging enzyme that is activated under heat stress conditions. It was observed that the thermal induction of the *APX2* gene was more prominent in light-pre-treated plants than in dark-pre-treated plants. Notably, the light-gated *APX2* gene induction was compromised in Arabidopsis mutants lacking the red light photoreceptor phytochrome B (phyB). Furthermore, exogenous application of the antioxidant ascorbate recovered the heat-sensitive phenotype of the *phyB* mutant. These observations indicate that light-primed ROS-detoxifying capability is intimately linked with the induction of thermotolerance. We propose that the phyB-mediated light priming of ROS detoxification is a key component of thermotolerant adaptation in plants.

Keywords: *Arabidopsis thaliana* • Photoreceptor • ROS detoxification • Thermotolerance.

Introduction

Plants are continuously exposed to unfavorable environmental changes in natural habitats. Temperature is one of the major environmental factors that profoundly affect plant growth and development, metabolism and physiology, and seed productivity (Quint et al. 2016). In particular, it has been widely documented in recent decades that global warming, a gradual increase of global average temperature, and associated climate changes, such as extreme cold and heat spells in local areas, broadly influences vegetation on Earth and crop productivity (Zhao et al. 2017). In this sense, it is necessary to extend our understanding of the molecular mechanisms that direct plant adaptation processes to thermal stresses in order to develop means of improving crop cultivation capacity.

Plants possess versatile protection systems to cope with high temperature stresses (Larkindale et al. 2005, Morimoto et al. 2017). One such adaptive mechanism is the co-ordinated regulation of biosynthesis and metabolism of reactive oxygen species (ROS), which accumulate to a high level in plants at unfavorable high temperatures (Lee et al. 2015). High-level accumulation of ROS is harmful to plant growth and survival because they cause bleaching of Chls and destruction of cellular components (You and Chan 2015, Zhao et al. 2017). It has been reported that three major scavenging enzymes, i.e. ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT), co-ordinate ROS detoxification, while superoxide dismutase (SOD) mediates ROS production (Mittler et al. 2004). Therefore, a physiological balance among the ROS metabolic enzyme activities is crucial for maintaining ROS homeostasis. In particular, ASCORBATE PEROXIDASE 2 (APX2) is one of the central ROS detoxification enzymes that function during development of thermotolerance in plants (Panchuk et al. 2002). APX enzymes utilize ascorbate as an electron donor to reduce hydrogen peroxide to water (Karyotou and Donaldson 2005), thereby reducing the risk of oxidative damage (Caverzan et al. 2012). Heat shock proteins (HSPs) also contribute to the protection of plant cells from heat-induced cellular damage. They act as heat-induced molecular chaperons, which facilitate and correct protein folding, assembly and stabilization so that protein substrates are protected from thermo-triggered denaturation (Perez et al. 2009). Consequently, overexpression of *HSP101* in Arabidopsis plants enhances thermotolerance (Queitsch et al. 2000).

It is notable that temperature responses are closely linked with light signaling. In plants, light acts as a pivotal environmental

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signaling inducer as well as the ultimate source of photosynthesis (Lee et al. 2017). Thus, plants possess multiple photoreceptors that are capable of efficiently sensing specific ranges of light wavelengths (Lau and Deng 2012). The phytochrome (phy) photoreceptors have two interchangeable forms, Pr and Pfr, the interconversion of which is regulated by the red to far-red light ratio in nature (Xu et al. 2015). Upon photoactivation, the phy proteins enter the nucleus, where they interact with a group of PHYTOCHROME-INTERACTING FACTORs (PIFs) to trigger a wide range of light responses (Zhang et al. 2013). Interestingly, the phy photoreceptors also function as thermosensors: the light-activated reversion of Pfr to Pr is accelerated at warm temperatures (Jung et al. 2016, Legris et al. 2016). The blue light-sensing cryptochrome (CRY) photoreceptors are known to interact with PIF4, which is a key component of thermomorphogenesis (Ma et al. 2016). The phototropin (PHOT) photoreceptors were initially identified as the modulator of phototropic plant growth (Zhao et al. 2013). Later, it has been proven that they also sense temperature changes to rearrange chloroplasts in the cytoplasm for optimal photosynthesis (Fujii et al. 2017). Meanwhile, it is known that thermoinduced plant growth is attenuated under UV-B light conditions (Hayes et al. 2017). In contrast, Arabidopsis mutants lacking the UV-B photoreceptor are still sensitive to warm temperatures. These reports indicate the integration of light and temperature signals in regulating plant growth, morphogenesis and environmental adaptation.

In nature, plants utilize light information to anticipate upcoming environmental fluctuations that occur regularly on a daily basis (Griebel and Zeier 2008), among which diurnal rhythms of ambient temperature fluctuations have been most comprehensively studied in recent years. Of particular interest is the light gating of temperature responses (Lee and Thomashow 2012, Dickinson et al. 2018). The transcription of genes that mediate freezing tolerance is accelerated when the photoperiod is short (Lee and Thomashow 2012). Thus, plants exhibit enhanced freezing tolerance under short days. In addition, hypocotyl thermomorphogenesis is more prominent during the daytime under long-day conditions, when the temperature is typically high (Park et al. 2017). Furthermore, it has been recently reported that a retrograde chloroplast signaling gates the thermotolerance response (Dickinson et al. 2018), perhaps providing a molecular link between photosynthetic activity and development of thermotolerance. However, it is not clear how light signaling is associated with temperature responses at the molecular level in most cases.

In this work, we demonstrated that phyB-mediated light priming is essential for development of thermotolerance in Arabidopsis. Our data illustrate that light primes the HEAT SHOCK FACTOR A1 (HSFA1)-mediated thermal induction of *APX2* gene expression under high temperature conditions. While the *APX2* gene was induced to some extent by high temperatures in darkness, the thermal induction of the *APX2* gene was more prominent in plants that are exposed to light prior to heat treatment in a phyB-dependent manner. We also demonstrated that the light-gated *APX2* expression enhances ROS-scavenging capacity, and exogenous application of ascorbate is sufficient to mimic the light priming of thermotolerance. In conjunction with the direct role of the HSFA1 transcription factor in inducing *APX2* transcription, it is evident that the HSFA1-mediated temperature signals and the phyB-mediated light signals converge at APX2, constituting a light priming mechanism of ROS detoxification for the induction of thermotolerance.

Results

Development of thermotolerance is diurnally regulated in Arabidopsis

In plants, many temperature responses, such as cold and freezing tolerance, heat tolerance and thermomorphogenesis, are rhythmic across the day/night cycle (Lee and Thomashow 2012, Dickinson et al. 2018, Park and Park 2018). These rhythmic behaviors enable plants to anticipate upcoming temperature changes and prepare for timely responses to temperature environments (Zhu et al. 2016). Owing to the wide-ranging effects of global warming on ecological vegetation and crop agriculture, plant thermotolerance is emerging as a key issue in the field in recent decades. It is known that phytochromes, which otherwise act as photoreceptors, sense ambient temperatures through the photochemical conversion step, and its interacting partner PIF4 plays a central role in thermal adaptation (Jung et al. 2016). These observations, together with the well-known cross-talk between light and temperature signals (Park et al. 2017), suggest that development of thermotolerance would be rhythmic in parallel to rhythmic light responses (Dickinson et al. 2018).

To investigate systemically the potential rhythmicity of induction of thermotolerance during the day, Columbia (Col-0) seedlings were exposed to heat (45°C) for 45 min at different Zeitgeber time (ZT) points for up to 48 h under either long days (LDs; 16 h light and 8 h dark) or constant light (**Fig. 1A**). Heat treatments were performed in complete darkness to remove any indirect effects of light illumination during heat exposure. It was found that development of thermotolerance, as analyzed by measurements of survival rates and Chl contents, was rhythmic under LDs, reaching a peak at noon (ZT8 and ZT32) and a trough at dawn (ZT0, ZT24 and ZT48) (**Fig. 1B–D**). This observation is consistent with the diurnal rhythmicity of thermotolerance under short days (SDs) in a previous report (Dickinson et al. 2018). It was notable that Col-0 seedlings were most sensitive to heat at the end of the dark period, whereas they were thermoresistant during the light period, implying that light would be important for thermotolerance.

In plants, daily rhythmicity is generally derived from either light effects or circadian effects (Schaffer et al. 2001). To examine whether the rhythmicity of thermotolerance is associated with circadian rhythms, Col-0 seedlings entrained under LDs were subjected to heat treatments under constant light (**Fig. 1E**). It was found that the daily rhythms of survival rate and Chl contents disappeared under constant light (**Fig. 1F–H**). Instead, seedlings exhibited relatively high resistance to heat in the light, indicating that development of thermotolerance is not circadian gated but rather diurnally regulated.

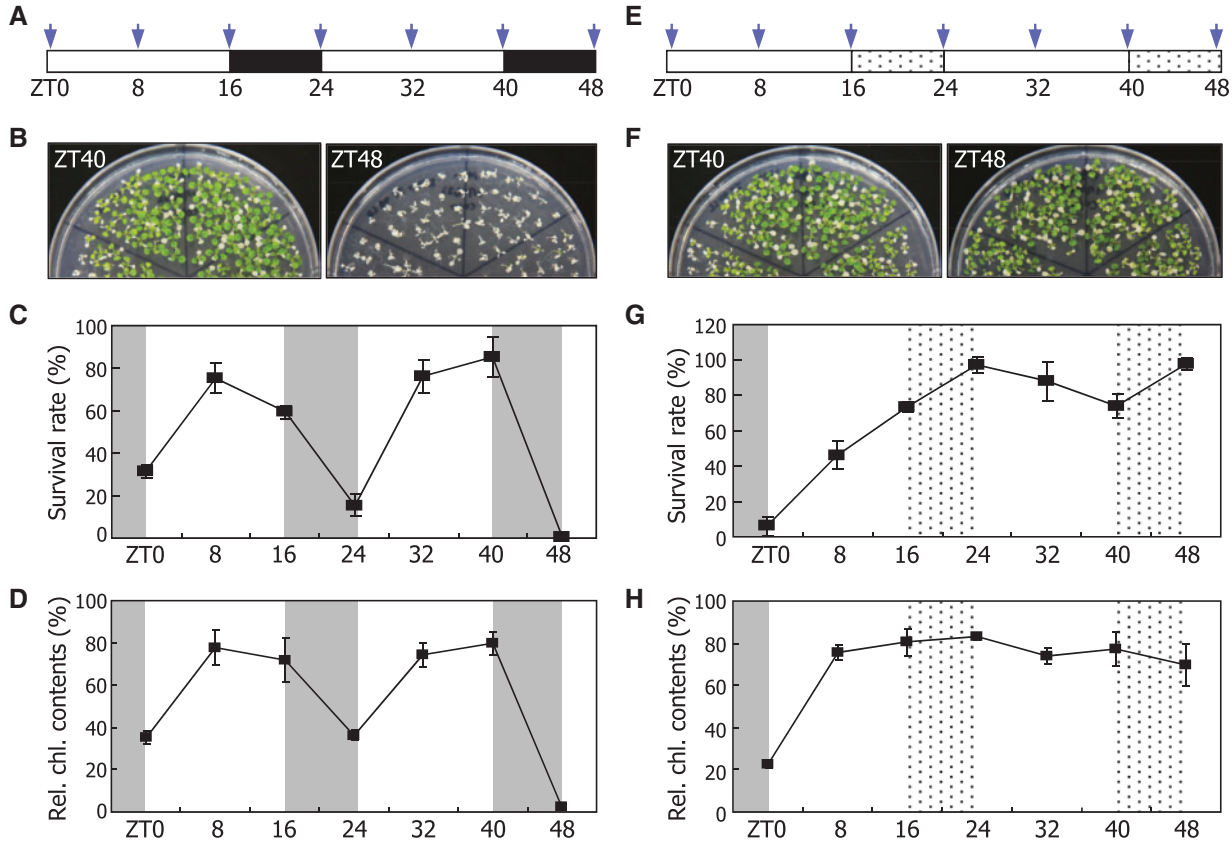

Fig. 1 Development of thermotolerance is diurnally regulated in Arabidopsis. Seven-day-old Col-0 seedlings grown under LDs were transferred to either LDs or constant light conditions at 23°C, during which seedlings were subjected to heat treatment (45°C/45 min in darkness) at the indicated ZT points for up to 48 h. The heat-treated seedlings were allowed to recover under constant light at 23°C for 5 d. Biological triplicates, each consisting of approximately 20 seedlings, were statistically analyzed in each assay. Bars indicate the SEM. (A–D) Devlopment of thermotolerance under LDs. Scheme of heat treatments (A), thermotolerance phenotypes (B), survival rates (C) and relative Chl contents (D) were analyzed. In (A), arrows mark the ZT points of heat treatments. (E–H) Development of thermotolerance under constant light. Scheme of heat treatments (E), thermotolerance phenotypes (F), survival rates (G) and relative Chl contents (H) were analyzed as described above.

It was also likely that light is required for the induction of thermotolerance.

Light priming is essential for development of thermotolerance

We found that the diurnal rhythms of development of thermotolerance were evident, with a peak at noon and a trough at dawn under LDs. Under constant light, thermotolerance was strongly induced at all ZT points tested. These observations suggest that light is required for the induction of thermotolerance. Since heat treatments were carried out in darkness, we ruled out the possibility of direct effects of light on the induction of thermotolerance. The remaining possibility was that light priming prior to heat exposure would affect the induction of thermotolerance.

We first carried out dark transfer experiments, in which seedlings were incubated for different times in darkness before heat treatment at ZT8 (**Fig. 2A**). It was found that the survival rate and Chl contents of seedlings were significantly reduced in seedlings that were incubated in darkness for longer than 2 h (**Fig. 2B, C**). On the other hand, the light transfer experiment, in which seedlings pre-incubated for different times in the light were exposed to heat treatment, revealed that light pre-incubation for longer than 2 h is sufficient for the enhancement of thermotolerance (**Fig. 2D–F**). Together, these data indicate that light pre-incubation for a certain period of time prior to heat treatment is essential for development of thermotolerance.

To confirm the requirement for light pre-incubation for thermotolerance, we examined the expression of temperature-responsive genes in seedlings following dark transfer treatments. It is known that ROS rapidly accumulate in heat stress-exposed plants, and production of antioxidants and ROS-scavenging enzymes underlies the induction of thermotolerance (Lee et al. 2015). For example, the *APX2* gene encoding ascorbate peroxidase is quickly induced after heat exposure, and the production of APX2 enzyme is a prerequisite for development of thermotolerance (Panchuk et al. 2002). Gene expression analysis by reverse transcription-mediated quantitative real-time PCR (RT–qPCR) showed that the thermal induction of the *APX2* gene was most prominent in light-pre-incubated seedlings but its induction was abruptly reduced in seedlings that are exposed to darkness for longer than 2 h prior to heat treatment (**Fig. 2G**). Genes encoding HSP70 and HSP101, representative molecular

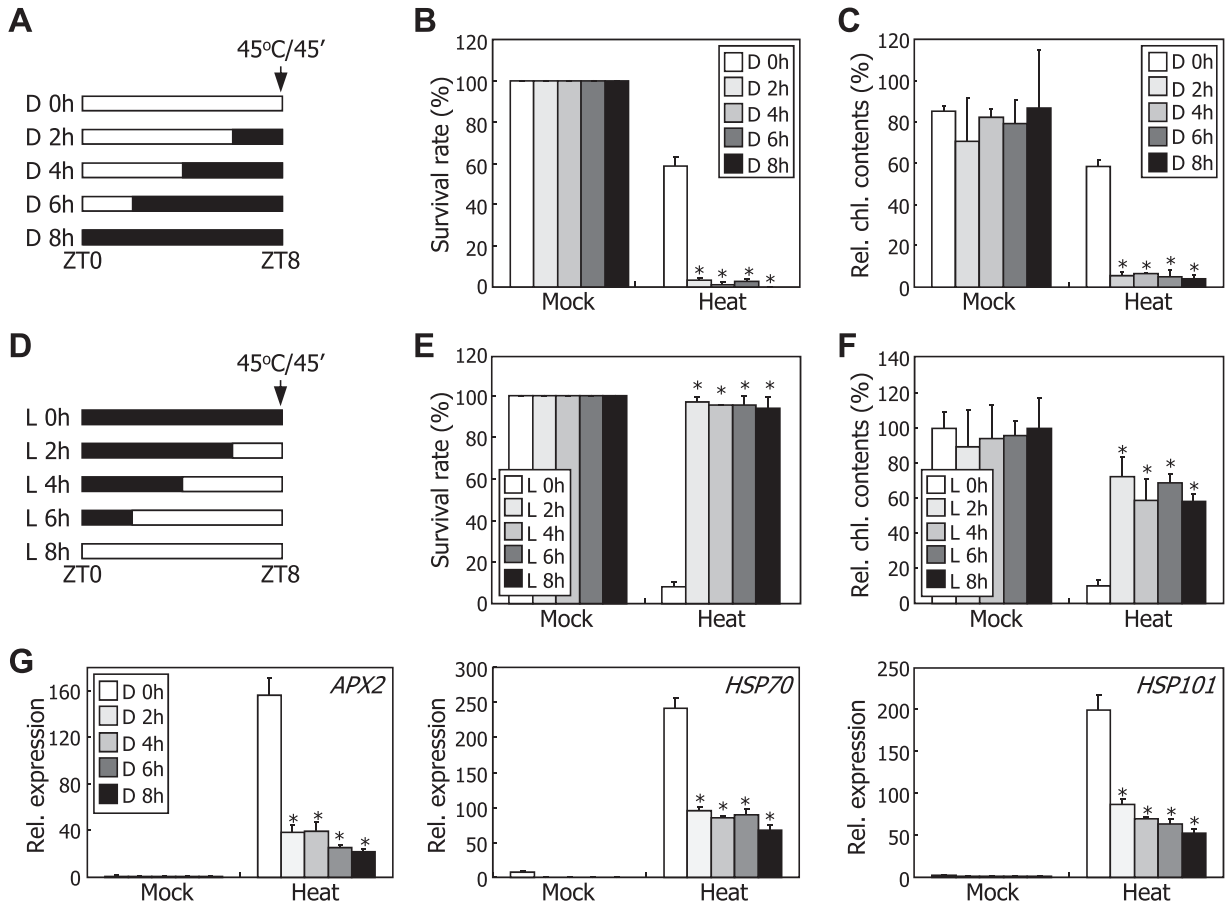

Fig. 2 Light illumination prior to heat treatment is essential for development of thermotolerance. Seven-day-old Col-0 seedlings grown under LDs at 23°C were then incubated in either light or darkness for up to 8 h prior to heat treatment. The heat-treated seedlings were allowed to recover under constant light at 23°C for 5 d. Biological triplicates, each consisting of approximately 20 seedlings, were statistically analyzed using Student's *t*-test (*$P < 0.01$, difference from 0 h). Bars indicate the SEM. L and D, light and dark, respectively. (A–C) Development of thermotolerance in dark-pre-treated seedlings. Scheme of heat treatments (A), thermotolerance phenotypes (B) and relative Chl contents (C) were assayed. (D–F) Development of thermotolerance in light-pre-treated seedlings. Scheme of heat treatments (D), thermotolerance phenotypes (E) and relative Chl contents (F) were assayed as described above. (G) Effects of different light–dark combinations on heat-responsive gene expression. RT–qPCR was employed for gene expression analysis. Biological triplicates were statistically analyzed (*t*-test, *$P < 0.01$).

chaperones functioning during development of thermotolerance (Queitsch et al. 2000, Su and Li 2008), were significantly induced in the light-pre-incubated seedlings but their induction was largely attenuated in dark-pre-incubated seedlings (Fig. 2G). Taken together, these observations further support that light exposure prior to heat treatment is necessary for enhancement of thermotolerance.

Light priming triggers ROS detoxification

Since ROS are rapidly produced under heat stress conditions, causing thermobleaching of Chls and eventual cell death (You and Chan 2015), it is anticipated that ROS-scavenging systems should be promptly activated in heat-stressed plants (Lee et al. 2015). It has been reported that light signaling is tightly associated with ROS metabolism in developing seedlings (Zhong et al. 2009). In addition, we observed that the thermal induction of *APX2* gene expression is gated by light (Fig. 2G), raising the possibility that light would prime the ROS detoxification in the course of development of thermotolerance.

APX enzymes catalyze the conversion of hydrogen peroxide to water using ascorbate (Caverzan et al. 2012). It is thus well known that exogenous application of ascorbate reduces ROS accumulation in heat-stressed plants, thus enhancing thermotolerance (Lee et al. 2015). On the basis of the previous observations, we hypothesized that APX-mediated ROS detoxification would be important for the light-gated establishment of thermotolerance. To examine this hypothesis, we analyzed the thermotolerance phenotypes of Col-0 seedlings in the presence of ascorbate. Light-pre-incubated seedlings were resistant to heat regardless of ascorbate application (Fig. 3A, B). In contrast, while dark-exposed seedlings were susceptible to heat, their thermotolerance was enormously enhanced in the presence of ascorbate, supporting that light-gated ROS detoxification via APX2 underlies development of thermotolerance.

Next, we directly monitored endogenous ROS contents in seedlings that were similarly treated with light and ascorbate. ROS facilitate the conversion of non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to fluorescent

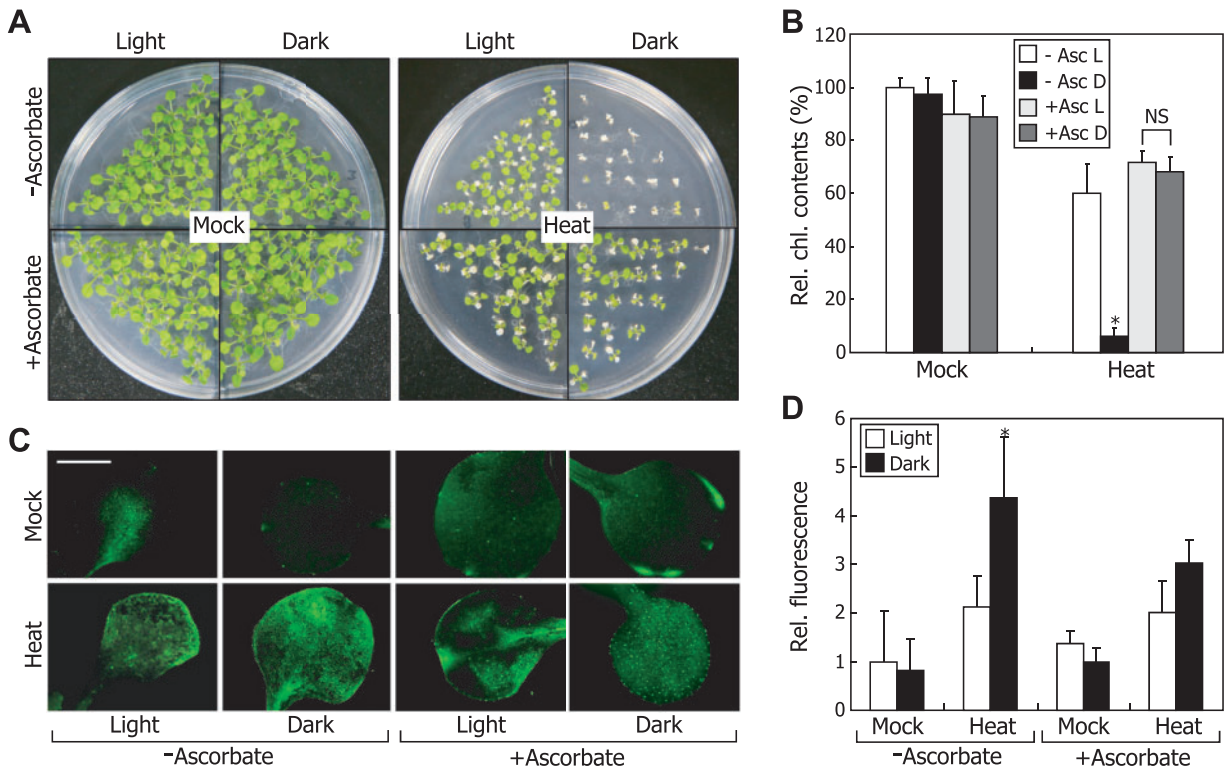

Fig. 3 Light priming enhances ROS detoxification during development of thermotolerance. Seven-day-old Col-0 seedlings grown under LDs at 23°C were further incubated in either light or darkness for 2 h. At ZT8, seedlings were subjected to heat treatment at 45°C for 45 min. Biological triplicates, each consisting of approximately 20 seedlings, were statistically analyzed (*t*-test, $*P < 0.01$). Bars indicate the SEM. (A, B) Effects of ascorbate (Asc) on development of thermotolerance. Seedlings grown in either the presence or absence of 0.5 mM Asc for 7 d were heat treated. Thermotolerance phenotypes (A) and relative Chl contents (B) were assayed. NS, non-significant. (C, D) Effects of light priming on ROS accumulation. Seedlings were treated with H_2DCFDA for fluorescent detection of ROS (C). Relative fluorescence intensity was quantitated (D). Scale bar = 600 mm.

2′,7′-dichlorofluorescein (DCF), and thus relative fluorescence intensities reflect relative contents of endogenous ROS (Zhong et al. 2009). H_2DCFDA-mediated fluorescent staining assays revealed that ROS accumulated to a higher level in dark-exposed seedlings than in light-exposed seedlings at high temperatures (**Fig. 3C, D**). Notably, exogenous application of ascorbate reduced ROS contents in dark-exposed seedlings to a level comparable with that in light-exposed seedlings (**Fig. 3C, D**), which is consistent with the effects of ascorbate on the induction of thermotolerance (**Fig. 3A, B**).

We also analyzed the effects of light priming on the thermal elevation of APX enzyme activity. It was found that the thermo-elevated APX enzyme activity was more prominent in light-pre-incubated seedlings than in dark-pre-incubated seedlings (Supplementary Fig. S1). Together, these observations indicate that light priming of the APX2-mediated ROS detoxification is intimately associated with the induction of thermotolerance.

Light primes HSF-mediated ROS detoxification

Our data indicate that light priming is required for the thermal induction of *APX2* gene expression. Thus, a question was how the light priming event is related to upstream factors that regulate the thermal induction of the *APX2* gene. It has been previously shown that HSF transcription factors directly bind to the specific *cis*-element in the promoters of heat-responsive genes, which is often termed the heat shock element (HSE) (Guo et al. 2008). The *APX2* gene promoter contains a copy of the HSE, and HSFA1b is shown to bind to the HSE sequence (Panchuk et al. 2002). There are four members of HSFA1 transcription factors, HSFA1-a, -b, -d and -e, in the Arabidopsis genome, and they function redundantly during thermotolerance responses (Liu et al. 2011).

We therefore employed the quadruple *hsfa1-a, -b, -d* and *-e* knockout mutant (*QK*) (Liu et al. 2011) in order to investigate the roles of HSFA1 transcription factors in light-gated ROS detoxification and development of thermotolerance. Thermotolerance assays revealed that light priming of development of thermotolerance was compromised in the light-exposed *QK* seedlings (**Fig. 4A, B**). Accordingly, light-gated induction of *APX2* gene expression was also impaired in the *QK* seedlings (**Fig. 4C**), supporting the critical roles of HSFA1 transcription factors in mediating the light-primed induction of development of thermotolerance.

We next asked whether the HSFA1-mediated regulation of *APX2* gene expression is involved in the light-enhanced ROS detoxification. H_2DCFDA staining assays revealed that ROS accumulated to similar levels in both light-exposed and dark-exposed *QK* seedlings, unlike the efficient light priming of ROS

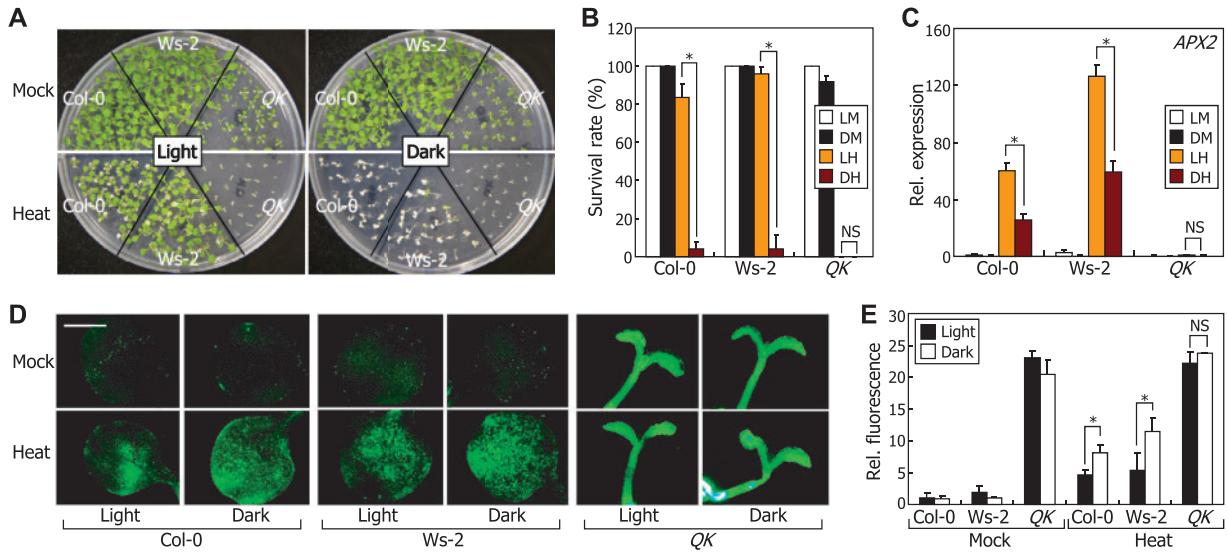

Fig. 4 Light-gated ROS detoxification requires HSFs during development of thermotolerance. Seven-day-old seedlings grown under LDs at 23°C were further incubated in either light or darkness for 2 h. At ZT8, seedlings were subjected to heat treatment. Biological triplicates, each consisting of appromiately 50 seedlings, were statistically analyzed (*t*-test, $^*P < 0.01$, difference from light). Bars indicate the SEM. The quadruple *hsfa1* mutant (*QK*) was included in the assays. (A) Thermotolerance of Col-0, Ws-2 and *QK* seedlings under either light or dark conditions. (B) Survival rate. (C) Transcript levels of the *APX2* gene. Whole seedlings were used for total RNA extraction. (D) H_2DCFDA staining. Scale bar = 600 mm. (E) Relative fluorescence intensity. NS, non-significant.

detoxification in light-exposed wild-type seedlings (**Fig. 4D, E**). It is notable that ROS also accumulated to a relatively high level in *QK* seedlings even at 23°C, implying that HSFA1 transcription factors function in maintaining ROS homeostasis under normal growth conditions and their activity is rapidly elevated under heat stress conditions (Liu et al. 2011).

Photoreceptor-mediated light signaling primes development of thermotolerance

Plant photoreceptors sense distinct ranges of light wavelengths (Lau and Deng 2012). To explore specific light wavelengths and associated photoreceptors that mediate the light priming of induction of thermotolerance, we performed thermotolerance assays under different light wavelengths. Seedlings were exposed to different light wavelengths for 2 h prior to heat treatment. It was found that red and blue light efficiently enhanced development of thermotolerance, as analyzed by measurements of the survival rate and Chl contents in heat-treated seedlings (**Fig. 5A, B**). Far-red light pre-incubation also enhanced thermotolerance, but the extent of enhancement was much weaker than that by red and blue light. These observations suggest that various photoreceptors would be involved in the light priming event of development of thermotolerance.

We found that the effectiveness of far-red light was relatively weaker compared with those of red and blue light in priming the onset of thermotolerance. The phyA photoreceptor is responsible for sensing far-red light, and the phyA-defective mutant exhibits skotomorphogenesis under far-red light conditions (Casal et al. 2014). To examine further the functional linkage between development of light-primed thermotolerance and far-red light, the light priming of *APX2* gene expression was assayed in *phyA* mutant seedlings that were pre-incubated under far-red light conditions. The result showed that thermal induction of *APX2* gene expression did not occur in either Col-0 or mutant seedlings (Supplementary Fig. S2A), indicating that far-red light and phyA do not play a primary role in the light-gated ROS detoxification. In contrast, blue light priming was able to induce *APX2* expression during development of thermotolerance, while it still occurred in the *cry1cry2* double mutant at high temperatures (Supplementary Fig. S2B). Together, these data support that phyA, CRY1 and CRY2 are not the primary photoreceptors that mediate the light priming of development of thermotolerance.

Our data on the priming of development of thermotolerance by different light wavelengths showed that red light is the most effective among the light wavelengths tested. Notably, thermotolerance assays revealed that red light-primed development of thermotolerance was greatly compromised in phyB-defective mutant seedlings (**Fig. 5C, D**). Accordingly, the light-gating effects of red light on the thermal induction of *APX2* gene expression were significantly impaired in the mutant seedlings (**Fig. 5E**), unlike *phyA* and *cry1 cry2* mutant seedlings.

There are eight *APX* genes in the Arabidopsis genome (Panchuk et al. 2005). Gene expression analysis showed that the expression of the *APX2* gene was elevated by 50-fold at high temperatures (**Fig. 5E**). The transcript levels of *APX1*, *APX3*, *APX4* and *APX6* genes were also induced slightly under identical conditions (Supplementary Fig. S3). In addition, gene expression assays revealed that genes encoding GPX and CAT enzymes detoxifying ROS were not significantly influenced by heat treatments and light priming pre-treatments (Supplementary Fig. S4). Together, it is evident that among the known ROS-detoxifying enzymes, APX2 plays a primary role in the light priming of thermotolerance.

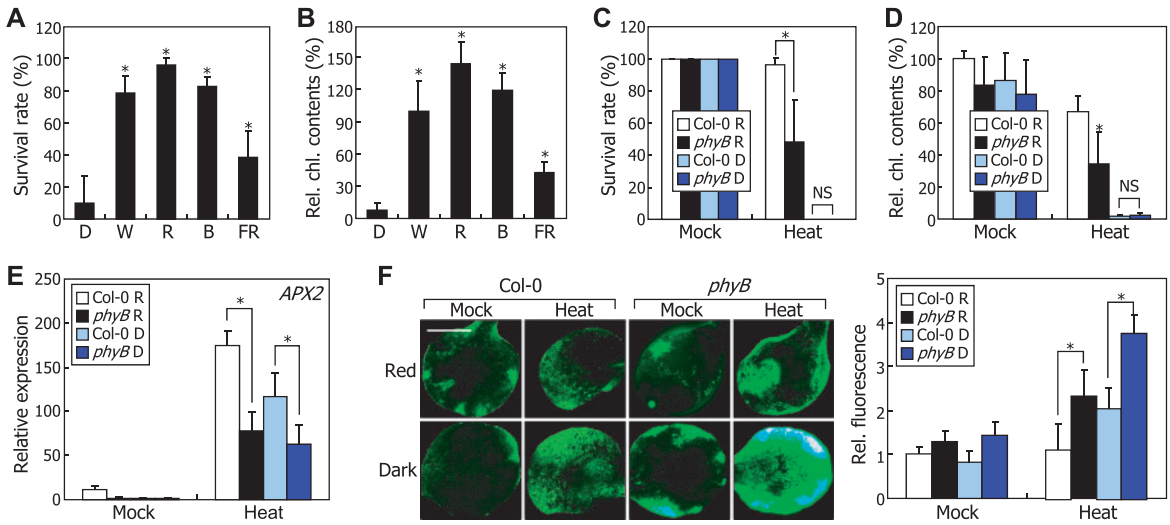

Fig. 5 Different light wavelengths prime development of thermotolerance. Seven-day-old seedlings grown under LDs at 23°C were further incubated in different light regimes at ZT6 for 2 h. Seedlings were then subjected to heat treatment. The heat-treated seedlings were allowed to recover under constant light at 23°C for 5 d. Biological triplicates, each consisting of approximately15 seedlings, were averaged and statistically analyzed (*t*-test, $*P < 0.01$). Bars indicate the SEM. (A, B) Thermotolerance of Col-0 seedlings under different light wavelengths. Survival rates (A) and relative Chl contents (B) were analyzed. D, dark; W, white light; R, red; B, blue; FR, far-red. (C, D) Thermotolerance of *phyB* mutant seedlings. Survival rates (C) and relative Chl contents (D) were analyzed. NS, non-significant. (E) Levels of *APX2* transcripts in heat-treated seedlings. Transcript levels were analyzed by RT–qPCR. (F) ROS accumulation in heat-treated seedlings. After heat treatment, seedlings were subjected to H_2DCFDA staining (left panel). Relative fluorescence intensity was measured (right panel). Scale bar = 600 mm.

We next examined ROS accumulation in red light-pre-incubated *phyB* mutant seedlings at high temperatures. H_2DCFDA-mediated fluorescent staining revealed that ROS levels were higher in the red light-primed *phyB* mutant seedlings than in Col-0 seedlings at high temperatures (**Fig. 5F**). Collectively, these observations indicate that the light priming of APX2-mediated ROS detoxification and development of thermotolerance is mediated primarily by phyB, although roles of other photoreceptors, such as phyD and phyE, could not be excluded.

The phyB photoreceptor exerts its role in governing the red light-responsive gene transcription through interactions with PIF transcription factors (Xu et al. 2015), raising the question of whether PIFs are involved in the light priming event of development of thermotolerance. It was found that red light-gated thermotolerance was still observed in the quadruple *pifq* mutant (Supplementary Fig. S5A, B), which harbors quadruple mutations in *PIF1*, *PIF3*, *PIF4* and *PIF5* genes (Zhang et al. 2013). In addition, thermal induction patterns of *APX2* gene expression were not discernibly different between Col-0 and the *pifq* mutant seedlings at high temperatures (Supplementary Fig. S5C). Thus, it is evident that PIF-mediated light signaling is not directly involved in the light priming of thermotolerance.

It has been recently reported that retrograde chloroplast signals gate the thermal induction of *HSP* gene expression (Dickinson et al. 2018). We observed that light primes the thermal induction of at least *HSP70* and *HSP101* genes during development of thermotolerance (**Fig. 2G**). We therefore asked whether phyB-mediated light priming of development of thermotolerance is linked with chloroplast-mediated gating of thermotolerance. Gene expression studies revealed that light-gated induction of *HSP* gene expression at high temperatures still occurred in *phyB* mutant seedlings (Supplementary Fig. S6A), suggesting that the signaling pathway governing the phyB-mediated light priming of thermotolerance is functionally separated from that directing the chloroplast-mediated gating of thermotolerance.

We next asked whether chloroplast signals are involved in the light priming of *APX2* gene expression at high temperatures. It is known that DCMU triggers the photosynthetic electron flow from PSII to plastoquinone, leading to accumulation of the oxidized plastoquinone pool, whereas 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) triggers the electron flow from plastoquinone to the Cyt b_6f complex, resulting in accumulation of the reduced plastoquinone pool (Sherameti et al. 2002). It has been previously shown that while the thermal induction of *HSP70* expression is increased by DBMIB treatment, it is suppressed by DCMU treatment (Dickinson et al. 2018). Our chemical treatment assays revealed that the thermal induction of *APX2* gene expression is increased by both DCMU and DBMIB (Supplementary Fig. S6B). It has been reported that H_2O_2 activates the HSFA1 transcription factors (Dickinson et al. 2018), which directly target the *APX2* gene, suggesting that the DBMIB-induced ROS production leads to the HSFA1-mediated induction of the *APX2* gene. These observations indicate that the phyB-gated thermal acceleration of ROS detoxification is distinct from the retrograde chloroplast-mediated gating of *HSP* gene induction.

PhyB-mediated priming of ROS detoxification leads to induction of thermotolerance

Our data show that the red light photoreceptor phyB mediates the light priming of ROS detoxification and thus

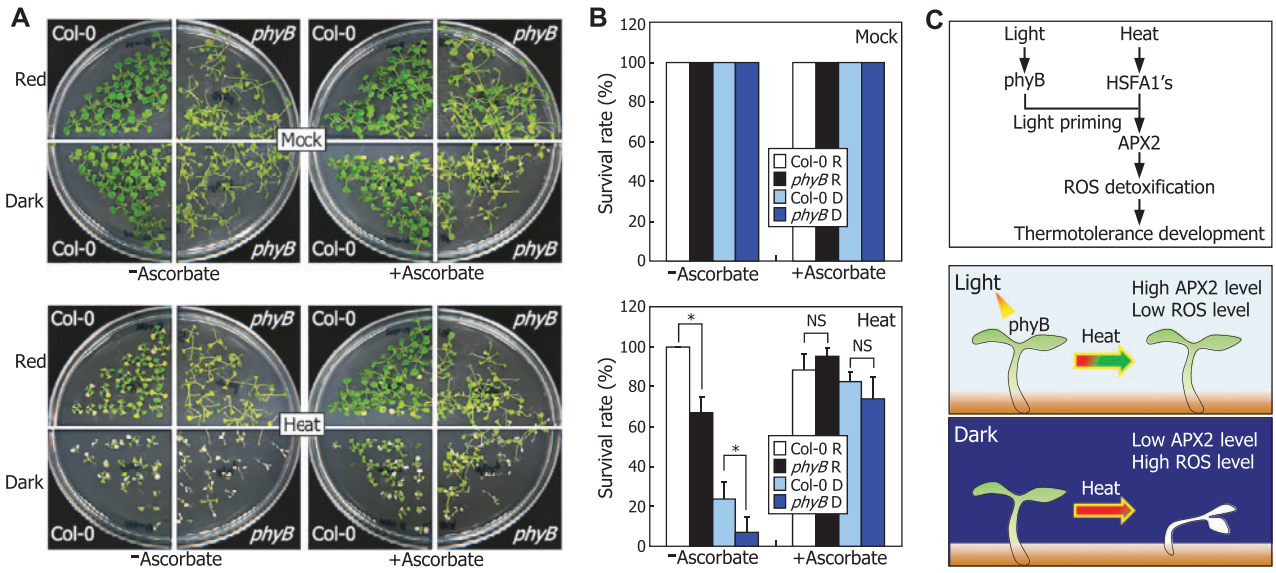

Fig. 6 PhyB-mediated light signals prime ROS detoxification during development of thermotolerance. Seven-day-old seedlings grown under LDs at 23°C in the presence or absence of 0.5 mM ascorbate (Asc) for 7 d were exposed to either red light or darkness for 2 h. At ZT8, the seedlings were subjected to heat treatment. Biological triplicates, each consisting of 42 seedlings, were analyzed statistically (*t*-test, $^*P < 0.01$, difference from Col-0). (A, B) Effects of Asc on the induction of thermotolerance in *phyB* mutant seedlings. Heat-treated seedlings were allowed to recover at 23°C under constant light for 5 d (A). Survival rates were analyzed (B). Bars indicate the SEM. (C) Working scheme for the light priming of development of thermotolerance. Red light-activated phyB primes the thermoinduced *APX2* expression, resulting in a reduction of ROS accumulation in light-grown seedlings.

development of thermotolerance at high temperatures (**Fig. 5F**). Thus, we finally asked whether the thermosensitive phenotype of the *phyB* mutant is associated with disruption of ROS-scavenging capability in the mutant. To answer this question, we examined thermotolerance phenotypes of the mutant in the presence of ascorbate. The thermosusceptible phenotype of the *phyB* mutant was efficiently recovered in the presence of ascorbate, as verified by measurements of survival rates (**Fig. 6A, B**). This observation confirms that phyB-gated activation of APX enzymes is functionally linked with light priming of development of thermotolerance. Meanwhile, thermotolerance assays revealed that the effects of light priming was still evident in the presence of the GPX substrate glutathione (GSH) (Supplementary Fig. S7). Taken together, these observations indicate that phyB-mediated priming of APX function leads to ROS detoxification, thereby enhancing thermotolerance.

Altogether, our data illustrate that phyB-mediated light signaling, and perhaps that mediated by other photoreceptors as well, primes the development of thermotolerance via APX2-mediated ROS detoxification (**Fig. 6C**). The phyB-mediated light priming depends on the HSFA1 transcription factors that directly regulate the expression of the *APX2* gene, showing that the phyB-mediated light signals and the HSFA1-mediated temperature signals converge through the APX2-mediated ROS detoxification process. In nature, plants often experience high temperature conditions during the light period (Park et al. 2017). Therefore, the phyB-mediated light priming of development of thermotolerance would provide an adaptation strategy that ensures plant survival under heat stress conditions.

Discussion

Signaling cross-talk between light and temperature cues during thermotolerance

Complicated functional relationships between light and temperature signaling events have been described extensively in plant environmental adaptation responses (Lee and Thomashow 2012, Dickinson et al. 2018, Park and Park 2018). One typical example is cold acclimation, in which cold activation of C-repeat-binding factors (CBFs) plays central roles (Lee and Thomashow 2012). It has been reported that plants grown under SDs exhibit enhanced freezing tolerance compared with those grown under LDs, and *CBF* gene expression is markedly elevated under SDs (Lee and Thomashow 2012). This acclimation mechanism enables plants to prepare for forthcoming cold temperatures under SDs, which prevail in the autumn. Warm temperature responses are also linked to light signaling. Phytochromes, which are otherwise well-characterized red/far-red light photoreceptors, have been recently reported to function as thermosensors (Jung et al. 2016, Legris et al. 2016). Furthermore, circadian rhythms help thermomorphogenic responses to occur at specific time periods during the day (Zhu et al. 2016, Park et al. 2017). It is believed that these molecular and physiological mechanisms as a whole enable plants to enhance plant adaptation capability to high temperatures.

In this work, we showed that light primes the ROS detoxification reaction during the acquisition of thermotolerance capacity at high temperatures, providing an additional example of light–temperature signaling cross-talk. Our data are also

supported by the recent report that describes the gating of development of thermotolerance by chloroplast-derived signals (Dickinson et al. 2018). Sunlight is the ultimate source of energy on the Earth, and temperature is considered as a typical type of energy. In this sense, it is easily predictable that average temperature is relatively higher when the photoperiod is longer (Park et al. 2017). It is therefore natural that plants have evolved a light priming system, with which they are able to prepare for possible heat stress in the near future. This interpretation is also consistent with the previous view that light conditions enable plants to anticipate upcoming temperature fluctuations to prepare for appropriate temperature responses that would be required for optimal plant growth and performance (Griebel and Zeier 2008, Lee and Thomashow 2012). We believe that the light–temperature signaling cross-talk uncovered in this work would provide an excellent example for understanding the evolutionary relationship between light and temperature responses in plants.

Photoreceptor-mediated light signaling primes development of thermotolerance

We found that the red light photoreceptor phyB mediates the light-gated thermotolerance response. The APX2 enzyme catalyzes the conversion of hydrogen peroxide to water, thus reducing ROS accumulation at high temperatures (Panchuk et al. 2002). The thermal induction of *APX2* gene expression was impaired in the *phyB* mutant. It has been recently reported that chloroplast signals also gate thermotolerance (Dickinson et al. 2018). Thus, a question was whether the phyB-mediated priming of thermotolerance is related with the chloroplast-mediated gating of thermomorphogenesis.

HSPs act as molecular chaperones during development of thermotolerance (Queitsch et al. 2000). Light activates photoreceptors and enhances chloroplast signaling (Lee et al. 2017). In addition, common signaling mechanisms have been identified between photoreceptors and chloroplast retrograde signaling (Martín et al. 2016). Furthermore, treatment with chemicals that affect the redox status of the plastoquinone pool modulated the *APX2* expression in a pattern that is distinct from the mode of *HSP* gene expression in response to the chemicals during chloroplast-derived signaling. Therefore, it is postulated that light primes development of thermotolerance by at least two distinct signaling pathways: phyB-gated ROS detoxification and chloroplast-gated protein quality control.

Various priming mechanisms have been described widely in various environmental adaptation processes, and their fundamental scheme has been considered as a plant memory process (Mauch-Mani et al. 2017). Epigenetic regulation is tightly linked with diverse memory processes in plants (Iwasaki and Paszkowski 2014). In particular, hypermethylation events, such as H3K4me2 and H3K4me3, are known to be associated with transcriptional memory during development of thermotolerance (Lämke et al. 2016). In addition, the distribution patterns of H3K9me2 and H3K27me3 in the promoter of the *FLOWERING LOCUS C* (*FLC*) gene are responsive to long-term exposure to low temperatures, simply termed vernalization (Kim and Sung 2013). It is possible that epigenetic regulation might also underlie the phyB-mediated priming of ROS detoxification and development of thermotolerance.

There are several reports supporting that phyB-mediated signaling is involved in epigenetic regulation. Red light illumination promotes seed germination by suppressing the expression of a gene encoding the zinc-finger transcription factor *SOMNUS* (*SOM*) via the phyB–PIF1 module (Kim et al. 2008). Notably, the Jumonji C-containing histone arginine demethylases have been identified as the epigenetic regulators working downstream of SOM to promote gibberellic acid biosynthesis (Cho et al. 2012). Studies on natural genetic variations in Arabidopsis have also shown that phyB and histone deacetylase 6 are involved in light-controlled chromatin compaction events (Tessadori et al. 2009). It will be interesting to examine whether these epigenetic regulators also mediate the phyB-gated *APX2* gene expression at high temperatures.

Light-gated ROS detoxification and development of thermotolerance

ROS function as critical signaling mediators in various cellular pathways and in adjusting redox status in plant cells (Mubarakshina et al. 2010, Noctor and Foyer 2016). However, overaccumulation of ROS often causes oxidative damage on biological molecules and cellular structures, and Chl bleaching (Zhong et al. 2009), indicating that endogenous levels of ROS should be tightly regulated. It is known that high temperatures trigger ROS production (Panchuk et al. 2002). Accordingly, thermal adaptation mechanisms in plants possess efficient ROS-scavenging systems to cope with the harmful chemicals (Lee et al. 2015).

Our findings describe that light primes development of thermotolerance by activating the APX2-mediated ROS detoxification reaction. Conversely, exogenous application of the ROS scavenger ascorbate mimics the light gating effects on the induction of thermotolerance. Genes encoding ascorbate peroxidase enzymes are widely conserved in virtually all higher plant species (Caverzan et al. 2012). Considering the critical role of APX2 enzymes in triggering the thermotolerance response, it would be possible to apply the light-gated ROS detoxification mechanism to enhance sustainable growth in crop plants under heat stress conditions. It will also be interesting to examine whether the phyB-mediated light priming mechanism would also mediate plant adaptation to global warming, which is emerging as a worldwide ecological issue in recent decades.

Materials and Methods

Plant materials and growth

All *Arabidopsis thaliana* lines used were in the Col-0 background, except for the quadruple *hsfa1* mutant (*QK*) (Liu et al. 2011), which was generated from both the Col-0 and Ws-2 background. The *QK* mutant was kindly provided by Dr. Yee-Yung Charng. The *phyA-211*, *phyB-9*, *cry1cry2* and the quadruple *pifq* mutants have been described previously (Wang et al. 2010, Zhang et al. 2013). The *pifq* mutant was obtained from the Arabidopsis Biological Resource Center.

Sterilized Arabidopsis seeds were cold-imbibed at 4°C for 3 d in complete darkness to synchronize germination. Plants were grown on Murashige and Skoog (MS)-agar plates under LDs (16 h light and 8 h dark) with white light illumination (150 μmol m^{-2} s^{-1}), which was provided by fluorescent FLR40D/A tubes (Osram), in a controlled culture room set at 22°C with 60% humidity.

Thermotolerance assay

Seven-day-old seedlings grown on MS-agar plates under LDs were exposed to 45°C for 45 min at ZT8 in darkness and allowed to recover at 23°C for 5 d under continuous light. For light quality assay, 7-day-old seedlings were exposed to specific wavelength ranges of light for 2 h before heat treatment, as described above. For chemical treatment, seedlings were grown on MS-agar containing 0.5 mM ascorbate (Sigma) or 1 mM GSH (Sigma). For the examination of the effect of chloroplast-gated light signaling on the induction of thermotolerance, either 30 μM DCMU (Sigma) or 50 μM DBMIB (Sigma) was exogenously applied to 7-day-old seedlings for 2 h before heat treatment (Sherameti et al. 2002).

For the determination of the effects of light transfer and dark transfer on the induction of thermotolerance, 7-day-old seedlings were pre-incubated for different lengths of either light periods or dark periods before heat treatments (45°C, 45 min) at ZT8, respectively. The presence of newly developing leaves after 5 d of recovery following heat treatment was considered as the sign of survival.

Measurement of Chl contents

Heat-treated seedlings were allowed to recover under constant light at 23°C for 5 d. Seedlings were then harvested for the extraction of Chls with 100% methanol and were incubated at 4°C for 2 h in complete darkness. Chl contents were analyzed by measuring absorbance at 650 and 660 nm using a Mithras LB940 multimode microplate reader (Berthold Technologies). The contents of Chl *a* and Chl *b* were calculated from the absorbance values at 650 and 660 nm, as described previously (Lee et al. 2015). Total Chl contents indicate the sum of Chl *a* and Chl *b*. Relative Chl contents were calculated by dividing total Chl contents of heat-treated seedlings by those of seedlings treated under control conditions.

Light quality assay

For light quality assays, 7-day-old seedlings were exposed to white, red, blue or far-red light for 2 h. The *phyB* mutant, the *cry1 cry2* mutant or the *phyA* mutant was included as the photoreceptor mutant in the assays using red light, blue light or far-red light illumination, respectively. The *pifq* mutant was also included in the red light assays. At ZT8, seedlings were exposed to 45°C for 45 min in darkness. Total RNA samples were extracted from whole seedlings, and ROS quantification was performed immediately after heat treatment.

Gene expression analysis

Transcript levels were analyzed by RT–qPCR. All RT–qPCRs were performed following the principles that have been proposed to guarantee reproducible and accurate measurements of transcript levels (Udvardi et al. 2008). Total RNAs were extracted from whole seedlings, unless otherwise specified. RT–qPCRs were conducted in 384-well blocks with an QuantStudio 6 Flex (Life Technologies) using the SYBR Green I master mix in a volume of 10 μl. The two-step thermal cycling profile employed was 15 s at 95°C for denaturation and 1 min at 60–65°C for annealing and polymerization. An *eIF4A* gene (At3g13920) was included as internal control in each PCR to standardize the differences in the amounts of cDNA samples used (Supplementary Table S1).

To ensure the reproducibility of RT–qPCRs, they were performed in biological triplicate using total RNA samples prepared separately from three independent plant materials that were grown under identical conditions. The comparative $\Delta\Delta C_T$ method was employed to evaluate relative quantities of each amplified product in the samples. The threshold cycle (C_T) was automatically determined for each reaction by the system set with default parameters.

Fluorescence microscopy

The cell-permeant H_2DCFDA (Sigma) was used to stain ROS in plant tissues. Plant samples were incubated in 10 μM H_2DCFDA solution for 1 h at 23°C in complete darkness. It is known that the non-fluorescent H_2DCFDA is converted to the fluorescent DCF upon chemical reactions with ROS (Zhong et al. 2009). A fluorescence microscope (Olympus) equipped with the GFP (green fluorescent protein) filter was used to image DCF fluorescence in whole seedling. A ×5 objective lens was used, and fluorescence exposure time was set to 30 ms. The Image J public domain was used to quantify fluorescence intensity. Relative fluorescence quantification was determined using the whole leaf.

Measurement of APX enzyme activity

Seven-day-old seedlings grown at 23°C under LDs were pre-incubated in the light or in darkness for 2 h before heat treatments. Whole seedlings were used for the assays. APX enzyme activity was measured as described previously (Verma and Dubey 2003). The 2 ml reaction mixture contained 500 μl of 0.2 mM ascorbic acid, 100 μl of 0.2 mM EDTA, 300 μl of 6% H_2O_2 and 100 μl of leaf extract in 100 mM pH 7.0 potassium phosphate buffer. Leaf extract was added to the mixture, and the decrease in absorbance was recorded at 290 nm using a UV-Vis spectrophotometer at 15 s intervals for up to 3 min. The relative enzyme activity of light-primed seedlings under mock conditions was set to 1.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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