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ORIGINAL ARTICLE

Functional characterization of key structural genes in rice flavonoid biosynthesis

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Abstract Rice is a model system for monocot but the molecular features of rice flavonoid biosynthesis have not been extensively characterized. Rice structural gene homologs encoding chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) were identified by homology searches. Unique differential expression of *OsF3H*, *OsDFR*, and *OsANS1* controlled by the Pl^{w} locus, which contains the R/B-type regulatory genes *OSB1* and *OSB2*, was demonstrated during light-induced anthocyanin accumulation in T65-Plw seedlings. Previously, F3H genes were often considered as early genes co-regulated with CHS and CHI genes in other plants. In selected non-pigmented rice lines, *OSB2* is not expressed following illumination while their expressed *OSB1*sequences all contain the same nucleotide change leading to the T^{64} M substitution within the conserved N-terminal interacting domain. Furthermore, the biochemical roles of the expressed rice structural genes (*OsCHS1*, *OsCHI*, *OsF3H*, and *OsF3′H*) were established in planta for the first time by complementation in the appropriate Arabidopsis *transparent testa* mutants. Using yeast two-hybrid analysis, OsCHS1 was demonstrated to interact physically with OsF3H, OsF3′H, OsDFR, and OsANS1, suggesting the existence of a macromolecular complex for anthocyanin biosynthesis in rice. Finally, flavones were identified as the major flavonoid class in the non-pigmented T65 seedlings in which the single-copy *OsF3H* gene was not expressed. Competition between flavone and anthocyanin pathways was evidenced by the significant reduction of tricin accumulation in the T65-Plw seedlings.

Keywords Rice · Flavonoid structural genes · Anthocyanin · Flavones

Abbreviations

AD	Activation domain
ANS	Anthocyanidin synthase
BD	Binding domain
bHLH	Basic helix-loop-helix
CHS	Chalcone synthase
CHI	Chalcone isomerase
DFR	Dihydroflavonol 4-reductase
F3H	Flavanone 3-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
FGT	Flavonoid 3-*O*-glucosyltransferase
ONPG	*o*-Nitrophenyl-*β*-D-galactopyranoside

Introduction

Flavonoids play important roles in plants including UV protection, defense against pathogens and pests, pollen fertility, signaling with microorganisms, auxin transport

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regulation, and pigmentation (Winkel-Shirley 2001). Food products containing flavonoids are increasingly popular due to their antioxidant and anticancer properties. Despite their diversity of functions and structures, all flavonoids are derived from the general phenylpropanoid pathway and most enzymes involved in the pathways leading to major flavonoid classes have been determined (Fig. 1). For example, the reactions to anthocyanin synthesis are catalyzed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3-*O*-glucosyltransferase (FGT). Genes encoding anthocyanin biosynthesis enzymes were initially isolated from maize, snapdragon, and petunia (Holton and Cornish 1995). Depending on plant species, flavonoids can be extensively modified through hydroxylation, *O*-methoxylation, *C*- or *O*-glycosylation, and a variety of other biochemical conversions.

The dicot model plant Arabidopsis has facilitated the understanding of flavonoid metabolism from gene expression, assembly of enzyme complexes, to the establishment of functional roles of flavonoids. Most of the key flavonoid enzymes are encoded by a single gene in Arabidopsis (Winkel 2006). The *transparent testa* (*tt*) mutants have helped define the roles of flavonoids in UV protection (Li et al. 1993) and auxin transport (Buer and Muday 2004). However, flavonoids do not have functions in male fertility in Arabidopsis, unlike maize and tobacco (Winkel-Shirley 2001). The hypothesis that flavonoid enzymes assemble as a macromolecular complex was also supported by studies in Arabidopsis. Direct associations between CHS, CHI, F3H, and DFR have been demonstrated, suggesting that the

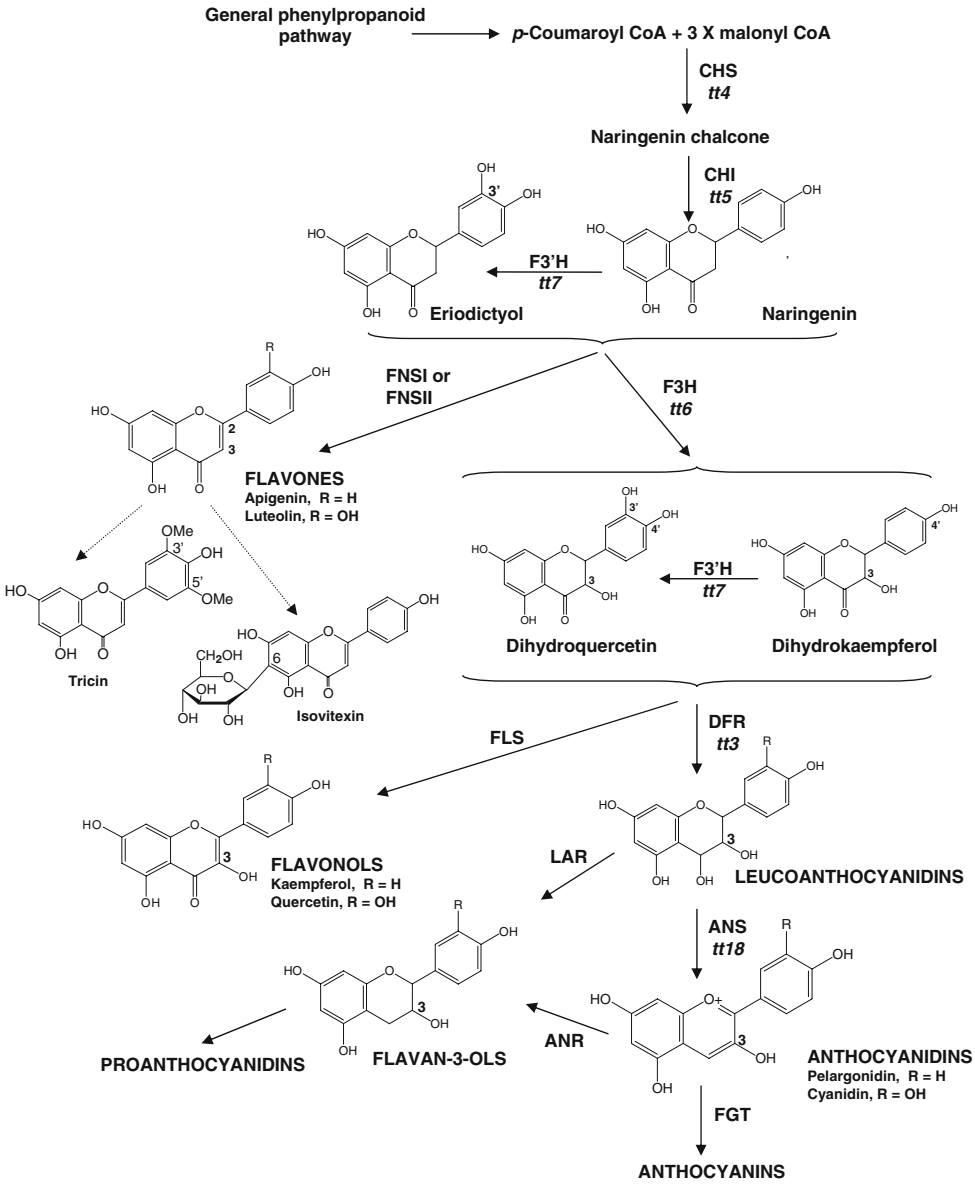


Fig. 1 Pathways of flavonoid biosynthesis starting with the general phenylpropanoid pathway and leading to the major flavonoid classes. The first committed step is catalyzed by chalcone synthase (*CHS*) using malonyl CoA and *p*-coumaroyl CoA as substrates. Enzymes are abbreviated as follows: anthocyanidin synthase (*ANS*), anthocyanidin reductase (*ANR*), chalcone isomerase (*CHI*), dihydroflavonol 4-reductase (*DFR*), flavanone 3-hydroxylase (*F3H*), flavone synthases I or II (*FNS I* or *FNS II*), flavonoid 3′hydroxylase (*F3′H*), flavonoid 3-*O*-glycosyltransferase (*FGT*), flavonol synthase (*FLS*), leucoanthocyanidin reductase (*LAR*). Arabidopsis *tt* mutations in different enzymatic steps are indicated. Isovitexin and tricin are two flavones identified in rice seedlings in this study (Fig. 6). Both of them are derived from apigenin and the biosynthetic steps are still unknown (indicated as *broken arrows*)

flux of intermediates into different flavonoid products could be controlled by the formation of metabolons (Burbulis and Winkel-Shirley 1999).

Recently rice has become an experimental system for monocot. Hundreds of rice sequences could be retrieved when keyword or BLAST searches using the known flavonoid enzyme names or sequences are performed in different public databases. In fact, flavonoids with significant biological activities have been described in rice, such as isovitexin in hull, tricin in bran, proanthocyanidins in pericarp of pigmented grains, and anthocyanins in purple-leaf cultivars. Previously, rice genes encoding homologs of CHS (Reddy et al. 1996) and CHI (Druka et al. 2003), which were used in physical mapping analysis, were identified only based on sequence homology. On the other hand, a rice ANS gene was demonstrated to complement the *a2* mutation in maize kernels by transient assays (Reddy et al. 2007). Similarly, a functional DFR gene was revealed when transgenic rice over-expressing the gene was found to produce red-colored pericarp (Furukawa et al. 2007). Two major classes of transcription factors, namely the basic helix-loop-helix (bHLH) type R/B family and the MYB type C1 family, are known to regulate anthocyanin biosynthesis pathway in many plants. Physical interactions between the R/B and C1 proteins are required to activate the expression of the key structural genes. The *japonica* rice line Taichung 65 (T65) carries a functional *OsC1* gene, which is a C1 homolog (Saitoh et al. 2004), but it lacks a dominant Purple Leaf (*Pl*) locus required for leaf and shoot pigmentation (Sakamoto et al. 2001). Pl^{w}, a *Pl* locus in the isogenic line T65-Plw generated using T65 as a recurrent parent, was found to harbor at least two *R/B* homologs, namely *OSB1* and *OSB2* (Sakamoto et al. 2001). *OSB1* is an allele of the functional rice *Ra1* gene previously reported by Hu et al. (1996). *OSB1* and *OSB2* appear to be functionally redundant since either gene was sufficient to induce anthocyanin pigments in rice aleurone cells when co-expressed with the maize *C1* gene in transient assays (Sakamoto et al. 2001). However, the regulation of different rice flavonoid structural genes by the Pl^{w} locus during anthocyanin biosynthesis remains uncharacterized. Ectopic expression of the maize C1 and R genes in rice endosperm failed to induce anthocyanin pigmentation (Shin et al. 2006), suggesting that a different regulatory mechanism is involved in rice.

The commonly grown and consumed rice varieties throughout the world produce green leaves and seeds with white pericarp. In addition, polished rice, which is primarily composed of endosperm tissue, is poor in phytochemicals. The perceived health benefits of flavonoids have made them attractive targets for metabolic engineering of food crops including rice (Shin et al. 2006). In this study, we attempted to characterize a collection of key flavonoid structural genes in rice identified through bioinformatics searches. Their expression was examined during light-induced anthocyanin accumulation in T65-Plw seedlings in comparison to the non-pigmented T65 seedlings. To establish their biochemical functions, the rice genes were over-expressed in the appropriate Arabidopsis *tt* mutants for complementation analysis. Potential interactions of the validated flavonoid enzymes were also investigated using yeast two-hybrid analysis. Finally, metabolite investigations demonstrated the accumulation of health-beneficial flavones (tricin and isovitexin) in both pigmented and non-pigmented rice seedlings. Our results provide the background for further molecular dissection of anthocyanin regulation and flavone biosynthesis in rice. The knowledge should be useful for metabolic engineering in edible tissues (such as the endosperm) which normally do not accumulate flavonoids.

Materials and methods

Plant materials

In this study, the following *japonica* rice (*Oryza sativa* L.) lines were used for flavonoid and gene expression analysis: T65 and T65-Plw (Sakamoto et al. 2001), Nipponbare (NIAS, Japan), Zhonghua 11 (South China Agricultural University, China), and Tainong 67 (Chiayi Agricultural Experimental Station, Taiwan). All these rice lines produce green leaves and shoots except for T65-Plw which is a purple-leaf line. Pl^{w}, a *Pl* locus derived from a *javanica* rice variety (Pirurutong), was introduced into the parent line T65 by nine times of recurrent back-crossing to generate the isogenic line T65-Plw (Sakamoto et al. 2001). Etiolated rice seedlings (10 day-old) with elongated mesocotyls were placed under constant light at room temperature. Arabidopsis *tt3*, *tt4*, *tt5*, *tt6*, *tt7*, and *tt18* mutants (Arabidopsis Biological Resource Center, OH, USA) defective in genes encoding DFR, CHS, CHI, F3H, F3'H, and ANS, respectively, were used for complementation analysis.

Identification of rice flavonoid genes and cDNA clones

Amino acid sequences of known CHS, CHI, F3H, F3'H, DFR, and ANS enzymes were used to conduct tBlastN searches against the NCBI database and Rice Genome Annotation (http://rice.plantbiology.msu.edu/). Full-length rice cDNA clones showing the highest identity over the entire length of the translated sequences were identified and subsequently requested from different laboratories (*OsCHS1*, *OsCHI*, *OsF3'H*: National Institute of Agrobiological Sciences, Tsukuba, Japan; *OsDFR* : S. Iida, National Institute for Basic Biology, Okazaki, Japan). *OsF3H* and *OsANS1* clones were not available and their full-length coding regions were amplified by reverse transcription

polymerase chain reactions (RT-PCR) using RNA samples prepared from pigmented T65-Plw seedlings (see below).

RT-PCR experiments

Total RNA was extracted from rice mesocotyl tissues using the Trizol method (Invitrogen, CA, USA). DNase I-treated RNA samples (4 μg) were reversed transcribed by M-MLV reverse transcriptase (Promega, WI, USA). Gene-specific primers were designed from the 3′-UTR regions of the different rice flavonoid structural genes. Amplification of target cDNA was performed with the GoTaq Flexi DNA Taq polymerase (Promega, WI, USA) using the following program: 94°C (10 min); 30 cycles of 94°C (30 s), 55°C (30 s), 72°C (1 min); 72°C (7 min). To obtain full-length coding regions of T65-Plw *OsF3H*, T65-Plw *OsANS1*, and T65 *OSB1*, Pfx DNA polymerase (Invitrogen) was used for high-fidelity amplification. A list of primers used in different RT-PCR experiments is shown in Electronic supplementary material Table S1.

Arabidopsis complementation analysis

Coding regions of the identified rice genes were cloned into an over-expression vector (Yu et al. 2005) containing the CaMV 35S promoter and the nopaline synthase 3′-terminator. The resulting plasmids were introduced into the binary vector pCAMBIA 1300 (CAMBIA, Australia). *Agrobacterium*-mediated transformation of the appropriate Arabidopsis mutants was performed by the floral dip method (Clough and Bent 1998). Harvested seeds were surface-sterilized and germinated on Murashige and Skoog (MS) (Sigma, MO, USA) agar plates containing 3% (v/v) sucrose and 25 μg ml^{-1} hygromycin (Sigma). Resistant seedlings were transplanted and placed in a growth chamber (22°C; 16 h light, 8 h dark). At least three independent lines with strong transgene expression for each construct were selected for phenotypic analysis. For staining of proanthocyanidins (condensed tannin), T_1 seeds were treated with dimethylaminocinnaldehyde (DMACA) (Sigma) reagent (2% w/v DMACA in 3 M HCl/50% w/v methanol) for 1 week, followed by washing in 70% ethanol for three times. To induce anthocyanin accumulation, seeds were germinated on MS plates without nitrogen sources. For analysis of flavonols by HPLC (see below), leaf tissues (0.5–1.0 g) were collected from 14-day-old T_1 seedlings.

Yeast two-hybrid analysis

The Matchmaker GAL4 Two-Hybrid System 3 was purchased from Clontech Laboratories (Palo Alto, CA, USA) and used essentially as described in the protocol handbook. Full-length coding sequences of *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsDFR*, and *OsANS1* were PCR-amplified from the corresponding cDNA clones. The products were subcloned in-frame with the 3′-end of the GAL4 activation domain (AD) in the pGADT7 vector or the 3′-end of the GAL4 binding domain (BD) in the pGBKT7 vector. Using the lithium acetate method, the AD and BD fusion vectors were transformed into the *Saccharomyces cerevisiae* strains AH109 and Y187, respectively. Pair-wise combinations of the fusion constructs were introduced into the same yeast cells by mating as described in the protocol handbook. Mated yeast colonies carrying the interacting fusion proteins were selected by growth on leu^-, trp^-, his^- minimum medium containing 5 mM 3-aminotriazole (Sigma). Five independent yeast colonies for each pair of potentially interacting proteins were analyzed for *β*-galactosidase activities which were quantified using the substrate *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) according to the protocol handbook.

HPLC-UV-MS analysis of plant metabolites

Plant tissues ground to fine powder in liquid nitrogen were extracted in 100% methanol. For acid hydrolysis, an equal volume of 2 N HCl was added to the samples for incubation at 90°C for 1 h. The acid-hydrolyzed samples were dried under nitrogen and resuspended in 100 μl of methanol for HPLC analysis. Authentic standards of cyanidin 3-*O*-rutinoside (Alexis, CA, USA), quercetin and kaempferol (Sigma), isovitexin and tricin (Apin Chemicals, Oxfordshire, UK) were used for metabolite identification. Filtered samples (20 μl) were injected onto a HP 1100 series HPLC system (Agilent Technologies, CA, USA) connected with a Nucleosil 100-5 C18 column (5 μm, 150 × 2 mm, Agilent Technologies). Separation was performed using a solvent system of 0.5% formic acid (v/v) (A) and acetonitrile (B) with a linear gradient of 15–60% B over 25 min. Flow rate was maintained at 0.2 ml min^{-1} and the elution monitored by a diode-array detector (200–600 nm) in tandem with an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, CA, USA) operating in positive ion mode. Full scan and product ion spectra were acquired using parameters optimized for maximum sensitivities: capillary temperature, 350°C; sheath gas (N_2), 70 (arbitrary unit); auxiliary gas (N_2), 40 (arbitrary unit); ESI spray voltage, 4.8 kV; capillary voltage, 18 V; multipole 1 offset voltage, –8 V; multipole 2 offset voltage, –8.5 V; inter-multipole voltage, –10 V.

Results

Rice structural genes for flavonoid biosynthesis

Flavonoid structural gene homologs were identified by querying the Rice (Nipponbare) Genome Annotation with known flavonoid enzyme sequences from maize and

sorghum. Rice gene homologs encoding CHS, CHI, F3H, F3′H, DFR, and ANS were found to have at least 66% amino acid identity to the respective maize or sorghum flavonoid enzymes (Table 1). It should be noted that *OsDFR* was found to be non-functional in Nipponbare rice due to a premature stop codon in the second exon (Furukawa et al. 2007). Hence, a published DFR sequence of a purple rice cultivar (AB003496) was used instead in this study. Each rice gene was subsequently searched against the entire genome to identify potential gene family members. Both CHS and ANS appear to be each encoded by at least two genes in the rice genome. There are two highly conserved ANS-like genes located on different chromosomes, *OsANS1* and *OsANS2*, with over 93% amino acid sequence identity. Similarly, *OsCHS1* and *OsCHS2* are located on different chromosomes and their amino acid sequence identity is over 80%.

Expression analysis of flavonoid structural and regulatory genes in rice seedlings

The expression patterns of different structural and regulatory genes during light-induced anthocyanin accumulation were investigated in the rice line T65-Plw and the isogenic parent line T65. Etiolated T65-Plw seedlings with elongated mesocotyls appeared purple while the T65 seedlings turned green at 48 h after illumination (Fig. 2a). Cyanidin 3-*O*-rutinoside was identified as the major anthocyanin pigment in the T65-Plw seedlings (Electronic supplementary material Fig. S1). Gene expression experiments were performed by semi-quantitative RT-PCR using RNA samples prepared from the seedlings harvested at different time points. As shown in Fig. 2b, *OsF3H*, *OsDFR*, and *OsANS1* were differentially expressed in the pigmented T65-Plw seedlings following light exposure. The expression patterns for *OsF3H* and *OsDFR* were similar and their transcripts were not detected until 12 h. In the case of *OsANS1*, expression was induced as early as 4 h after illumination and the transcript levels remained relatively constant throughout the 24-h period. On the other hand, *OsCHS1*, *OsCHI*, and *OsF3′H* gene expression was light-inducible and occurred in similar patterns in both T65 and T65-Plw seedlings (Fig. 2b). Expression of the other members of the CHS and ANS families, i.e. *OsCHS2* and *OsANS2*, was not detected in the rice seedlings (data not shown).

OSB1 and *OSB2* are two bHLH *R*/*B*-type regulatory genes identified in the Pl^{w} locus of T65-Plw plants (Sakamoto et al. 2001). On the other hand, *OsC1* is a functional homolog of maize *C1* described previously in rice cultivars with colored apiculus, such as T65 (Saitoh et al. 2004). As shown in Fig. 2b, *OSB2* expression was restricted to the T65-Plw seedlings and it appeared to be light-independent. On the other hand, *OSB1* and *OsC1* expression was light-inducible in both T65-Plw and T65 seedlings. Transcripts of *OSB1* and *OsC1* were detected as early as 4 h following illumination and their levels gradually declined during the rest of the 24-h period. We isolated the full-length *OSB1* cDNA from T65 plants and compared the coding region with the functional *Ra1* and T65-Plw *OSB1* sequences. Two unique changes in the T65 *OSB1* sequence could be identified (ESM Fig. S2). The first nucleotide change results in a predicted non-conservative amino acid substitution at position 64 (T^{64} M) located within the conserved N-terminal interacting domain (Goff et al 1992). The T-64 residue is strictly conserved in Ra1, OSB2, maize Lc and B, and Arabidopsis TT8 (Fig. 3). The second difference is the deletion of a nucleotide (G) altering the reading frame at amino acid 574 and terminating the protein sequence immediately (ESM Fig. S2).

A series of similar RT-PCR experiments were also performed with other non-pigmented *japonica* rice cultivars,

Table 1 Rice structural gene homologs of flavonoid enzymes

Flavonoid enzymes	Query sequence[a]	Rice gene homologs	TIGR Locus[c]	Identity[d] (%)
Chalcone synthase	Maize C2 (P24824)	*OsCHS1*	LOC_Os11g32650	91.7
		OsCHS2	LOC_Os07g11440	79.2
Chalcone isomerase	Maize CHI (Q08704)	*OsCHI*	LOC_Os03g60509	74.0
Flavanone 3-hydroxylase	Maize F3H (AAA91227)	*OsF3H*	LOC_Os04g56700	78.0
Flavonoid 3′-hydroxylase	Sorghum F3′H1[b] (DQ787855)	*OsF3'H*	LOC_Os10g17260	82.8
Dihydroflavonol reductase	Maize A1 (P51108)	*OsDFR*	LOC_Os01g44260	77.1[e]
Anthocyanidin synthase	Maize A2 (CAA39022)	*OsANS1*	LOC_Os01g27490	66.4
		OsANS2	LOC_Os06g42130	66.2

[a] Accession numbers are shown in parenthesis

[b] Maize F3′H sequence was not available in the public databases

[c] Locus identifier assigned at the rice genome annotation (Nipponbare cultivar)

[d] Amino acid sequence identity to the query protein

[e] A full-length DFR sequence of a purple rice cultivar (AB003496) was used for sequence comparison

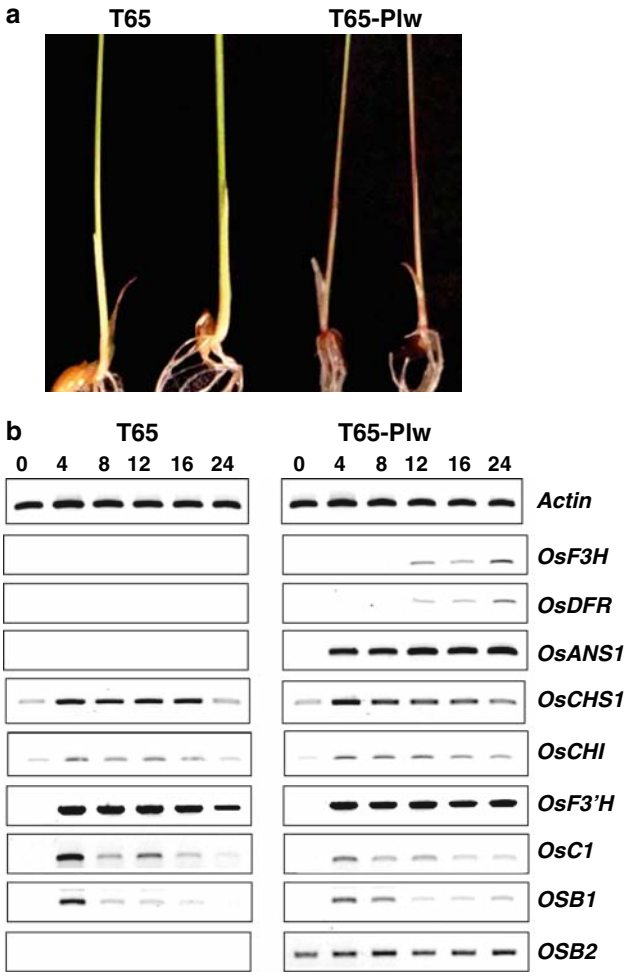


Fig. 2 Accumulation of anthocyanin and expression of flavonoid structural and regulatory genes in illuminated rice seedlings. **a** Seedlings of T65 and T65-Plw at 48 h after light exposure. Rice seeds were germinated in dark for 10 days and then placed under continuous light. Note the anthocyanin pigmentation in the T65-Plw seedlings. **b** Semi-quantitative RT-PCR (28 PCR cycles) expression analyses of flavonoid structural and regulatory genes in rice seedlings at different time points after illumination. Actin expression was used as the internal control. Note the differential light-induced expression of the structural genes *OsF3H*, *OsDFR*, and *OsANS1* in the T65-Plw seedlings. Similarly, *OSB2* was only expressed in the pigmented seedlings, but the expression was light-independent. Expression of *OsCHS1*, *OsCHI*, *OsF3′H*, and *OSB1* were light-inducible. Both T65 and T65-Plw seedlings showed similar expression patterns for these genes

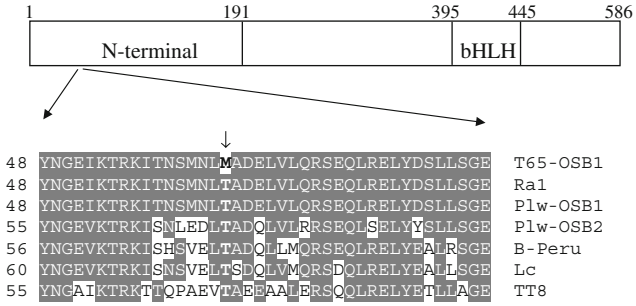


Fig. 3 Alignment of a selected region in the N-terminal interacting domains from different plant anthocyanin-related bHLH regulatory proteins. Amino acid numbering in the schematic diagram is based on the rice Ra1 sequence (AAC49219) and residues conserved in at least three sequences are highlighted. Note the T^{64} M substitution in the T65 OSB1 sequence. The T-64 residue is strictly conserved in maize B-Peru (CAA40544) and Lc (AAA33504), rice Ra1, T65-Plw OSB1 (BAB64301), and T65-Plw OSB2 (BAB64302), and Arabidopsis TT8. The same substitution was also found in the Nipponbare, Zhonghua 11 and Tainong 67 OSB1 sequences (not shown)

including Nipponbare, Tainong 67, and Zhonghua 11. The pigmentation-specific genes *OsF3H*, *OsDFR*, *OsANS1*, and *OSB2* were not expressed in seedlings of all these lines (ESM Fig. S3). On the other hand, *OSB1* expression occurred in a light-inducible manner as observed in T65 seedlings. Similarly, the two unique nucleotide changes identified in T65 *OSB1* are found in the *OSB1* sequences of Nipponbare, Tainong 67, and Zhonghua 11 (data not shown). These results suggested that the lack of anthocyanin accumulation in these non-pigmented rice cultivars is caused by a common molecular background for *OSB1* and *OSB2* genes in the *Pl* locus.

Complementation of Arabidopsis flavonoid mutants

The light-induced expression of *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3′H*, *OsDFR*, and *OsANS1* implicated their roles in the accumulation of flavonoid metabolites in rice seedlings. Most of these flavonoid gene homologs (i.e. *OsCHS1*, *OsCHI*, *OsF3H*, and *OsF3′H*) have not been functionally characterized in planta previously. In this study, the above six rice genes were transformed into the appropriate Arabidopsis *tt* mutants for complementation analysis. Results showed that the abilities of the different transgenic Arabidopsis mutants to accumulate proanthocyanidins (DMACA-positive) in seed coat and anthocyanin pigment under nitrogen deficiency were rescued (Fig. 4a). In addition, transgenic expression of *OsCHS1*, *OsCHI*, and *OsF3H* restored the accumulation of the flavonols quercetin and kaempferol in *tt4*, *tt5*, and *tt6* mutants, respectively (Fig. 4b). On the other hand, transgenic *tt7* mutants over-expressing *OsF3′H* resulted in the accumulation of quercetin (3′,4′-hydroxylated) as the major flavonol while only kaempferol (4′-hydroxylated) was detected in the *tt7* seedlings (Fig. 4b). Taken together, our Arabidopsis complementation analysis provided conclusive evidences that the expressed *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3′H*, *OsDFR*, and *OsANS1* genes all encode functional flavonoid enzymes in planta.

Yeast two-hybrid analysis of interactions among rice flavonoid enzymes

The possibility that an anthocyanin multienzyme complex exists in rice was investigated by yeast two-hybrid analysis

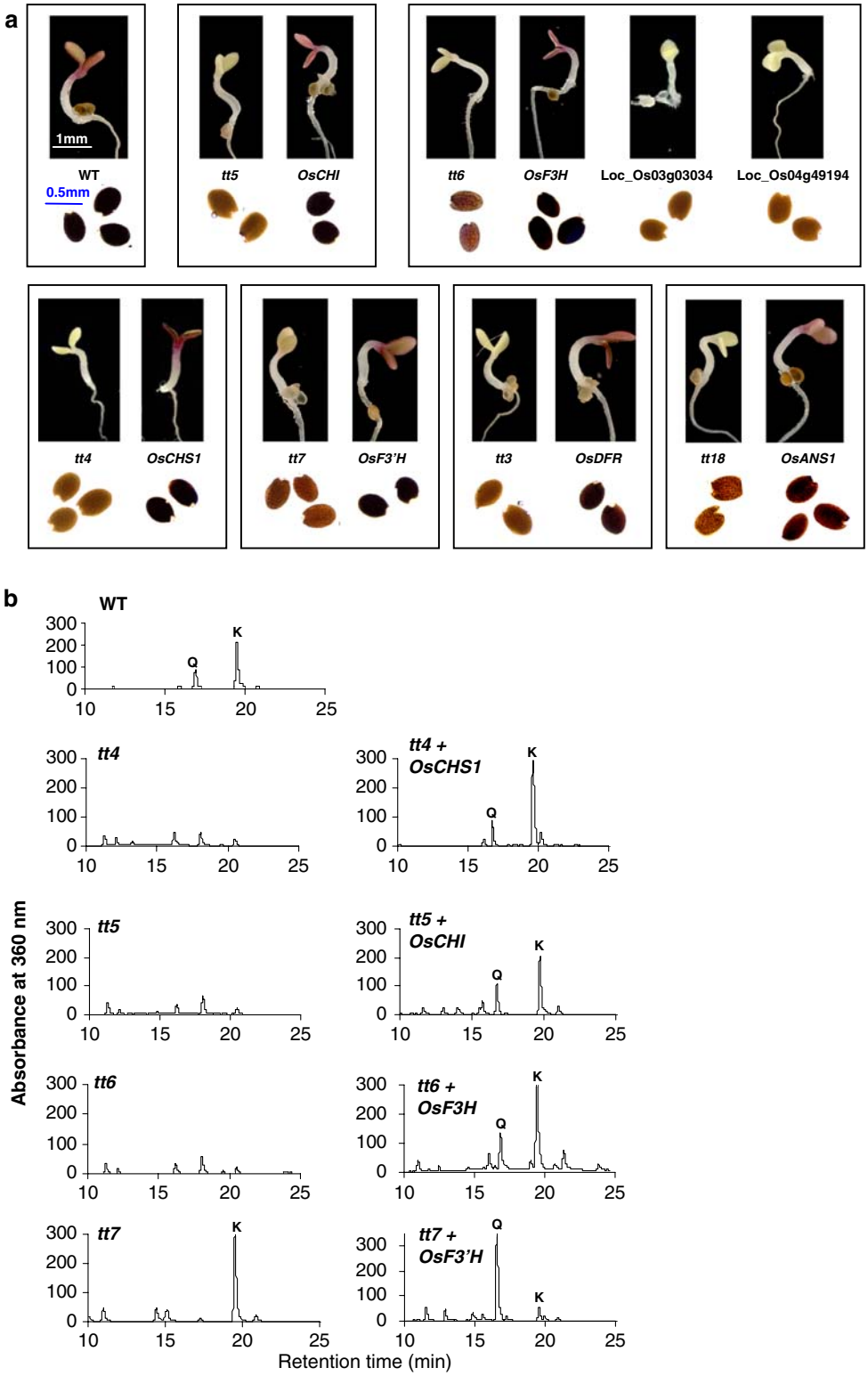
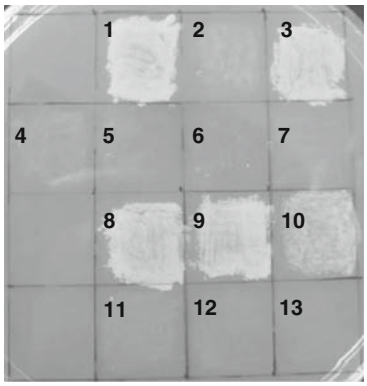


Fig. 4 Complementation analyses of the expressed rice flavonoid structural genes in transgenic Arabidopsis *tt* mutants. **a** Restoration of anthocyanin pigmentation and seed coat color in the transgenic *tt3*, *tt4*, *tt5*, *tt6*, *tt7*, and *tt18* mutants over-expressing *OsDFR, OsCHS1*, *OsCHI*, *OsF3H*, *OsF3′H*, *OsANS1*, respectively. Seedlings were grown in low nitrogen medium to induce anthocyanin accumulation. Seeds were stained with DMACA for detection of condensed tannin. Two closest rice homologs of *OsF3H*, LOC_Os03g03034 and LOC_Os04g49194, were included in the analysis. Both of them failed to complement the *tt6* mutation, indicating that they do not encode functional F3H enzymes. *WT* wild type. **b** HPLC profiles of flavonols (*Q* quercetin; *K* kaempferol) detected in plant extracts. Accumulation of flavonols was restored in transgenic *tt4*, *tt5*, and *tt6* plants over-expressing *OsCHS1*, *OsCHI*, and *OsF3H*, respectively. Quercetin (3′, 4′-hydroxylated) was detected as the predominant flavonol in transgenic *tt7* plants over-expressing *OsF3′H*

of potential interactions of the functional enzymes OsCHS1, OsCHI, OsF3H, OsF3′H, OsDFR, and OsANS1. The rice proteins were tested in all possible pair-wise combinations of GAL4 AD and GAL4 BD fusions. Expression of the reporter gene *His3* was examined by screening the yeast colonies on medium lacking histidine

and supplemented with 3-aminotriazole as described previously (Burbulis and Winkel-Shirley 1999; Owens et al. 2008). Subsequently, the expression of another reporter gene *LacZ* was determined in the histidine prototrophic colonies by quantitative assays using ONPG. To summarize, our results demonstrated the formation of OsCHS1 homodimer since clone 1 (BD-OsCHS1/AD-OsCHS1) showed growth on his$^-$ medium and highest LacZ activities (Fig. 5). In addition, OsCHS1 was found to interact with OsF3H, OsF3′H, OsDFR, and OsANS1 in a specific direction, i.e. only when OsCHS1 was fused with the GAL4 BD while the other four proteins were fused to the GAL4 AD. In contrast, no interactions were observed between OsCHS1 and OsCHI (Fig. 5). Similarly, no reporter gene expressions were detected in yeast colonies carrying all the other combinations of fusion proteins (data not shown), suggesting that there are minimal interactions among OsCHI, OsF3H, OsF3′H, OsDFR, and OsANS1.

a



1: BD-CHS1/AD-CHS1
2: BD-CHS1/AD-CHI
3: BD-CHS1/AD-F3H
4: BD-CHS1/AD
5: BD/AD-CHS1
6: BD/AD-CHI
7: BD/AD-OsF3H
8: BD-CHS1/AD-F3'H
9: BD-CHS1/AD-DFR
10: BD-CHS1/AD-ANS1
11: BD/AD-F3'H
12: BD/AD-DFR
13: BD/AD-ANS

b

Clone	AD fusion	Activity (UβGal)
1	OsCHS1	7.98 ±0.23*
2	OsCHI	4.77 ±0.24
3	OsF3H	7.07 ±0.17*
4	AD vector	5.00 ±0.35
8	OsF3'H1	6.81 ±0.38*
9	OsDFR	6.83 ±0.20*
10	OsANS1	6.17 ±0.45*

Fig. 5 Yeast 2-hybrid analysis of interactions among the functionally validated rice flavonoid enzymes. **a** Growth of yeast cells was assayed on leu$^-$, trp$^-$, his$^-$ medium containing 5 mM 3-aminotriazole for 4 days at 30°C. The mated yeast strains carried the indicated combinations of flavonoid enzymes fused to GAL4 AD or GAL4 BD. **b** Quantification of *β*-galactosidase activity in the mated yeast strains. The activities are shown in mean ± standard deviation of 5 independent yeast colonies. *These values are deemed significantly higher than that obtained for the AD vector control as determined by the Student's *t* test ($P < 0.05$)

Accumulation of flavones in rice seedlings

The expression of *OsCHS1* and *OsCHI* in the non-pigmented T65 seedlings indicated that flavonoids other than anthocyanin pigments are also synthesized. Searching of the rice genome database with the OsF3H sequence suggested that F3H is encoded by a single gene (data not shown). We over-expressed two closest homologs of *OsF3H*, Loc_Os03g03034 (37% identical to OsF3H) and Loc_Os04g49194 (35% identical to OsF3H), in Arabidopsis *tt6* mutants, but neither of them were able to complement the flavonoid deficiency (Fig. 4a). Thus, similar to the *TT6* gene in Arabidopsis, *OsF3H* represents the only F3H-encoding gene in rice. The absence of *OsF3H* expression in T65 seedlings suggested that 3-hydroxylated flavonoids, such as dihydroflavonols, flavonols, and flavan-3-ols, were not present. Consistent with this, these classes of flavonoids could not be detected in seedling extracts by LC-MS/MS (data not shown). In contrast, the flavones tricin and isovitexin were found in acid-hydrolyzed extracts of T65 seedlings, as confirmed by LC retention times and MS/MS spectra in comparison with the authentic standards (Fig. 6a, b). Flavones are one of the few flavonoid classes that do not require F3H activities (Fig. 1). Isovitexin is a C-glucoside of apigenin at position 6 and the glucosidic linkage is resistant to acid hydrolysis treatments. On the other hand, tricin is a 3′, 5′-dimethoxylated flavone. These flavones were also detected in extracts prepared from the pigmented T65-Plw seedlings (data not shown). Time-course metabolite analysis was subsequently performed to compare the accumulation of flavones in the two rice lines. The amounts of isovitexin were similar in both rice lines over time (Fig. 6c). However, tricin levels in the pigmented T65-Plw seedlings were significantly reduced when compared to the non-pigmented T65 seedlings at 72 and 96 h following illumination (Fig. 6c).

Discussion

In Arabidopsis and other dicot plants, expressions of the early biosynthesis genes (EBGs) encoding CHS, CHI and F3H and the late biosynthesis genes (LBGs) encoding DFR, ANS and FGT are regulated separately (Quattrocchio et al. 2006). The same expression pattern was also reported in pigmented wheat coleoptiles following light exposure and the LBGs are controlled by the *Rc* locus (Ahmed et al. 2006). In contrast, all the known flavonoid structural genes in maize are coordinately activated by the regulator proteins C1 and R/B (Irani et al. 2002). Ectopic expression of the maize *C1* and *R/B* genes have been used as a "standard" strategy to activate flavonoid biosynthesis in diverse plant species. Our investigations in pigmented rice seedlings

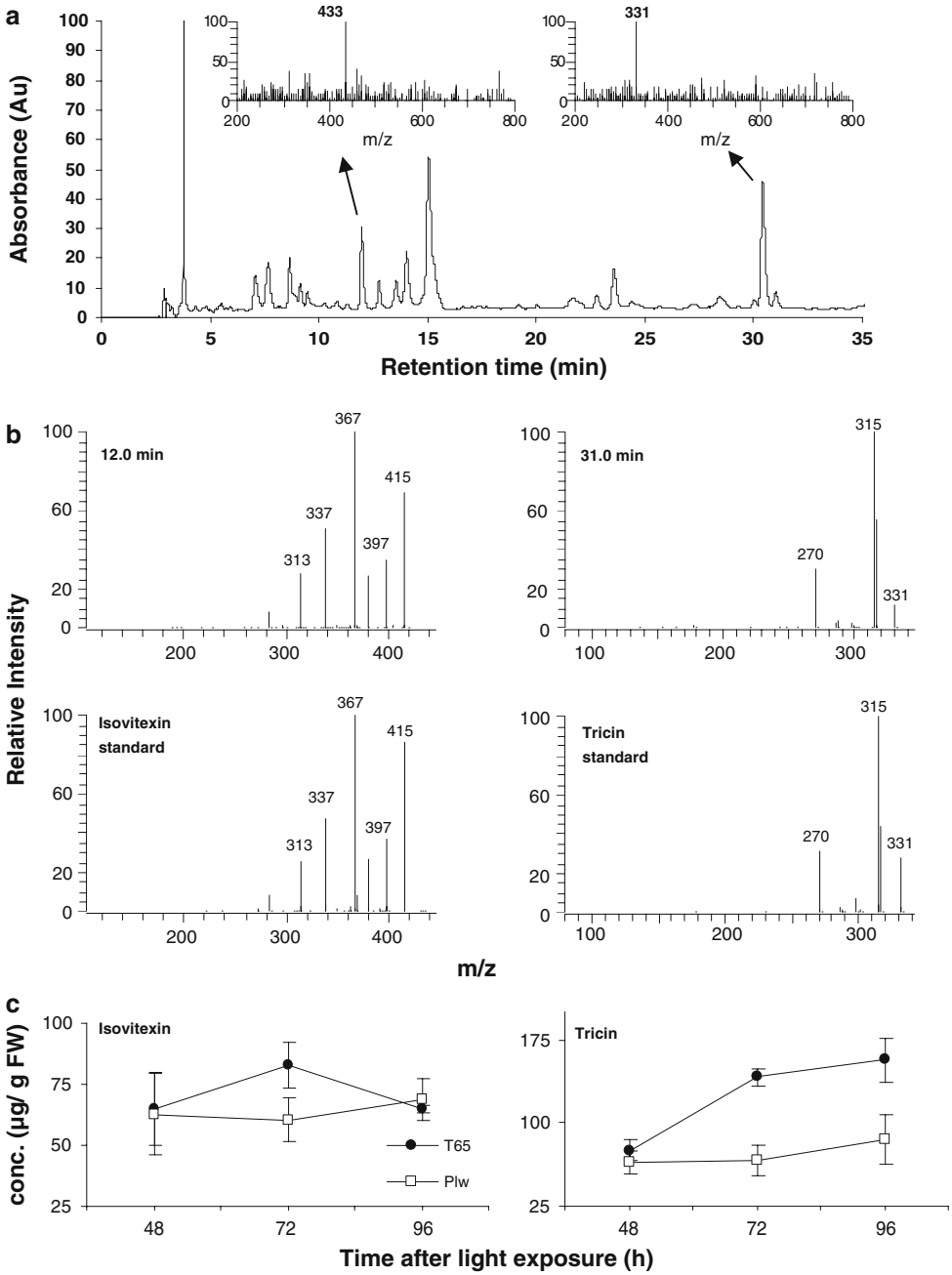


Fig. 6 Accumulation of flavones in rice seedlings. **a** HPLC-MS profile of acid-hydrolyzed extract prepared from 2-week-old T65 seedlings. Major $[M + H]^+$ ions at *m/z* 331 (12.0 min) and *m/z* 433 (31.0 min) consistent with the protonated ions of tricin and isovitexin, respectively, were detected. **b** MS/MS spectra for the *m/z* 331 and 433 ions and the corresponding authentic standards. **c** Time-course accumulation of tricin and isovitexin in T65 and T65-Plw seedlings after illumination. Tricin levels were reduced by at least 50% in T65-Plw seedlings compared to T65 seedlings at 72 and 96 h after light exposure. Isovitexin levels were approximately the same in both rice seedlings

revealed expression patterns of flavonoid structural genes that have not been observed in other plants. Using the isogenic rice lines T65-Plw and T65, we demonstrated that the Pl^w locus, which confers anthocyanin pigmentation, activated only a subset of structural genes in seedlings, namely *OsF3H*, *OsDFR*, and *OsANS1* (Fig. 2b). Interestingly, *OsF3H*, which has been classified as an EBG in dicot plants and wheat, is instead differentially up-regulated with the "LBGs" (i.e. *OsDFR* and *OsANS1*) by the Pl^w locus (Fig. 2b), suggesting a different regulatory mechanism for anthocyanin biosynthesis in rice. On the other hand, the light-induced expression of *OsCHS1*, *OsCHI*, and *OsF3′H* is likely to be controlled by regulatory proteins not directly related to pigmentation but are common in both T65 and T65-Plw seedlings.

The rice Pl^w locus was found to harbor at least two *R*/*B*-type bHLH genes, *OSB1* and *OSB2*, co-segregating with pigmentation in T65-Plw plants (Sakamoto et al. 2001). Transient expression assays showed that either OSB1 or OSB2 could interact with maize C1 to induce pigmentation in rice aleurone cells (Sakamoto et al. 2001). In addition, transgenic expression of *OSB2* alone in the parental line T65 resulted in pigmentation in shoots (Kawahigashi et al. 2007). Our results suggested that the non-pigmented rice

seedlings, including T65, Nipponbare, Tainong 67, and Zhonghua 11, shared a common molecular background in the *Pl* locus that may result in the absence of anthocyanin accumulation. For example, *OSB2* is not expressed in these cultivars while their expressed *OSB1* genes are likely to be non-functional. The single nucleotide deletion close to the 3′-end (ESM Fig. S2) in their *OSB1* coding sequences may not affect the protein function since the functional T65-Plw *OSB1* sequence (Sakamoto et al. 2001) contains a 2-bp deletion further upstream (ESM Fig. S2). However, the T^{64} M substitution (Fig. 3) is located within the N-terminal interaction domain demonstrated to interact with the R3 repeat of the MYB protein C1 for transcriptional activation of flavonoid structural genes (Goff et al. 1992; Grotewold et al. 2000; Hernandez et al. 2004). Multiple alignment of plant anthocyanin-related bHLH sequences revealed that the T-64 residue is strictly conserved in this region (Fig. 3). Whether the single amino acid substitution affects the interaction of OSB1 with OsC1 for transcriptional activation remains to be investigated. Recently, a single amino acid change was found to convert the Arabidopsis bHLH factor AtATR2 from a tryptophan synthesis regulator to a pigmentation inducer (Smolen et al. 2002). In contrast, the ability of a grapevine MYB regulator to activate anthocyanin biosynthesis was abolished due to an amino acid substitution (Walker et al. 2007).

Further dissection of the molecular regulation of anthocyanin biosynthesis controlled by the Pl^{w} locus will be facilitated by the presence of the functionally defined structural genes. Previously *OsCHS1* (Reddy et al. 1996) and *OsCHI* (Druka et al. 2003) were described only based on sequence homology. *OsDFR* (Furukawa et al. 2007) and *OsANS1* (Reddy et al. 2007) were the only rice flavonoid structural genes that have been functionally characterized in transgenic plants. To our knowledge, the biochemical roles of *OsCHS1*, *OsCHI*, *OsF3H*, *OsF3′H* are established in planta for the first time in this work. In addition, we demonstrated that the *tt6* and *tt18* mutants are effective for defining the functions of plant genes encoding F3H and ANS, respectively (Fig. 4). Our results also provided additional evidences that all the flavonoid key enzymes are functionally conserved and exchangeable (Dong et al. 2001) in evolutionary distinct plant species. Moreover, complementation in Arabidopsis *tt* mutants offers a rapid and conclusive analysis for defining the functions of flavonoid structural genes from different plants before meaningful interpretations of their expression profiles and prediction on metabolic flow could be made.

Enzymes that catalyze sequential reactions in primary metabolism (e.g. glycolysis, TCA cycle, fatty acid oxidation, Calvin cycle) are often organized in complexes to facilitate metabolite channeling and sequester toxic intermediates (Winkel 2004). The occurrence of macromolecular assemblies of flavonoid, sinapate, and lignin enzymes was first proposed by Stafford (1974) to explain the control of flux among these competing branch pathways. Direct associations among flavonoid enzymes were first demonstrated in Arabidopsis (Burbulis and Winkel-Shirley 1999). CHS, CHI, and DFR were shown to be involved in orientation-dependent interactions by yeast two-hybrid analysis. In addition, interactions between CHI and F3H in plant cells were shown by affinity chromatography and immunoprecipitation. These findings suggested the existence of a globular complex of flavonoid enzymes with multiple points of contact in Arabidopsis. However, the ability of the rice enzymes to complement Arabidopsis mutations (Fig. 4) opens the argument again as to whether the formation of macromolecular complexes has high influence on flavonoid synthesis (Dong et al. 2001), since conservation between some enzymes in these two plants was only moderate (e.g. 49% identity for ANS). Thus, the physiological and biochemical significance of this organization is worthy of more vigorous investigations in different plant systems. Results from our yeast two-hybrid analysis inferred a different organization of a multienzyme assembly for anthocyanidin biosynthesis in rice. The OsCHS1 homodimer is likely to serve as a common platform for attachment of OsF3H, OsF3′H, OsDFR, and OsANS1 in specific orientations, while physical interactions among the latter enzymes are minimal. The P450 OsF3′H protein may anchor the macromolecular complex to the cytosolic surface of the endoplasmic reticulum (Winkel 2004). Interestingly, OsCHI was not involved in any forms of interactions with the other enzymes, and this is reminiscent of the fact that chalcone isomerization occurs non-enzymatically in some plants (Holton and Cornish 1995). Structural elucidation of the different flavonoid enzymes should facilitate the prediction of protein surfaces and domains that could provide for interactions among the putative multienzyme complex members (Winkel 2004).

F3H is a key branch-point enzyme necessary for the synthesis of 3-hydroxylated flavonoids including dihydroflavonols, flavonols, anthocyanins, flavon-3-ols, and proanthocyanidins (Fig. 1). Thus, the absence of expression of the single-copy *OsF3H* gene in non-pigmented seedlings would limit the types of flavonoids accumulated. In fact, the flavones isovitexin and tricin are the major flavonoids detected (Fig. 6). These flavones have been widely reported as healthy phytochemical constituents in rice hull and bran. Evidently the expression of *OsCHS1* and *OsCHI* provided the flavanone substrates for flavone biosynthesis in both T65 and T65-Plw seedlings. The reduced accumulation of tricin in the pigmented T65-Plw seedlings (Fig. 6c) could be explained by the presence of the competing anthocyanin pathway. Physical association of OsF3H with OsCHS1 would potentially channel more intermediates for the

formation of the 3-hydroxylated anthocyanins. On the other hand, a separate complex composed of different combinations of enzymes may exist for flavone biosynthesis, but the committed reaction step has not been characterized in rice. Two independent flavone synthase (FNS) enzyme systems (Martens and Mithöfer 2005) have been described in dicot for converting flavanones to flavone by introducing a C2–C3 double bond (Fig. 1). The soluble dioxygenase FNS I is found exclusively in members of Apiaceae whereas the cytochrome P450 monooxygenase FNS II is more widespread among other plants. Interestingly, FNS-encoding genes are not found in the genome of the dicot model Arabidopsis, consistent with the nearly absence of flavones in the Brassicaceae (Martens and Mithöfer 2005).

In summary, our work has revealed some unique molecular features of flavonoid biosynthesis in rice. *OsF3H*, *OsDFR*, and *OsANS1* were activated by the Pl^{w} locus, which contains the *OSB1* and *OSB2* regulatory genes, during light-induced anthocyanin accumulation in the T65-Plw seedlings. F3H genes in other plants are often coordinately regulated with CHS and CHI genes as the EBGs. All the non-pigmented rice seedlings investigated showed the absence of *OSB2* gene expression and they contain the same nucleotide changes in the *OSB1* gene. Our yeast two-hybrid results suggested a novel organization of an OsCHS1 homodimer interacting in specific orientations with OsF3H, OsF3′H, OsDFR, and OsANS1 in a macromolecular complex for anthocyanin biosynthesis. Further understanding of the molecular regulation and cellular organization of the flavonoid enzymes is important for engineering pigmentation in rice tissues that normally do not synthesize anthocyanins. In addition, rice seedlings, instead of bran and hull, can be used conveniently for molecular dissection of flavone biosynthesis which remains largely unknown in monocot.

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