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Research article

Characterization of phytochrome C functions in the control of de-etiolation and agronomic traits in rice

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ABSTRACT

Although phytochrome A (phyA) and phyB have been functionally characterized, functions of phyC in rice growth and development have remained elusive because of the functional dependency of phyC on the phyB protein. In this study, we introduced *PHYB(C364A)*, in which the chromophore attachment site cysteine 364 was converted to alanine, into the *phyAphyB* double mutant (*aabb*) and the *phyAphyBphyC* triple mutant (*aabbcc*) to produce *PHYB(C364A)/aabb* lines and *PHYB(C364A)/aabbcc* lines, respectively. *PHYB(C364A)/aabbcc* lines were insensitive to red light (R) and far-red light (FR), suggesting that PHYB(C364A) protein was biologically inactive. Functions of phyC were characterized using the *PHYB(C364A)/aabb* lines, without the functional interference of phyA or phyB. Phytochrome C responded to R and FR to trigger de-etiolation in the very-low-fluence response and low-fluence response in the *PHYB(C364A)/aabb* lines. Compared with the *aabb* mutant, seedlings of *PHYB(C364A)/aabb* lines showed higher chlorophyll content and reduced leaf angle. The *PHYB (C364A)/aabb* lines also showed a delayed heading date under long-day conditions. Phytochrome C-regulated agronomic traits were measured at the mature stage. The *PHYB(C364A)/aabb* lines showed significantly increased plant height, panicle length, grain number per main panicle, seed-setting rate, grain size, and grain weight, compared with those of the *aabb* mutant. Taken together, the present findings confirm that phyC perceives R and FR, and plays an important role in photomorphogenesis and yield determination in rice.

1. Introduction

Light is among the most important ambient signals for plants. A plant monitors the external light conditions, including the presence or absence of light as well as the duration, wavelength, and intensity of incident light, and makes light-specific adjustments in physiological and developmental processes to adapt to the changing environment (Noriyuki and Masamitsu, 2007; Bae and Choi, 2008). To accomplish this vital task, plants have evolved multiple photoreceptor systems, including phytochromes (phy), cryptochromes, phototropin, ZTL/FKF1/LKP2 proteins, and UVR8 (Lin and Shalitin, 2003; Christie, 2007; Kim et al., 2007; Sawa et al., 2007; Bae and Choi, 2008; Rizzini et al., 2011). Phytochromes in higher plants are encoded by small gene families and mainly perceive and respond to red (R) and far-red (FR) wavelengths (Clack et al., 1994; Mathews and Sharrock, 1997; Takano et al., 2005, 2009; Bae and Choi, 2008). The complete genome sequences of *Arabidopsis* and rice have revealed that *Arabidopsis* contains five PHY genes, *PHYA* to *PHYE* (Sharrock and Quail, 1989; Clack et al., 1994), whereas the rice genome contains only three genes, *PHYA*, *PHYB*, and *PHYC* (Kay et al., 1989; Dehesh et al., 1991; Basu et al., 2000).

The generation and characterization of rice phytochrome single, double, and triple mutants have revealed that individual members of the rice phytochrome family function in the control of responses to R and FR in terms of de-etiolation and flowering processes (Takano et al., 2001, 2005, 2009; Osugi et al., 2011). Phytochrome-mediated responses can be classified into three different types, namely the low-fluence response (LFR), the very-low-fluence response (VLFR), and the FR-high-irradiance response, in accordance with their energy requirements (Briggs et al., 1984). Several reports have shown that phyA is the principal photoreceptor involved in VLFRs, such as photo-inhibition of coleoptile growth and *light-harvesting chlorophyll a/b-binding protein*

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(*Lhcb*) gene expression (Takano et al., 2001, 2005; Xie et al., 2007). In contrast, phyB-specific photo-inhibition of coleoptile growth requires the photon fluence of LFR (Xie et al., 2007).

Phytochromes A and B perceive continuous red light (Rc) to control de-etiolation responses in a highly redundant manner in rice (Takano et al., 2005). Under continuous far-red (FRc), the *phyA* mutant exhibited partially impaired de-etiolation, and the *phyAphyC* double mutant did not exhibit significant light responses, indicating that phyC perceives FR to trigger light responses in rice (Takano et al., 2005). However, the responses to FR were completely abolished in *phyAphyB* double mutants (hereafter *aabb*), indicating that phyB affects the photosensing of FR by phyC. Under Rc, the *phyC* single mutant showed de-etiolation responses identical to those of the wild type (WT); seedlings of the *aabb* mutant are blind to R (Takano et al., 2005). Moreover, *phyB* single and *phyBphyC* double mutant (hereafter *bbcc*) showed similar sensitivity to R. In this context, phyC is not indicated to be involved in R-triggered light responses. However, additional studies have shown that rice phyC is unstable and functionally inactive unless it forms heterodimers with phyB (Xie et al., 2014).

Phytochrome molecules function as a dimer in two stable photo-interconvertible forms, Pr and Pfr (Rockwell et al., 2006). Red light induces a Pr-to-Pfr conformational shift that promotes translocation of Pfr into the nucleus. Phytochromes in the nucleus, interact with other proteins to trigger light-responsive gene expression (Chen et al., 2004; Chen and Chory, 2011). Phytochrome C predominantly exists as phyB/phyC heterodimers and much less as monomers in rice plant (Xie et al., 2014), and no phyA/phyC heterodimer has been observed (X. Xie, unpublished). The same phenomena are observed in *Arabidopsis* (Sharrock and Clack, 2004). However, *Arabidopsis* phyC is not able to form heterodimers with other type II phytochromes, including phytochromes D and E, nor with type I phyA in *Arabidopsis* (Sharrock and Clack, 2004). In both rice and *Arabidopsis*, homodimer forms of phyC have not been observed (Sharrock and Clack, 2004; Xie et al., 2014). To the best of our knowledge, only in wheat has a phyC homodimer been reported (Chen et al., 2014). Although the reason that phyC exists in the phyB/phyC heterodimer is unknown, the phyB/phyC heterodimer is indispensable for maintenance of phyC protein levels and functions in both *Arabidopsis* and rice (Sharrock and Clack. 2004; Monte et al., 2003; Clack et al., 2009; Xie et al., 2014). Thus, phyC functions are poorly characterized due to the functional dependency of phyC on phyB protein in rice. In *Arabidopsis*, *PHYC* alleles are under diversifying selection and play important roles in mediating natural variation in flowering and growth responses (Balasubramanian et al., 2006). In addition, *PHYC* plays an important role in determining heading time under inductive long-day (LD) photoperiods in barley (Nishida et al., 2013) and wheat (Chen et al., 2014). Thus, it is invaluable to characterize the function of phyC in rice growth and development.

In addition to the dependency of phyC on phyB, the redundancy of the photosensory abilities of phyC to R and FR, in relation to those of phyA and phyB, renders use of a phyC-null single mutant insufficient to characterize the functions of phyC. In a previous report, the mutated gene *PHYB(C364A)*, where the chromophore attachment site cysteine (C) 364 was converted to alanine (A), was introduced into the *aabb* mutant and the homozygous transgenic line *PHYB(C364A)/aabb* was generated (Xie et al., 2014). In that study, the phyC protein was restored to the level of the WT and showed a direct physical interaction with PHYB(C364A) in *PHYB(C364A)/aabb* lines. Moreover, *PHYB (C364A)/aabb* exhibited de-etiolation responses to both FR and R, whereas the *aabb* mutant was unresponsive to both FR and R. In the present study, we introduced *PHYB(C364A)* into the rice phytochrome null mutant, *phyAphyBphyC* triple mutant (hereafter *aabbcc*), and confirmed that PHYB(C364A) protein is biologically inactive in *PHYB (C364A)/aabbcc* lines. Thus, phyC is the only functional photoreceptor in *PHYB(C364A)/aabb* lines. Using *PHYB(C364A)/aabb* lines, we defined the photosensory specificities of phyC and characterized the functions of phyC in regulating rice de-etiolation and agronomic traits.

2. Materials and methods

2.1. Plant materials

Plant materials used in this study were as follows: WT, *Oryza sativa* L. cv. Nipponbare; *bbcc* mutant, *phyB-1phyC-1*; *aabb* mutant, *phyA-4 phyB-1*; *aabbcc* mutant, *phyA-4phyB-1phyC-1* (Takano et al., 2005, 2009). To generate *PHYB(C364A)/aabb* transgenic lines, the mutant *PHYB(C364A)*, in which the codon (TGC) encoding the chromophore-binding site (cysteine) was substituted with GCC (encoding alanine), was inserted between the constitutive *Cauliflower mosaic virus* 35 S promoter and the Nos terminator of the pPZP2Ha3 vector (Fuse et al., 2001) in the sense orientation. Then *PHYB(C364A)* was introduced into the *aabb* mutant by *Agrobacterium tumefaciens*-mediated transformation method as described previously (Xie et al., 2014). Three independent *PHYB(C364A)/aabb* lines (#27, #69, and #72) were used in this study. The background of these mutants and transgenic lines is Nipponbare.

2.2. Immunoblotting analysis

Soluble protein was extracted from above-ground parts of etiolated seedlings as described by Takano et al. (2005). Protein extracts were separated by SDS-PAGE and blotted on to polyvinylidene difluoride membranes (Immobilon-P, Millipore, MA, USA). The PHYB(C364A) protein was detected immunochemically using ECL chemiluminescence kits (GE Healthcare, Uppsala, Sweden). The PHYB antibody used was identical to that of a previous study (Takano et al., 2005).

2.3. Construction of PHYB(C364A)/aabbcc plants

To obtain *PHYB(C364A)/aabbcc*, *PHYB(C364A)/aabb* #27 or #69 was crossed with the *bbcc* mutant. The genotypes of the F_2 segregants were analyzed by PCR. The mutant alleles of *phyA-4* and *phyC-1* were detected in accordance with the method described by Takano et al. (2005). *PHYB (C364A)* was detected in accordance with the method described by Xie et al. (2014).

2.4. Measurement of coleoptile length

For continuous light treatments, sterilized seeds of the WT, *aabbcc* mutant, and *PHYB(C364A)/aabbcc* lines (#27 and #69) were sown on 0.4% (w/v) agar, and seedlings were grown in the dark, under FRc, or under Rc at 28 °C for 9 d. Images of seedlings were captured using a digital camera, and coleoptile length was measured with a ruler.

For pulse light treatments, sterilized seeds of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27 and #69) were sown on 0.4% (w/v) agar and seedlings were grown in the dark. Seedlings were exposed to a pulse of either R with a fluence rate of 15 μmol $m^{-2} s^{-1}$ for 2 min or FR with a fluence rate of 15 μmol $m^{-2} s^{-1}$ for 10 min at 80 h after induction of germination. After light treatment, the seedlings were maintained in the dark at 28 °C until day 9 when coleoptile length was measured with a ruler.

2.5. Reverse transcription-quantitative PCR analysis of Lhcb genes

Seedlings of the WT, *aabb* mutant and *PHYB(C364A)/aabb* lines (#27 and #69) were grown in darkness for 7 d, then irradiated with a pulse of either R with a fluence rate of 15 μmol $m^{-2} s^{-1}$ for 2 min or FR with a fluence rate of 15 μmol $m^{-2} s^{-1}$ for 10 min. After light treatment, the seedlings were kept in darkness for an additional 4 h and subsequently harvested for RNA extraction from shoots (above-ground parts). Reverse transcription–quantitative PCR (qRT-PCR) was used to examine the expression levels of two *Lhcb* genes, *Os03g0592500* and *Os09g0346500*, in accordance with the method described by Xie et al. (2014).

2.6. Measurement of leaf angle

Sterilized seeds of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69, and #72) were sown on 0.4% (w/v) agar and then grown under continuous white light (Wc) at 28 °C for 9 d. Images of the seedlings were captured, and the angle between the blade and sheath of the second leaf was measured from the images.

2.7. Measurement of chlorophyll content

After being dehusked and surface-sterilized, seeds of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69 and #72) were sown on 0.4% agar and incubated overnight at 4 °C. The seedlings were grown under Wc or Rc at 28 °C for 9 d. The above-ground parts were used for measurement of the chlorophyll content based on the methods described by Zhang (1985) with a slight modification. Fresh tissue (0.2 g) was cut into pieces in a mortar with a small amount of quartz sand and calcium carbonate powder, and then ground well in 10 mL of 95% ethanol. After filtration into a brown volumetric flask, the extracts were brought to a total volume of 25 mL with 95% ethanol. Absorbance values were measured at the wavelengths of 665 nm and 649 nm, recorded as A665 and A649, respectively. The concentrations (mg/L) of chlorophyll *a* (*Ca*) and chlorophyll *b* (*Cb*) were calculated as follows: $Ca = 13.95 \times A665–6.88 \times A649$; $Cb = 24.96 \times A649–7.32 \times A665$. The content of chlorophyll (mg/g) was calculated as $(Ca + Cb) \times$ the volume of extracts/sample fresh weight.

2.8. Measurement of heading date and agronomic traits

Seeds of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69 and #72) were sown on May 10, 2016, and the seedlings were transplanted with a distance of 15 cm × 25 cm on June 14, into paddy fields in Jinan (36°84′N, 117°12′E), Shandong province with routine field management. The cultivation period was typical of the growing season for rice in Jinan, Shandong province with sunrise at about 05:00–05:30 and sunset at about 18:30–19:00. Thus, daylengths during the cultivation period exceeded 13 h and the natural daylength was considered to be equivalent to LD conditions in this study. The heading date was monitored for the appearance of the first panicle.

Agronomic traits, consisting of plant height, internode length, panicle length, number of grains, number of filled grains, grain length, grain width, grain thickness, and 1000-grain weight, were measured when the seeds were harvested. For each line, approximately 20 individual plants were used for statistical analyses.

2.9. Light sources and light intensities

Monochromatic light sources and W in this study were identical to those described by Takano et al. (2005). The fluence rates were 15 μmol $m^{-2} s^{-1}$ for R and FR, and 50 μmol $m^{-2} s^{-1}$ for W.

2.10. Data analysis

All results are expressed as mean values ± standard error (SE) based on either three biological replicates or more than 20 seedlings. Statistical significance was assessed using Student's *t*-test. Probability values of less than 0.05 were considered to be statistically significant. A single asterisk (*) and double asterisks (**) represent significance at the levels of 0.05 and 0.01, respectively.

3. Results

3.1. PHYB(C364A) is biologically inactive

Given that the chromophore attachment site of the PHYB(C364A) derivative was alanine, instead of cysteine in the intact PHYB protein, the PHYB(C364A) protein is expected to be inactive, as observed for the PHYB(C357S) derivative in *Arabidopsis* (Wagner et al., 1996). To confirm this expectation, we produced *PHYB(C364A)/aabbcc* plants by crossing *PHYB(C364A)/aabb* and the *bbcc* mutant and screening the F_2 segregants. Immunoblot analyses showed that the expression level of PHYB(C364A) in the *PHYB(C364A)/aabbcc* lines was comparable with that of the WT in the etiolated seedlings (Fig. 1A). To examine the biological activities of the PHYB(C364A) protein in the *aabbcc* triple mutant, phenotypes of *PHYB(C364A)/aabbcc* seedlings grown under FRc or Rc were analyzed. When grown in darkness, the coleoptile length showed no significant differences among the genotypes tested (Fig. 1B and C). Under either Rc or FRc, coleoptile elongation of the WT was distinctly inhibited (0.54 ± 0.02 cm and 0.64 ± 0.03 cm under Rc and FRc, respectively) compared with the coleoptile length of 4.08 ± 0.13 cm for etiolated seedlings. In contrast, *PHYB(C364A)/aabbcc* and *aabbcc* mutants grown under Rc and FRc exhibited coleoptiles similar in length to those of etiolated seedlings (Fig. 1B and C). These results suggested that *PHYB(C364A)/aabbcc* were insensitive to both FR and R despite the presence of the PHYB(C364A) protein. Therefore, PHYB(C364A) is biologically inactive. It is reasonable to characterize the true function of phyC in rice growth and development without the functional interference of phyA or phyB in *PHYB(C364A)/aabb* lines.

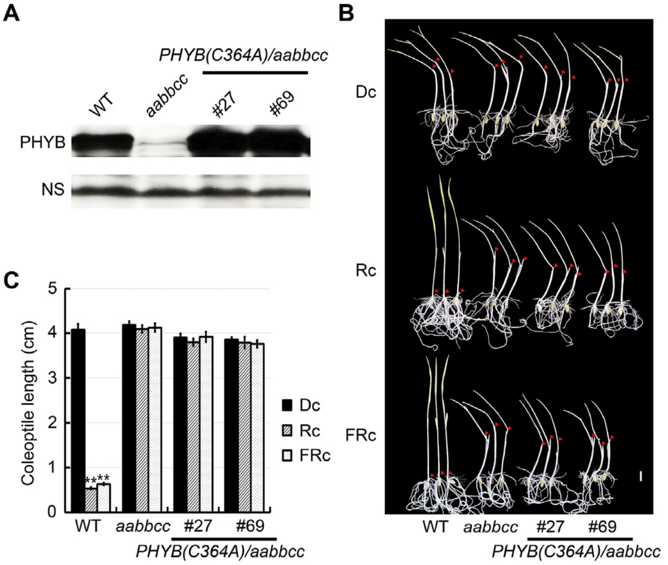

Fig. 1. PHYB(C364A) is biologically inactive in the *aabbcc* background. (A) Immunoblot analyses of PHYB in the wild type (WT), *aabbcc* mutant, and *PHYB (C364A)/aabbcc* transgenic lines using anti-PHYB antibodies. Protein extracts (50 μg) from etiolated seedlings were loaded to detect PHYB and PHYB (C364A). NS, non-specific band in the same blot. (B) Visual phenotypes of WT, *aabbcc* mutant, and *PHYB(C364A)/aabbcc* seedlings (#27 and #69) grown under continuous darkness (Dc), red light (Rc) or far-red light (FRc) for 9 days. Red arrow heads indicate apices of coleoptiles in the seedlings. Bar = 10 mm. (C) *PHYB(C364A)/aabbcc* were insensitive to Rc and FRc irradiation. Mean coleoptile lengths are shown for WT, *aabbcc*, and *PHYB(C364A)/aabbcc* seedlings (#27 and #69) grown in the dark (filled bars), under Rc (15 μmol $m^{-2} s^{-1}$, hatched bars) or FRc (15 μmol $m^{-2} s^{-1}$, dotted bars) for 9 days. The mean ± SE (standard error) for at least 20 seedlings is plotted. $^{**}P < 0.01$ compared with etiolated seedlings using Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Rice phyC is involved in LFR and VLFR

In a previous study, rice phyC was shown to be involved in the responses to Rc and FRc in the presence of phyB (Xie et al., 2014), suggesting that the photosensory specificity of phyC is similar to that of

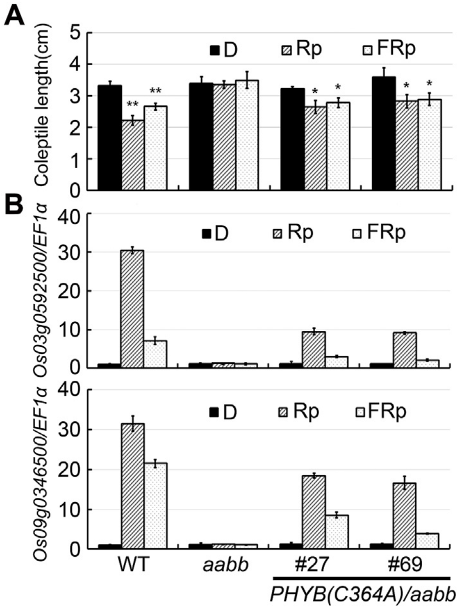

Fig. 2. Effects of a pulse of red light (Rp) or far-red light (FRp) on coleoptile growth and expression level of *Lhcb* genes. (A). Coleoptile length in the wild type (WT), *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27 and #69). Seedlings grown in the dark for 80 h were exposed to either Rp or FRp, then transferred back to darkness until day 9 for measurement of coleoptile length. Filled bars, coleoptile lengths on day 9 in the absence of light treatment; hatched and dotted bars, final coleoptile length on day 9 after exposure to a pulse of R or FR, respectively. The means ± SE for 30 seedlings are shown. *$P < 0.05$ and **$P < 0.01$ compared with etiolated seedlings of each material using Student's *t*-test. (B). Transcript levels of *Lhcb* genes in the WT, *aabb*, and *PHYB(C364A)/aabb* lines. Seedlings grown in the dark for 7 days were exposed to either Rp or FRp and then kept in darkness for an additional 4 h. The shoots were then harvested for analysis of transcript levels of two *Lhcb* genes (*Os03g0592500* and *Os09g034650*) by qRT-PCR.

phyA in rice. Rice phyA is reported to be involved in the VLFR and LFR, such as photo-inhibition of coleoptile elongation and *Lhcb* gene expression (Takano et al., 2005; Xie et al., 2007). Therefore, we measured coleoptile length and *Lhcb* gene expression in the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines under various light conditions. The fluence of R pulse (Rp) used in this study (1.8 mmol photons m^{-2}) could induce both the VLFR and LFR. The fluence of FR pulse (FRp) (9 mmol photons m^{-2}) was adjusted to induce a robust VLFR, as described by Takano et al. (2005). For analysis of coleoptile length, light treatments were applied at 80 h after induction of seed germination. After the light treatment, the seedlings were maintained in the dark at 28 °C until day 9 when the coleoptile length was recorded. When seedlings were exposed to either Rp or FRp, coleoptile length was significantly inhibited in the WT (2.2 ± 0.2 cm for Rp and 2.7 ± 0.1 cm for FRp) and in the *PHYB(C364A)/aabb* lines #27 (2.7 ± 0.2 cm for Rp and 2.8 ± 0.2 cm for FRp) and #69 (2.8 ± 0.2 cm for Rp and 2.9 ± 0.2 cm for FRp on average), compared with that of dark-grown seedlings (3.3 ± 0.1 cm, 3.2 ± 0.1 cm, and 3.6 ± 0.3 cm for the WT, #27, and #69 on average, respectively), although the inhibitory effects were weaker in *PHYB(C364A)/aabb* lines than in the WT (Fig. 2A). Seedlings of the *aabb* mutant grown under Rp and FRp showed a similar coleoptile length to those grown in the dark (Fig. 2A).

To examine expression of *Lhcb* gene, seedlings grown in the dark for 7 d were exposed to either Rp or FRp. After the light treatment, seedlings were kept in darkness for an additional 4 h and subsequently used for RNA extraction. Expression of *Lhcb* genes (*Os03g0592500* and *Os09g0346500*) was distinctly induced by FRp and Rp in *PHYB(C364A)/aabb* seedlings, but not in the *aabb* double mutant (Fig. 2B). These results suggested that, similar to phyA, rice phyC is also involved in the LFR and VLFR.

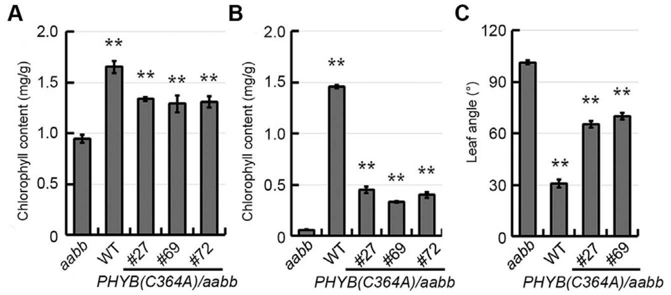

Fig. 3. Phytochrome C regulates chlorophyll content and leaf angle in *PHYB(C364A)/aabb* lines. (A) and (B) Chlorophyll content in seedlings of the wild type (WT), *aabb* mutant, and *PHYB(C364A)/aabb* lines. Seedlings were grown under continuous white light (Wc; 50 μmol m^{-2} s^{-1}) (A) or continuous red light (Rc; μmol m^{-2} s^{-1}) (B) for 10 days. The above-ground parts were used to measure chlorophyll content. The error bars represent the standard error. **$P < 0.01$ compared with the *aabb* mutant using Student's *t*-test. (C) Second-leaf blade declination in seedlings of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines. Seedlings were grown under Wc for 10 days at 28 °C. Declination angles were measured. The means ± SE for 20–30 seedlings are shown. **$P < 0.01$ compared with the *aabb* mutant using Student's *t*-test.

3.3. Rice phyC regulates chlorophyll content and leaf angle in rice seedlings

One de-etiolation response is the synthesis of chlorophyll. We compared chlorophyll contents between *PHYB(C364A)/aabb* and *aabb* seedlings grown under Wc and Rc. Chlorophyll contents were 1.34 ± 0.02 mg/g, 1.29 ± 0.08 mg/g, and 1.31 ± 0.05 mg/g on average in *PHYB(C364A)/aabb* lines #27, #69, and #72, respectively, which were notably higher than that of the *aabb* mutant (0.95 ± 0.04 mg/g on average) under Wc (Fig. 3A). A similar trend was observed in seedlings grown under Rc (Fig. 3B). These results suggested that phyC positively regulates chlorophyll synthesis during rice seedling growth. The WT seedlings contained much higher chlorophyll contents (1.65 ± 0.06 mg/g under Wc and 1.46 ± 0.02 mg/g under Rc on average) than those of *PHYB(C364A)/aabb* lines (Fig. 3A and B), which suggested that phyA and phyB are also involved in regulation of chlorophyll synthesis, consistent with a previous report (Zhao et al., 2013).

Phytochrome B is reported to participate in regulation of leaf angle in rice seedlings (Takano et al., 2005). In the present study, we measured the declination angles of the second-leaf blade in the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines grown under Wc. The leaf angle was about 101.3° in the *aabb* mutant, and introduction of *PHYB(C364A)* into the *aabb* mutant significantly reduced the leaf angle to 65.2° and 70.0° in *PHYB(C364A)/aabb* #27 and #69 lines, respectively (Fig. 3C). These results suggested that phyC is involved in regulation of the second-leaf declination under Wc. The WT seedlings showed smaller declination angles of the second-leaf blade (about 30.0°) than those of *PHYB(C364A)/aabb* lines #27 and #69. This result may be attributable to the positive function of phyB in the regulation of leaf angle as observed by Takano et al. (2005).

3.4. Rice phyC delays heading date and increases plant height

Plants of the *phyB* and *phyBphyC* mutants flower earlier than the WT under LD conditions (Takano et al., 2005). In the present study, to distinguish the roles of phyC from those of phyB in the regulation of floral initiation, we monitored heading date of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines in a paddy field under natural daylength in Jinan, Shandong, China. Given that daylengths during the cultivation period were more than 13 h, the natural daylength condition was considered to be equivalent to LD conditions. The *aabb* mutant flowered

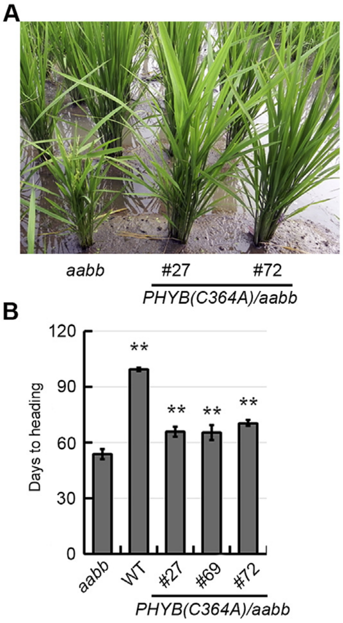

Fig. 4. Phytochrome C delays heading date in *PHYB(C364A)/aabb* lines. (A) Phenotypes of the *aabb* mutant and *PHYB(C364A)/aabb* lines (#27 and #72) of field-grown plants. (B) Heading date of the wild type (WT), *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69, and #72) grown in the paddy field under natural daylength in Jinan, Shandong, China. The means ± SE for 20 individual plants are displayed. $**P < 0.01$ compared with the *aabb* mutant using Student's *t*-test.

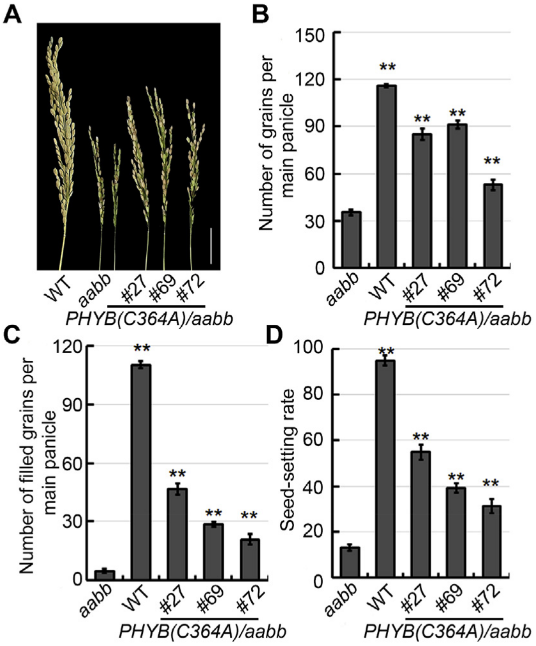

Fig. 6. Phytochrome C restores fertility in *PHYB(C364A)/aabb* lines. Plants of the wild type (WT), *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69, and #72) were cultivated in a paddy field under natural conditions in Jinan, Shandong, China. Panicle phenotypes were recorded by capturing images at the mature stage (A). Number of grains per main panicle (B), number of filled grains per main panicle (C), and seed-setting rate (D) were measured. The means ± SE for 20 individual plants are displayed. $**P < 0.01$ compared with the *aabb* mutant using Student's *t*-test.

after ~59 d, whereas plants of *PHYB(C364A)/aabb* lines flowered ~11 d later than the *aabb* mutant (Fig. 4A and B). These observations indicated that phyC is involved in the delay in heading date in response to LD conditions. The WT plants flowered after ~99 d, much later than the *PHYB(C364A)/aabb* lines (Fig. 4B). This result further suggested that phyA and/or phyB play an important role in determination of heading date under LD conditions as reported by Takano et al. (2005).

To evaluate quantitative differences in plant architecture, the plant height of the *aabb* mutant and *PHYB(C364A)/aabb* lines at the mature stage in field-grown plants was measured. The *PHYB(C364A)/aabb* lines showed significantly higher plant heights than the *aabb* mutant, as indicated by plant heights of 73.6 ± 0.8 cm, 70.0 ± 0.8 cm, and 75.7 ± 1.6 cm on average in *PHYB(C364A)/aabb* lines #27, #69, and #72, respectively, relative to that of 52.9 ± 1.3 cm in the *aabb* mutant (Fig. 5A). Panicle length was 17.2 ± 0.5 cm, 16.4 ± 0.4 cm, and 14.3 ± 0.4 cm on average for *PHYB(C364A)/aabb* lines #27, #69, and #72, respectively, which were much longer than that of the *aabb* mutant (11.9 ± 0.8 cm). The uppermost four internodes of *PHYB(C364A)/aabb* lines were longer than those of the *aabb* mutant (Fig. 5B). These results suggested that phyC positively increases plant elongation in rice. Of note, the plant height of the WT was 102.1 ± 2.2 cm, which was considerably higher than that of the *PHYB(C364A)/aabb* lines (Fig. 5A). The lengths of the panicle and the uppermost internode in the WT were distinctly higher than those of the *PHYB(C364A)/aabb* lines, whereas the lengths of the second to fourth internodes from the top of the plant in the WT were shorter than those of the *PHYB(C364A)/aabb* lines (Fig. 5B). These results suggested that other phytochromes (phyA and/or phyB) also act to control plant height and that individual phytochromes play diverse roles in regulating elongation of each internode.

3.5. Rice phyC positively regulates panicle architecture

The panicle architecture is a crucial agronomic trait that determines rice yield potential. We compared the main panicle features between the *aabb* mutant and *PHYB(C364A)/aabb* lines to analyze roles of phyC in regulating panicle architecture. The *PHYB(C364A)/aabb* lines showed an increased panicle length relative to that of the *aabb* mutant (Figs. 5B and 6A). The *aabb* mutant produced an average of 35.5 grains per main panicle, whereas *PHYB(C364A)/aabb* plants produced an average of 85.0, 69.2, and 52.9 grains per main panicle in lines #27, #69, and #72, respectively (Fig. 6B). The number of filled grains was

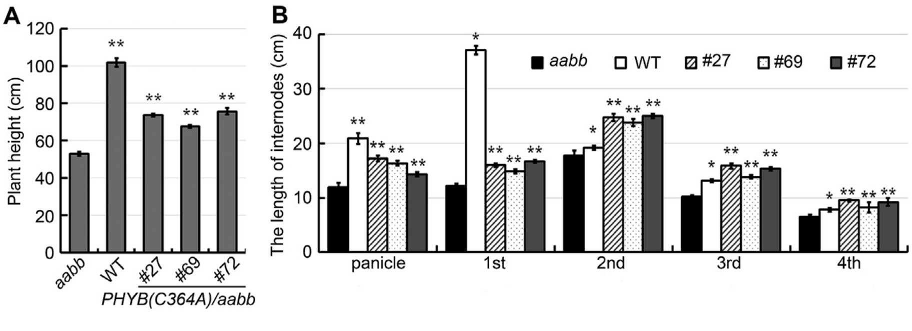

Fig. 5. Phytochrome C increases plant height in *PHYB(C364A)/aabb* lines. Plants of the wild type (WT), *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69, and #72) were cultivated in a paddy field under natural conditions in Jinan, Shandong, China. The plant height (A) and length of the panicle and the uppermost four internodes (B) were measured at the mature stage. Filled bars, *aabb* mutant; open bars, WT; hatched, dotted, and gray bars represent #27, #69, and #72, respectively. The means ± SE for 20 individual plants are displayed. $*P < 0.05$ and $**P < 0.01$ compared with the *aabb* mutant using Student's *t*-test.

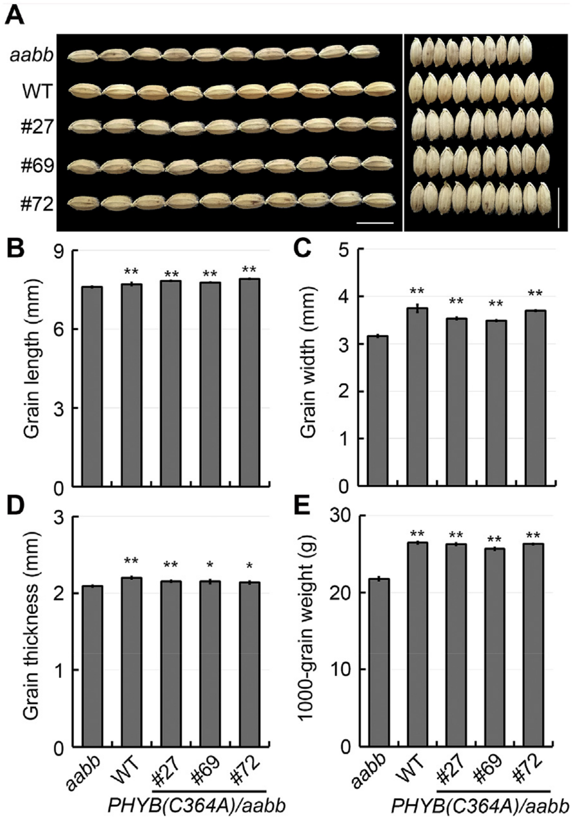

Fig. 7. Phytochrome C regulates grain size and weight in *PHYB(C364A)/aabb* lines. (A) Grain shape in the wild type (WT), *aabb* mutant, and *PHYB(C364A)/aabb* lines (#27, #69, and #72). Scale bars = 10 mm. (B–E) Grain length (B), grain width (C), grain thickness (D), and 1000-grain weight (E) were measured. The means ± SE for 20 individual plants are displayed. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the *aabb* mutant using Student's *t*-test.

lower in the *aabb* mutant (4.6 per main panicle) than those in the *PHYB(C364A)/aabb* lines (46.6, 37.8, and 20.8 in lines #27, #69, and #72) (Fig. 6C). As a result, the *PHYB(C364A)/aabb* lines showed higher seed-setting rates than the *aabb* mutant (Fig. 6D). Therefore, phyC positively influenced panicle development in rice. Although phyC acted to increase panicle traits, such as panicle length, grain number per panicle, and seed-setting rate in the *PHYB(C364A)/aabb* lines, the values of these panicle traits in the *PHYB(C364A)/aabb* lines were distinctly lower than those in the WT (Fig. 6). It is speculated that phyA and phyB are also involved in determination of rice panicle architecture.

We also quantified the grain size of the WT, *aabb* mutant, and *PHYB(C364A)/aabb* lines (Fig. 7A). Compared with those of the WT, the grain length, grain width, grain thickness, and 1000-grain weight of the *aabb* mutant were decreased by 2.4%, 15.8%, 5.0%, and 17.7%, respectively (Fig. 7B–E). The *PHYB(C364A)/aabb* lines showed significantly increased grain length, grain width, and grain thickness (Fig. 7A–D). As a result, compared with the *aabb* mutant, the 1000-grain weight was increased by 120.6%, 117.9%, and 120.8% in the *PHYB(C364A)/aabb* lines #27, #69, and #72, respectively, similar to the 1000-grain weight of the WT (Fig. 7E). These results indicated that phyC makes an important contribution to the control of grain size in rice.

4. Discussion

In this study, we confirmed that phyC is the only functional photoreceptor in *PHYB(C364A)/aabb* lines. Thus, it is reasonable to analyze the true photosensory specificities and roles of phyC in *PHYB(C364A)/aabb* lines. The present results reveal that the photosensory specificities of phyC are similar to those of phyA and that phyC plays important roles in regulating rice de-etiolation and agronomic traits.

4.1. Photosensory specificities of phyC in rice

In rice, phyA is reported to be involved in the responses to both R and FR, whereas phyB responds to R (Takano et al., 2001, 2005). In the current study, rice phyC perceived both R and FR to trigger de-etiolation responses (Fig. 2). Both Rp and FRp with the fluence to induce the VLFR and LFR triggered inhibition of coleoptile elongation and induction of *Lhcb* genes expression in *PHYB(C364A)/aabb* lines (Fig. 2), which suggested that rice phyC is involved in the LFR and VLFR. Rice phyA responds to R light in the VLFR and LFR (Takano et al., 2005). Thus, the photosensory specificities of phyC are similar to those of phyA, but differ from those of phyB in rice. By contrast, *Arabidopsis* phyC and wheat phyC do not participate in the control of seedling de-etiolation under FR, but are involved in the regulation of de-etiolation responses to R (Franklin et al., 2003; Monte et al., 2003; Chen et al., 2014).

4.2. Functions of phyC in regulation of agronomic traits

In a previous study, rice *phyB* and *phyBphyC* mutants exhibited similar phenotypes. For example, *phyB* and *phyBphyC* mutants similarly showed larger leaf declination angles and an earlier heading date under LD conditions compared with those of the WT (Takano et al., 2005). These results suggest that phyB and/or phyC are involved in the regulation of leaf angle and heading date. However, the function of phyB could not be distinguished from that of phyC owing to the dependence of phyC on phyB in the rice phytochrome mutants used. In the present study, we demonstrated that phyC functions not only in repression of leaf angle but also in floral initiation under non-inductive LD photoperiods (Figs. 3C and 4). The *PHYB(C364A)/aabb* lines showed a larger leaf angle and earlier heading date compared with those of the WT, suggesting that phyA and/or phyB play important roles in determination of leaf angle and heading date, consistent with the observations by Takano et al. (2005). Rice is a short-day (SD) plant. It has been reported that the *phyC* mutant flowers at the same time as the WT under SD conditions (Takano et al., 2005). In this context, phyC is indicated to have no effect on rice heading date under inductive photoperiods. However, wheat phyC promotes heading under inductive LD photoperiods (Chen et al., 2014). Thus, in a future study it is worth monitoring the heading dates of *PHYB(C364A)/aabb* lines grown under SD conditions to further confirm the role of rice phyC in the regulation of flowering time.

Similar to the *aabbcc* triple mutant, the *aabb* mutant showed a dwarf phenotype and reduced fertility at the mature stage in field-grown plants (Takano et al., 2009). Therefore, the function of phyC appeared to be negligible. In the present study, *PHYB(C364A)/aabb* lines showed increased plant height, panicle length, and internode length relative to those of the *aabb* mutant (Fig. 5), which suggested that phyC plays an important role in the control of plant growth. An additional distinct feature at the mature stage is that the *aabb* mutant produces small panicles and sets very few seeds (Fig. 6), similar to the observations of Takano et al. (2009). Compared with the *aabb* mutant, the panicle length, grain number per panicle, and seed-setting rate were significantly increased in *PHYB(C364A)/aabb* lines (Fig. 6). Therefore, phyC-mediated light responses positively regulated panicle architecture. Similarly, the wheat *phyC* null mutant shows altered spike development, reduced grain set, and elongated rachises, which suggests that wheat phyC regulates spike morphogenesis (Chen et al., 2014). Unexpectedly, introduction of *PHYB(C364A)* into the *aabb* mutant increased grain size and grain weight to the levels of the WT, which implied that phyC makes an important contribution to determination of rice grain development.

In the current study, for the first time phyC was shown to play important roles in rice de-etiolation responses and formation of agronomic traits. Although distinct and cooperative functions of rice phyA, phyB, and phyC in morphogenesis have been reported previously, limited information on the mechanisms underlying these functions is available. Asami et al. (2011) revealed that the phyA/phyA homodimer and phyB/phyC heterodimer can induce *Grain number, plant height and heading date 7* (*Ghd7*) expression. Phytochrome A influences flowering time mainly by affecting the expression of *GIGANTEA* under SD and *Ghd7* under LD in the absence of phyB (Lee et al., 2016). Additional studies by our research group contribute to understanding the underlying mechanism of phytochrome-mediated fertility and abiotic stresses. Analysis of transcript profiles revealed that altered carbohydrate metabolism is associated with the impaired fertility of stamens in the *aabb* mutant (Sun et al., 2017). Similarly, the rice *aabbcc* triple mutant shows higher daytime sugar contents compared with those of the WT (Jumtee et al., 2009). The molecular mechanisms of phyB-regulated drought and cold tolerance have been dissected (Liu et al., 2012; He et al., 2016). A recent study reports that *OsPIL15*, which encodes a phytochrome interacting factor-like protein, regulates grain size by directly targeting a purine permease gene, *OsPUP7*, in rice (Ji et al., 2019). Thus, it is possible that phytochrome-regulated grain size is mediated by the OsPIL15 pathway, although these authors did not investigate the relationship between phytochromes and OsPIL15. Therefore, it will be meaningful to explore the mechanism of phyC-regulated photomorphogenesis in future. In addition, rice phyC exists in phyB/phyC heterodimer and phyC monomer forms in the presence of phyB, but in a monomeric form in the absence of phyB (Xie et al., 2014). Considering together with evidence that the *aabb* double mutant showed the essentially same photomorphogenetic responses as the *aabbcc* triple mutant did (Takano et al., 2005, 2009), it can be concluded that monomeric phyC protein existing in the *aabb* mutant is biologically inactive. Then, one interesting question to be answered is what the biological significance for the monomeric phyC protein in rice is. By contrast, wheat phyC exists as both homodimers and monomers in the dark, but mainly homodimers in the light (Chen et al., 2014). Thus, an additional intriguing question to be answered is why rice phyC does not form homodimers.

Author contributions

X.X., H.Z., and C.Z. designed the experiments and analyzed the data. Y.L., C.Z., Z.Z., and J.Z. performed most experiments. X.X. wrote the manuscript. All authors read and commented on the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

Not applicable.

Declarations of interest

None.

Submission declaration

This work is original research that has not been published previously.

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