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Research Paper

Effect of Light Intensity and Wavelength on Anthocyanin Biosynthesis in Purple Rice Seedlings

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Abstract. Anthocyanin-rich purple rice is considered to be a particularly healthy natural food. To investigate anthocyanin biosynthesis in purple rice, two inbred lines (YDD1 and YDD2) were selected to undergo different light stress treatments. The accumulation of anthocyanin in purple rice leaves was mainly regulated by light intensity and wavelength. Compared to Nipponbare, the concentration of anthocyanin in purple rice was significantly induced under high light intensity (150 µmol m^{-2} s^{-1}) and blue light (470nm light wavelength). High light intensity and 470 light wavelength also induced the transcription of several genes, including the anthocyanin biosynthesis genes OsCHS, OsCHI, OsF3H, OsDFR, and OsANS. Two regulatory gene products, OsB1 and OsB2, were significantly and differentially expressed between YDD1 and YDD2, indicating a complex regulated pathway in purple rice.

Keywords: Anthocyanin, genes expression level, light intensity, light wavelength, purple rice.

INTRODUCTION

Rice is the primary staple food for more than half of the world's population. Compared to the widely consumed white rice, purple rice, which is the most frequently cultivated colored rice variety in Southeast Asia, has recently become popular because it is highly nutritious and has benefits for human health (Chen *et al.*, 2016; Wang *et al.*, 2007). The purple color present in rice is caused by the accumulation of anthocyanins in rice tissues (Reddy *et al.*, 1995). Accumulation of these anthocyanins in the pericarp gives distinctive purple grains, which can be exploited for consumption. These purple grains present with a distinctive color in the field, can be used as a morphological marker for selection and variety identification in breeding.

Rice anthocyanins also have many physiological functions against abiotic stresses (Kovinich *et al.*, 2015; Zhang *et al.*, 2014). For example, Chunthaburee *et al.* (2015) found that, under salinity stress of 150 mM NaCl, anthocyanin production was induced in rice seedlings, contributing to a greater antioxidant capacity than the control seedlings. In nature, anthocyanins are water-soluble pigments responsible for the red, purple and blue colors seen in plants (Zhang *et al.*, 2014). Their function in plants is to protect the photosynthetic apparatus and scavenge free radicals (Petroni and Tonelli, 2011; Gould, 2004). When consumed, anthocyanins can benefit human health by decreasing the risk of certain cancers, cardiovascular disease, diabetes, and other

chronic disorders (Sun *et al*., 2015; Zhu *et al.*, 2017).

The anthocyanin biosynthetic pathway has been well described, and most of the corresponding genes have been cloned (Hichri *et al.*, 2011; Zhang *et al.*, 2008; Shao *et al.*, 2013; Shih *et al.*, 2008). Anthocyanins are synthesized in three main phases: 1) the conversion of phenylalanine to p-coumaroyl CoA, which is mainly catalyzed by phenylalanine deaminase (PAL); 2) the flavonoid pathway phase, in which chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3' hydroxylase (F3H) catalyze the conversion of p-coumaroyl CoA to dihydroflavonol; and 3) the anthocyanin synthesis phase, in which dihydroflavonol reductase (DFR) and anthocyanin synthase (ANS) catalyze the conversion of dihydroflavonol to anthocyanins. Anthocyanin biosynthesis is regulated by several transcription factors, including those in the R/B gene cluster. In rice, *OsB1* and *OsB2* was found to change the color of rice leaves by regulating the structure of gene expression (Zhu *et al.*, 2017; Sakamoto *et al.*, 2001); *OsPAL* was highly expressed in rice stems and roots, and the expression levels of *OsCHS*, *OsF3H*, *OsDFR*, *OsANS* increased with increasing growing time (Kim *et al.*, 2007).

The biosynthesis of anthocyanins in rice accompanies photomorphogenesis, which suggests that light intensity and wavelength could affect anthocyanin biosynthesis and accumulation. Indeed, previous works has shown that different light wavelengths leads to different anthocyanins accumulation (Lichtenthaler *et al.*, 1981; Albert *et al.*, 2009). In lettuce leaves, the expression of three structural genes, *CHS*, *F3H* and *DFR*, was induced under UV-B light, leading to high accumulation of anthocyanins (Park *et al.*, 2007). In *Brassica rapa*, *PAL*, *CHS*, *F3H*, *DFR*, *ANS* are specifically expressed under UV-A light (Zhou *et al.*, 2007). In maize, the regulatory gene *c1*and *pl1* would adjust the accumulation of anthocyanins under white, blue and UvB light (Piazza *et al.*, 2002; Irani and Grotewold, 2005).

Studies of the biosynthesis of anthocyanins in purple rice have generally focused on the color of the grains rather than regulation of the biosynthestic pathway; thus, the regulation of anthocyanin biosynthesis remains poorly understood in this variety. This research used seedlings from two purple rice lines to explore the effects of different light intensities and wavelengths on anthocyanin concentration, and to determine the expression of genes associated with these effects. This research provides insight into the anthocyanin biosynthesis pathway.

MATERIALS AND METHODS

Rice materials and growth conditions

Seeds of YDD1, YDD2 and Nipponbare rice lines were sterilized and germinated at room temperature for 3 days. Individual healthy seedlings were then transferred to 0.15% agar medium. Seedlings were placed under one of three light intensities (5 μmol m^{-2} s^{-1}, 45 μmol m^{-2} s^{-1}, or 150 μmol m^{-2} $s^{-1)}$, or one of two light wavelengths (blue light, Bc: 470 nm; or far-red light, FRc: 730 nm) for 7 days. The light intensity of the Bc and FRc treatments was 45 μmol m^{-2} s^{-1}. Plants were grown under a light/dark period of 16 h at 30°C in the light and 8 h at 28°C in the dark.

Measurement of anthocyanin content

Seedling anthocyanin content was determined using the method described by Yuan *et al.* (2009), with minor alterations. Briefly, all fresh aboveground leaves were cut into 2 ml tubes, immediately placed into liquid nitrogen and ground into powders. Then, 1 ml HCl solution (pH 2) was added to each 0.2 g of powder and kept at room temperature for 10 minutes. Samples were centrifuged at 4°C and 16,000 rcf min^{-1} for 6 minutes, then the supernatants were removed. Compared with the darkest sample, supernatants were diluted with KCl (pH 1) by a factor of 5 or 10 for easy measurement. Finally, the diluted supernatants were measured at absorbance of 520 nm and 700 nm. The anthocyanin concentration in mg ml^{-1} was calculated as follows:

$(A \times MW \times DF \times V \times 10^{-3}) / (\varepsilon \times M)$

Where: $A = (A_{520}–A_{700})_{pH1} – (A_{520}–A_{700})_{pH4.5}$; MW = 449.2; DF = dilution factor; ε = 22,900; V = volume of HCl solution (ml); M = weight of each powdered sample.

All samples were determined in triplicate.

RNA extraction and cDNA preparation

Harvested seedlings were placed into liquid nitrogen, ground, and total RNA was extracted using the Total RNA Isolation Kit (Sangon, Shanghai, China). cDNA was synthesized by following the protocol described by the manufacturers of the PrimeScript^{TM}RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China).

Expression analysis

To study the gene expression patterns of each rice line under each light treatment, cDNA from each sample was diluted by a factor of 20 and used for qRT-PCR. qRT-PCR was performed using an ABI-7300 Fast Real-Time PCR system and the SYBR Premix Ex Taq Ⅱ (Tli RNaseH Plus) Kit, according to the manufacturer's instructions (TaKaRa, Dalian, China). Twenty microliter volumes of

Table 1. Primer sequences used for gene expression analysis.

Primer	Sequence (5′ to 3′)	No. of bases
OsCHS-F	TCCGAGTACGGCAACATGTC	20
OsCHS-R	GCGCATCTCGTCGAGGAT	18
OsCHI-F	GTTCACGAGGGTGACGATGA	20
OsCHI-R	GCAGTTCTCCGTCACCTTGTC	21
OsF3H-F	CATGCAGACGTGGATGTCAAG	21
OsF3H-R	CGTCCTGCTCCGAATGGT	18
OsDFR-F	GTGCACTTCTCGTCGTGGAA	20
OsDFR-R	TCCTCCAGCGTGTACCTGAAC	21
OsANS-F	CGTGCAACGCAAGCTGTT	18
OsANS-R	GCTGCGGCATTGTTGTCTT	19
OsB1-F	TCTCCTCGAGCTGCAATGC	19
OsB1-R	GCGTCGAACACTCTCGTCATC	21
OsB2-F	CTCGTGTTTCGTGTCATGGAA	21
OsB2-R	CACGGCCACCACCTTCTC	18

Table 2. Phenotypes of purple rice lines used in this study and their parents

Rice line	Plant height (cm)	Panicle length (cm)	Spikelets per panicle	Seed setting rate (%)	1000 grain weight (g)	Blast resistance*
YDD1	87.0	18.41	129.2	63.55	20.25	MR
YDD2	86.2	17.32	111.7	75.92	19.20	MR
P1	68.0	15.00	101.7	49.50	22.90	S
P2	100.0	18.00	156.5	80.60	25.50	MR

* Diease reactions have a range between 0-2 are scored as R; 3-4 are scored as MR; 5 or more scored as S.

each sample were run with the following program: 95°C for 30 seconds; then 40 cycles of 94°C for 5 s followed by 58°C for 30 seconds; then a melt curve at 95°C for 15 seconds, 58°C for 1 minute, and 95°C for 15 seconds. Primer sequences used for qRT-PCR are shown in Table 1.

RESULTS

Breeding of novel purple rice lines and pigment measurement

YDD1 and YDD2 are novel purple rice resources that were bred jointly by Yunnan and YuXi Academies of Agricultural Sciences in 2015. Briefly in 2001, scientists selected an elite *japonica* variety named YuYou-1 (P2), which had good rice quality and comprehensive characteristics, to improve a purple rice line named ZiXiangGeng (P1). By 2015, several stable lines (F_{15}) were selected through pedigree breeding; of these, the performance of YDD1 and YDD2 were deemed to be outstanding, giving plants with purple stems, leaves and pericarps, and dark purple panicles. Importantly, these two lines also performed well in the field, showing wide-ranging adaptation and comprehensive characteristics as shown in Table 2.

Anthocyanin content under high light intensity

To study the relationship between anthocyanin concentration and light intensity, seedlings (three leaves stage) from the two purple rice lines and a Nipponbare (control) line were grown in a growth chamber under light intensities of 5 µmol $m^{-2}s^{-1}$, 45 µmol $m^{-2}s^{-1}$ or 150 µmol $m^{-2}s^{-1}$ for 7 days.

As shown in Figure 1, Nipponbare seedlings were the normal green color under each of the three light intensities, while YDD1 and YDD2 were purple. In Nipponbare, the concentrations of anthocyanin under light intensities of 5 µmol $m^{-2}s^{-1}$, 45 µmol $m^{-2}s^{-1}$ and 150 µmol $m^{-2}s^{-1}$ were 1.808 mg kg^{-1}, 2.535 mg kg^{-1}, and 4.832 mg kg^{-1} respectively; in YDD1, anthocyanin

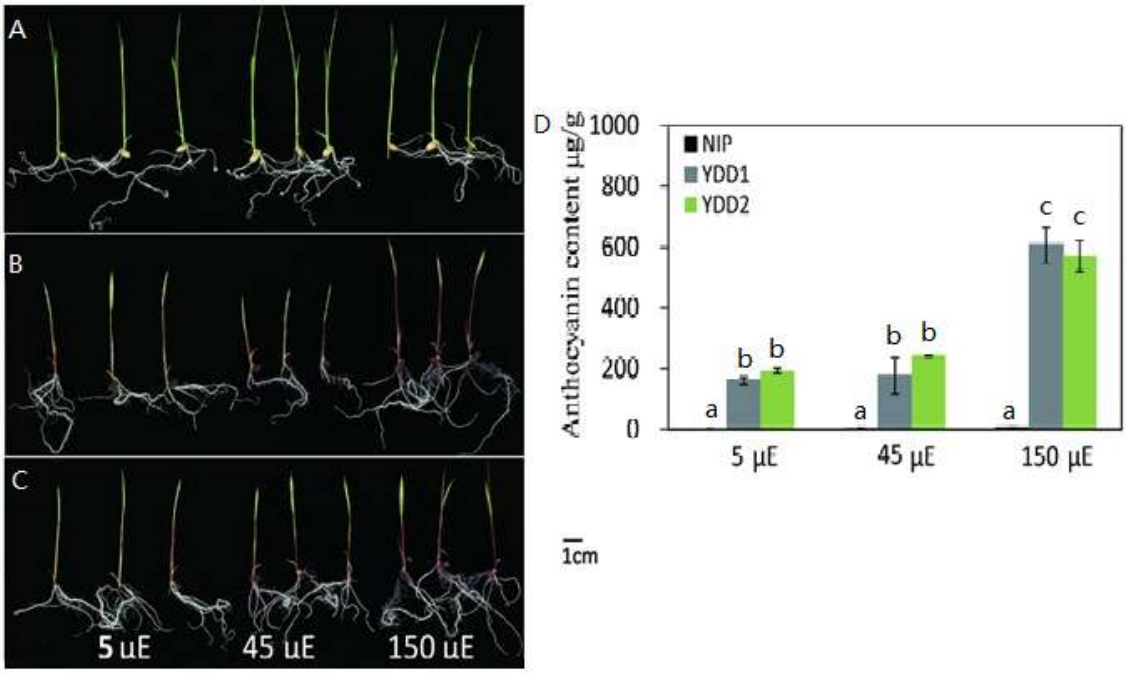

Figure 1. Effect of different light intensities on the colors and anthocyanin contents of two purple rice lines compared with Nipponbare.
Three leaves stage seedlings of Nipponbare (A), YDD1 (B) and YDD2 (C) grown under 5 μmol $m^{-2}s^{-1}$, 45 μmol m-2s-1 and 150 μmol $m^{-2}s^{-1}$ light intensity conditions for 7 days with a light/dark period of 28°C/16 h in the dark and 25°C/ 8 h in the light. (D) The anthocyanin contents of the three seedlings under each light intensity. a, b and c stands for significant differences at the levels of $p<0.01$ according to Student's t-test, the same letter indicate no statistical difference.

concentrations were 159.808 mg kg^{-1}, 177.017 mg kg^{-1} and 606.703 mg kg^{-1}, respectively; and in YDD2, they were 188.651 mg kg^{-1}, 239.777 mg kg^{-1} and 570.16 mg kg^{-1}, respectively.

Similarly to previous research (Albert *et al*., 2009; Wang *et al.*, 2010), the results showed that when the light intensity increased, anthocyanin concentrations increased particularly in the purple rice lines, in which the anthocyanin content increased significantly. However, anthocyanin concentrations differed between YDD1 and YDD2 at each light intensity. Under a light intensity of 45 μmol m^{-2} s^{-1}, the anthocyanin concentration of YDD2 was higher than in YDD1, but when it increased to 150 μmol m^{-2} s^{-1}, the anthocyanin concentration of YDD1 was higher. This phenomenon indicates that the metabolic pathway for anthocyanin synthesis is somewhat complex.

Long light wavelength (Far red light) suppresses anthocyanin biosynthesis

To reveal the relationship between light wavelength and anthocyanin accumulation, seedlings of both purple rice lines and the control line were grown under constant 730 nm light wavelength (constant far-red light, FRc) and 470 nm light wavelength (constant blue light, Bc), at a light intensity of 45 μmol m^{-2} s^{-1}.

As shown in Figure 2, the three lines accumulated significantly more anthocyanin under the wavelength of 470nm and 730nm. Leaves of Nipponbare seedlings stayed the normal green color under both 470nm and 730nm light wavelength treatments, but the concentration of anthocyanin was significantly higher under 470nm (0.3216 mg kg^{-1}) than under 730nm (0.0008 mg kg^{-1}). The leaves of both YDD1 and YDD2 were purple under Bc and the normal green color under FRc. The anthocyanin concentration was 62.52 mg kg^{-1} for YDD1 while 80.31 mg kg^{-1} for YDD2 under the shorter 470 nm wavelength, indicating that these purple lines have a mechanism to resist light damage.

Gene expression analysis

Previous studies have shown that anthocyanin biosynthesis is affected by several enzyme-catalyzed structural genes, including *OsCHS*, *OsCHI*, *OsF3H*, *OsDFR*, *OsANS*, and is also regulated by *OsB1* and *OsB2*. To study gene expression levels under different

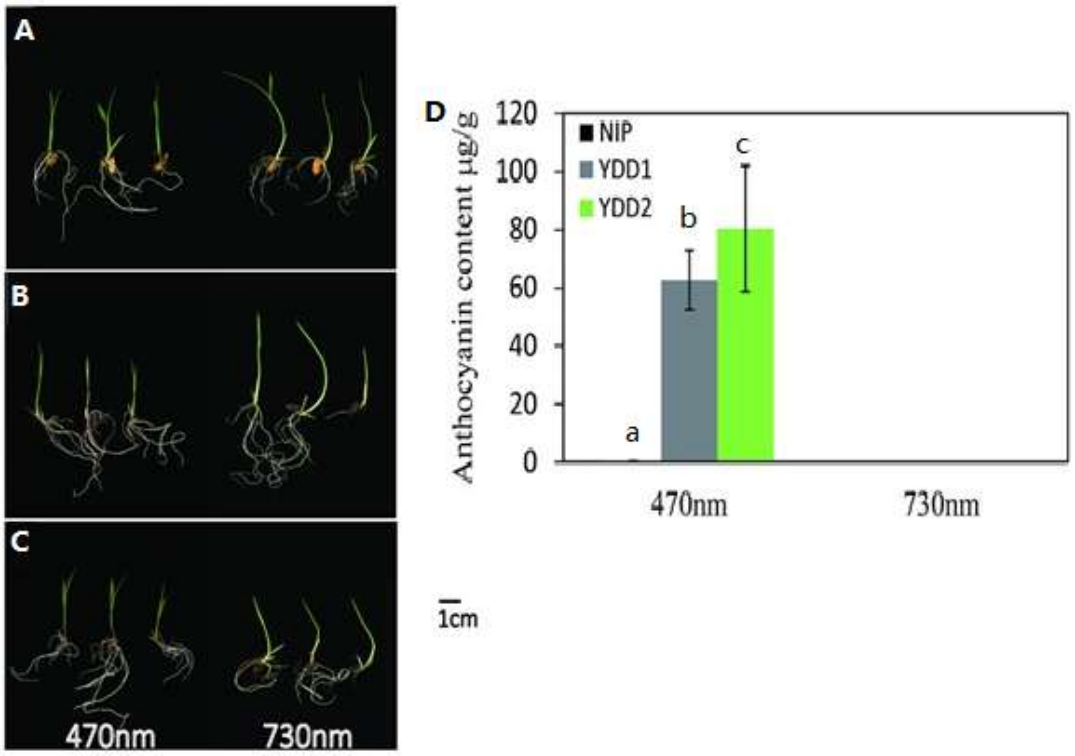

Figure 2. Effect of different light wavelength on the colors and anthocyanin contents of two purple rice lines compared with Nipponbare.

Three leaves stage seedlings of Nipponbare (A), YDD1 (B) and YDD2 (C) grown under 470nm and 730nm light wavelength conditions for 7 days with a light/dark period of 28°C/16 h in the dark and 25°C/ 8 h in the light. (D) The anthocyanin contents of the three seedlings under the two light wavelength respectively. a, b and c stands for significant differences at the levels of $p < 0.01$ according to Student's t-test.

light treatments, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was carried out.

Figure 3 shows the expression levels of seven genes under different light stresses. Interestingly, in *Nipponbare*, expression levels of structural genes always showed the opposite trend compared to the two purple lines. However, between the two purple lines, when treated with a light intensity of 5 μmol m^{-2} s^{-1}, expression levels of *OsCHS*, *OsCHI*, *OsF3H*, *OsDFR*, and *OsANS* were similar, while *OsB1* and *OsB2* varied significantly. When the light intensity was increased to 45 μmol m^{-2} s^{-1}, expression of five genes (all except *OsCHI* and *OsB2*) increased accordingly. And when the light intensity was increased to 150 μmol m^{-2} s^{-1}, all the six genes except *OsB1* show an accordingly higher expression level. The differing expression levels of *OsB1* and *OsB2* between YDD1 and YDD2 may also indicate a complex pathway for regulation of anthocyanin biosynthesis and accumulation. Also, in the purple lines, the expression levels of *OsDFR* and *OsANS* (which are involved in the last step of anthocyanin accumulation) were notably higher than the other genes, indicating that these two gene are key in the accumulation of anthocyanin.

Figure 4 shows the results of the analysis on the effects of light wavelength on gene expression. Compared with far-red light, blue light enhanced expression of the structural genes, and the expression levels of *OsDFR* and *OsANS* were significantly higher than those of *OsCHS*, *OsCHI* and *OsF3H*. The regulator *OsB2* may be key to the accumulation of anthocyanin in purple rice. These results suggest that Bc promotes anthocyanin accumulation by increasing the transcription of genes involved in anthocyanin biosynthesis and regulation.

DISCUSSION

Around the world, purple rice is considered to be particularly special for its health-promoting and medical benefits. Many previous studies have focused on the characteristics of its grain. For example, fine-mapping of the *Pa-6* gene, which is associated with the apiculus of purple rice, located it to a 41.7-kb interval bounded by L02

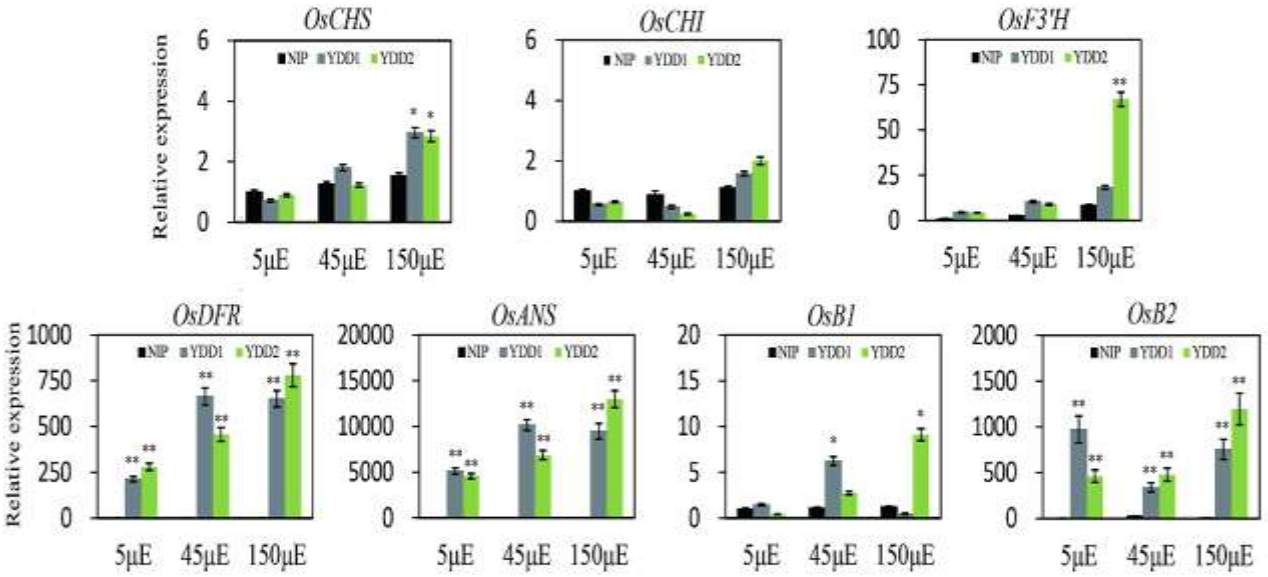

Figure 3. Gene expression levels under different light intensities.
Note: " * " and " ** "represent significant differences at P＜0.5 and P＜0.01 levels, respectively according to Student's t-test.

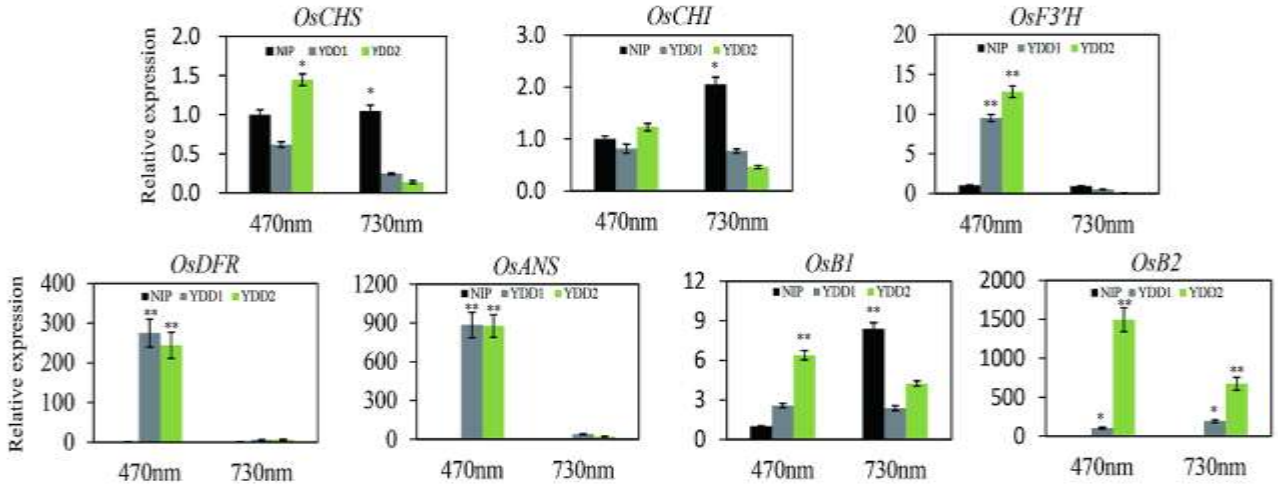

Figure 4. Gene expression levels under different light wavelengths.
Note: " * " and " ** "represent significant differences at P＜0.5 and P＜0.01 levels, respectively according to Student's t-test.

and RM19561 on chromosome 6 (Liu *et al.*, 2012). By exploring transcriptional and translational expression during grain development, Chen *et al.* (2016) found eight representative genes encoding different metabolic proteins. Using a construct containing eight anthocyanin-related genes, Liu's research group (Zhu *et al.*, 2017) generated a novel bio fortified 'purple endosperm rice' with high anthocyanin content and antioxidant activity in the endosperm.

However, the accumulation of anthocyanins has seldom been studied in detail in purple rice seedlings. Hence, the current study focused on the accumulation and biosynthesis of anthocyanins in seedlings of two purple rice varieties with significant purple coloration throughout the whole plant. Compared to Nipponbare (a green-colored control variety), changes in light intensity and light wavelength affected the anthocyanin concentrations of both purple rice lines in a similar way,

though there was variation between the two varieties. However, changes in the expression levels of two regulatory genes, *OsB1* and *OsB2*, were more complex between the two purple varieties. As shown in Figure 2, trends in the expression of *OsB1* under high light intensity were opposite between YDD1 and YDD2. *OsB1* and *OsB2* have been reported to be associated with rice leaf color, but further research is needed to determine the interactions between these two genes.

Light is an essential environmental factor for plant growth and development. Anthocyanin biosynthesis is affected by different light wavelengths (Brenda Winkel-Shirley, 2002; Cominelli *et al.*, 2018). Throughout their co-evolution, plants have developed sophisticated photoreceptor systems to enable their adaptation to variable light conditions. Earlier studies have shown that cryptochromes (CRYS) receive blue light, while phytochromes (PHYS) regulate plant responses to red and far-red light (Franklin and Quail, 2010), and anthocyanin accumulation is induced by both blue and far-red light (Ahmad *et al.*, 1995; Hoecker *et al.*, 1998). Indeed, the current study indicate that the induction of anthocyanin accumulation under blue light and high light intensity provides rice plants with protection against light-induced damage.

However, the results of the study indicated that far-red light negatively regulated the accumulation of anthocyanins. Compared with Nipponbare, trace amounts of anthocyanin were detected in the purple rice varieties under far-red light, indicating that the accumulation of anthocyanin in purple rice is much more sensitive to far-red light.

Although the mechanism underlying this finding needs further research, and it has practical significance for factories growing rice seedlings. In Yunnan province, China, it has become popular to eat purple or red rice – especially among celebrities. To meet this demand, around 100,000 hectares of purple rice is grown in the field. Avoiding the use of far-red light in industrialized breeding systems might therefore help to incubate strong and healthy seedlings for rice production systems in Yunnan.

Regulation of the accumulation of anthocyanin under light generally occurs at the transcriptional level of genes involved in anthocyanin biosynthesis, including *CHS*, *CHI*, *DFR* and *ANS* (Vandenbussche *et al.*, 2007; Shin *et al*., 2007). As PAL, CHS, CHI and F3H are substrates for enzymes involved in anthocyanin biosynthesis, the following biosynthesis processes are catalyzed by the two most important genes, *OsDFR* and *OsANS*, which ultimately leads to accumulation of a purple color in rice.

CONCLUSION

In this study, it is found that *OsCHS*, *OsCHI* and *OsF3H* have similar expression levels, but *OsDFR* and *OsANS* show extremely high expression under high light intensity and constant blue light. This leads us to speculate that *OsDFR* and *OsANS* are involved in the regulation of anthocyanin accumulation under light stress. Further research will shed light on this regulatory mechanism.

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