**Development of a visible marker trait based on leaf sheath–specific anthocyanin pigmentation applicable to various genotypes in rice**

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To overcome a limitation to the breeding of autogamous crops, recurrent selection using transgenic male sterility (RSUTMS) has been proposed. In this system, negatively or positively selectable marker traits are required along with dominant transgenic male sterility. Anthocyanin pigmentation is an excellent marker trait. Two regulatory genes for MYB and bHLH and a structural gene for DFR are required for anthocyanin pigmentation in rice. Therefore, to apply anthocyanin pigmentation as a marker trait in various rice genotypes, coordinated expression of the three genes is required. In this study, we developed a leaf sheath–specific promoter and introduced three genes—DFR and C1/Myb, driven by the 35S promoter, and OsB2/bHLH, driven by the leaf sheath–specific promoter—into the rice genome. Leaf sheath–specific pigmentation was confirmed in all seven genotypes tested, which included japonica and indica cultivars. Analysis of genome sequence data from 25 cultivars showed that the strategy of conferring leaf sheath–specific anthocyanin pigmentation by introduction of these three genes would be effective for a wide range of genotypes and will be applicable to RSUTMS.

**Key Words:** anthocyanin, *Oryza sativa*, DFR, Myb, bHLH, marker trait, leaf sheath–specific promoter.

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**Introduction**

Genetically fixed cultivars of autogamous crops are generally bred through two-way crosses and genetic fixation by selfing, followed by selection. However, the applicability of this method is critically limited by the number of available parental materials, the frequency of genetic recombination, and continuity of breeding effect (Fujimaki 1980). To overcome these limitations, recurrent selection using transgenic male sterility (RSUTMS) was proposed (Tanaka 2010). RSUTMS is a novel breeding system based on outcrossing in autogamous crop species that effectively makes use of genomic information, such as the genomic selection. To establish this breeding system, the development of dominant transgenic male sterility (TMS) is required to eliminate self-pollination. Application of dominant TMS has the advantage that efficient and continuous outcrossing can be realized due to the high frequency (theoretically 50%) of male sterile individuals. A gene cassette carrying a negatively or positively selectable marker with dominant TMS is required to eliminate colored diploids (Nanda and Chase 1965, Rotarenco et al. 2010). Because the mechanism of anthocyanin pigmentation is well understood (see below), pigmentation patterns could be controlled in an organ- and/or growth stage–specific manner. Anthocyanins are predominantly accumulated in epidermal cells of purple-leaf rice, and they absorb...
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visible radiation between 400 and 700 nm, with a maximum of about 530 nm (Peng et al. 2006). In maize, Pietrini and Massacci (1998) estimated that 43% of light energy was absorbed by the anthocyanin pigments in leaves with 35 times higher amount of anthocyanins than control leaves. Thus, anthocyanin pigmentation has a negative effect on photosynthesis and, therefore, plant growth in the field. Therefore, to use anthocyanin pigmentation as an easily recognized marker trait in rice, it is necessary to accumulate the pigments in organs other than the leaf blade, which is the main photosynthetic organ.

The anthocyanin pigmentation trait in maize has been well investigated over the last century (Emerson 1921). It is controlled by two regulatory gene families: C1/Purple plant (Pl), encoding R2R3 MYB-related transcription factors (TFs), and R/B, encoding basic helix-loop-helix (bHLH) MYC-type TFs. A combination of the MYB and bHLH proteins coordinately activates transcription of a set of genes for anthocyanin biosynthesis. Moreover, the expression patterns of both Myb and bHLH genes are tissue and developmental stage specific (Dooner et al. 1991, Grotewold et al. 2000, Petroni and Tonelli 2011, Schwinn et al. 2006). Although typical field-grown rice cultivars have green leaves, several accumulate anthocyanins and/or proanthocyanidins in various tissues, including leaf blades, sheaths, pericarps, and apiculi. Studies of the genetics of anthocyanin biosynthesis and accumulation in rice also have a long history (Morinaga 1938, Takezaki 1921); they have revealed that the pigmentation of various rice tissues is controlled mainly by three types of dominant genes: C (Chromogen), A (Activator), and their tissue-specific regulators including P (Purple), Pl (Purple leaf), etc. (Nagao and Takahashi 1963, Takahashi 1957). Further genetic analyses have uncovered additional loci that determine tissue-specific distribution and accumulation of anthocyanins and proanthocyanidins (Reddy 1996, Sakamoto et al. 2001, Takahashi 1982). In the C-A-P/Pl system of cultivated rice, the C gene corresponds to OsC1, a homolog of maize C1 (Reddy et al. 1998, Saitoh et al. 2004). Two independent genes from the Pl alleles have been identified; they encode R/B (bHLH) homologs (Hu et al. 1996, Sakamoto et al. 2001). Furukawa et al. (2007) demonstrated that the A gene encodes dihydroflavonol 4-reductase (DFR) in the anthocyanin biosynthesis pathway. Many modern Japanese rice cultivars, including ‘Nipponbare’, have a loss-of-function allele of the DFR gene (Furukawa et al. 2007, Nakai et al. 1998, Sun et al. 2018). Various mutations in the OsC1 gene have also been found in rice (Saitoh et al. 2004, Sun et al. 2018). Zhu et al. (2017) demonstrated the accumulation of anthocyanins in the endosperm of rice highly expressing six structural genes from coleus and the above two TF genes (C1 and R/B) from maize. In light of these facts, the accumulation of anthocyanins in the leaf blades and sheaths of rice is likely to require the introduction of at least three genes encoding DFR, MYB, and bHLH, preferably under the control of a tissue-specific promoter(s).

In this study, we first focused on the leaf sheath, as it is an organ in which it is easy to detect pigmentation and which can accumulate anthocyanins with little negative influence on photosynthesis. Next, we developed a leaf sheath–specific promoter by using an expression profile database of rice (Akasaka et al. 2018). Then, by simultaneously introducing three genes: the DFR and maize C1/Myb genes driven by the CaMV 35S promoter and the OsB2/bHLH gene driven by a leaf sheath–specific promoter into the rice genome, we conferred anthocyanin pigmentation of the target organ (leaf sheath) in various japonica and indica rice genotypes. Finally, we discuss the potential applications of this ‘three genes introduction strategy’.

Materials and Methods

Plant materials

Seven rice cultivars (japonica: ‘Koshihikari’, ‘Kusahonami’, ‘Momiroman’, ‘Nipponbare’, and ‘Tachioba’; indica: ‘Kasalath’ and ‘Tachisugata’) were used in this study. All plants were grown in commercial soil (Bonsol No. 1, Sumitomo Chemical, Tokyo, Japan) under simplified Biotron Breeding System conditions (Tanaka et al. 2016) or in a closed greenhouse, at 28°C in the daytime and 22°C at night.

Screening for leaf sheath–specific promoters

The leaf sheath–specific promoters were searched for using the method described in Akasaka et al. (2018). Sixteen candidate genes specifically expressed in the leaf sheath were selected in the rice expression profile database RiceXPro (Sato et al. 2011, 2013; http://ricexpro.dna.affrc.go.jp). From these candidates, three representative genes with no or extremely low expression levels in other tissues were selected. Approximately 2-kb sequences upstream of these three genes were cloned and the fragments were used as leaf sheath–specific promoters (LSSP) 01, 02, and 03 (Table 1).

Polymerase chain reaction

Genomic DNA was isolated from leaves according to Edwards et al. (1991). LSSP01, LSSP02, and LSSP03 sequences with restriction sites (AscI or HindIII and XbaI) were amplified from genomic DNA of ‘Nipponbare’ using the corresponding sets of specific primers (Supplemental Table 1). Point mutations were introduced in LSSP01 and LSSP02 PCR to eliminate restriction sites (Supplemental Table 1). PCR amplification was performed using PrimeSTAR GXL DNA Polymerase (Takara Bio, Shiga, Japan) in a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 98°C for 15 s, 55°C for 30 s, and 68°C for 2 min; and final extension at 68°C for 5 min.

To detect the inserted gene in transformants, PCR was performed using the primers HPT-F01 and HPT-R01 (Supplemental Table 1) under the following conditions: initial

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denaturation at 94°C for 5 min; 30 cycles of 98°C for 15 s, 55°C for 30 s, and 68°C for 50 s, and final extension at 68°C for 5 min.

**Vector construction and rice transformation**

ZmC1 (Acc. No. AF320613), a C1 family gene (Dooner et al. 1991), OsB2 (Acc. No. AB021080), an R family gene (Sakamoto et al. 2001) and OsDFR (Acc. No. AB003496), an anthocyanin synthesis gene (Furukawa et al. 2007, Nakai et al. 1998) were used in this study.

The LSSP01, LSSP02, and LSSP03 promoters were individually fused to OsB2. These fragments and the double terminator DT (35S terminator + nos terminator; Luo and Chen 2007) were introduced into the pUC198AM subplasmid (Kuroda et al. 2010). The CaMV 35S promoter (35SP) and DT were fused with each of OsB2, ZmC1, and OsDFR, and each of the resulting fragments was also introduced into pUC198AM. These gene cassettes were removed from the sub-plasmid using Ascl and MluI restriction enzymes and introduced into the pZH2B binary vector (Kuroda et al. 2010) in various combinations (Fig. 1). Vector p35SP::B2/C1 carried two gene cassettes for anthocyanin pigmentation: 35SP::OsB2::DT and 35SP::ZmC1::DT. Vector p35SP::DFR/B2/C1 carried three cassettes: 35SP::OsB2::DT, 35SP::ZmC1::DT, and 35SP::ZmDFR::DT. The pOsLSSP01, pOsLSSP02, and pOsLSSP03 constructs contained cassettes carrying 35SP::OsDFR::DT, 35SP::ZmC1::DT, and LSSP (01, 02, or 03, respectively)::OsB2::DT (Fig. 1). To generate pLSSP03::GUS, we fused the GUS gene from pIG121-Hm (Acc. No. AB489142; Ohta et al. 1990) with the LSSP03 promoter, and introduced this fragment and DT into pZH2B (Fig. 1).

All of the plasmids were introduced into Agrobacterium tumefaciens strain EHA105 (Hood et al. 1993), and rice was transformed as described in Ozawa (2009). The T1 seedlings were grown on MS medium (Murashige and Skoog 1962) under continuous white fluorescent light at 27°C; 3 weeks after germination, seedlings were transferred to a closed greenhouse.

**Table 1. Leaf sheath–specific promoters developed in this study**

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<thead>
<tr>
<th>Promoter</th>
<th>RAP locus ID</th>
<th>Accession no. of cDNA</th>
<th>Putative gene product</th>
<th>Level of gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSSP01</td>
<td>Os08g0460000</td>
<td>AK065284</td>
<td>Similar to Germin-like protein 1 precursor</td>
<td>200,920</td>
</tr>
<tr>
<td>LSSP02</td>
<td>Os02g0308400</td>
<td>AK104655</td>
<td>Beta-lg-H3/fasciclin domain containing protein</td>
<td>62,520</td>
</tr>
<tr>
<td>LSSP03</td>
<td>Os06g0639800</td>
<td>AK106068</td>
<td>Cytochrome P450 family protein</td>
<td>35,004</td>
</tr>
</tbody>
</table>

Fig. 1. Structure of the T-DNA region in the binary vectors p35SP::B2/C1, p35SP::DFR/B2/C1, pOsLSSP01, pOsLSSP02, pOsLSSP03, and pLSSP03::GUS. 35SP: promoter from the CaMV 35S RNA gene; mHPT: modified hygromycin phosphotransferase gene with deleted restriction enzyme sites; Tnos: terminator from the nopaline synthase gene; LSSP: leaf sheath–specific promoter; OsB2: B2 gene from O. sativa; DT: double terminator from the CaMV 35S RNA and nopaline synthase genes; ZmC1: C1 gene from Z. mays; OsDFR: dihydroflavonol 4-reductase gene from O. sativa; GUS: β-glucuronidase gene; LB and RB: left and right borders, respectively, of the T-DNA; XbaI: XbaI restriction enzyme sites used in Southern blot analysis. Arrows indicate HPT-specific primers (HPT-F01 and HPT-R01).
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**GUS assay**

GUS assay was performed as described in Konagaya et al. (2008) with minor modifications; plant tissues were incubated for 24 h at 37°C in GUS assay solution and bleached with 70% ethanol.

**Southern blot analysis**

Southern blot assay was performed as in Abe et al. (2018) with minor modifications. Genomic DNA was isolated from leaves of ‘Nipponbare’ and T₀ plants carrying pOsLSSP03 using the CTAB method (Murray and Thompson 1980). Genomic DNA (10 µg) was digested with XbaI. A 529-bp fragment of the HPT gene was amplified with HPT-F01 and HPT-R01 primers (primer positions are indicated in Fig. 1) and used as a probe.

**Genome sequence comparison of structural genes for anthocyanin biosynthesis**

The presumed anthocyanin synthesis pathway is shown in Fig. 2. The Rice Annotation Project Database (RAP-DB, Sakai et al. 2013; http://rapdb.dna.affrc.go.jp/) was used for searching the genes, and the open reading frame (ORF) sequences of retrieved genes were compared in TASUKE (Kumagai et al. 2013; http://ricegenomes.dna.affrc.go.jp/RiceGenomes/) with the next generation sequence (NGS) data (Table 2). The NGS data from five genotypes—‘Kasalath’, ‘Koshihikari’, ‘Nipponbare’, ‘Tachiaoba’, and ‘Tachisugata’—are available and were used in this analysis. In addition, the NGS data from ‘Taichung 65’ (‘T65’), which has anthocyanin pigmentation owing to the introgression of DFR, OsC₁, and OsB₂ (Hirose et al. 2008), and *Oryza rufipogon* ‘W0120’, which has anthocyanin pigmentation in the anthers (Kurata and Yamazaki 2006; http://www.shigen.nig.ac.jp/rice/oryza/base/top/top.jsp), were used. The NGS data from 16 rice genotypes (11 temperate *japonica*, 2 tropical *japonica*, 3 indica; selected on the basis of NGS data quality (average sequencing depth > 25)) were used to check the natural variation of the ORFs of the structural genes related to anthocyanin biosynthesis (Table 2). In addition, NGS data from ‘IR 8’ and ‘IR 64’ were used despite their lower read depth because these cultivars are historically important.

**Results**

**Anthocyanin pigmentation in calluses with introduced OsDFR, ZmC₁, and OsB₂ genes driven by the CaMV 35S promoter**

First, we introduced each construct (OsDFR, ZmC₁, and OsB₂) under the control of the CaMV 35S promoter (35SP) into ‘Nipponbare’ separately. However, no anthocyanin pigmentation was observed in any calluses or regenerated plants. We also introduced the tandem cassette vector p35SP::B2/C1 (Fig. 1), in which ZmC₁ and OsB₂ are driven by 35SP, but no anthocyanin pigmentation was observed (Fig. 3). However, when we introduced the p35SP::DFR/B2/C1 vector, which contains the OsDFR, ZmC₁, and OsB₂ cassettes in tandem, all controlled by 35SP, calluses with and without anthocyanin appeared 2 weeks after *Agrobacterium* infection. However, anthocyanin-containing calluses transferred onto regeneration medium rapidly turned brown, stopped cell division, and did not re-differentiate.

The p35SP::B2/C1 vector was also introduced into six other cultivars (‘Kasalath’, ‘Koshihikari’, ‘Kusahonami’, ‘Momiroman’, ‘Tachiaoba’, and ‘Tachisugata’). Only the indica cultivars (‘Kasalath’ and ‘Tachisugata’) accumulated anthocyanin in calluses (Fig. 3). On the other hand, when the p35SP::DFR/B2/C1 vector was introduced into these six cultivars, the calluses of all of them accumulated anthocyanin (Fig. 3). However, similar to ‘Nipponbare’, no regenerated plants could be obtained from anthocyanin-containing calluses.

**Features of T₀ transgenic calluses and regenerated plants**

To avoid anthocyanin pigmentation in calluses and to prevent the negative effect of accumulated anthocyanin on photosynthesis, we explored novel leaf sheath–specific promoters, which we named LSSP01, 02 and 03 (Table 1), and used them to construct the binary vectors pOsLSSP01, pOsLSSP02, and pOsLSSP03.
Table 2. Estimation of the functions of the enzyme genes related to anthocyanin biosynthesis on the basis of NGS data

<table>
<thead>
<tr>
<th>Cultivar/strain</th>
<th>Variety</th>
<th>PAL</th>
<th>C4H</th>
<th>4CL</th>
<th>CHS</th>
<th>CHS</th>
<th>CHS2</th>
<th>CHI</th>
<th>CHI</th>
<th>F3H</th>
<th>F3H</th>
<th>F3H</th>
<th>F3H</th>
<th>F3H</th>
<th>DFR</th>
<th>ANS</th>
<th>Accession number</th>
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a Cultivars in which anthocyanin accumulation was recovered by the introduction of three genes, DFR, maize C1, and OsB2, in this study.

b Hirose et al. (2008) achieved anthocyanin accumulation by introducing OsB2 into an isogenic line in the T65 genetic background that contained the DFR and OsC1 loci from ‘Murasakiine’ (a purple-leaf rice cultivar). Anthocyanin pigmentation was observed in the stigma of this accession (https://shigen.nig.ac.jp/oryzabase/imageGallery/list?fqTags=tagForFacet%3AFLOWER).

c Anthocyanin pigmentation in these strains, which have three isoforms, indicates that at least one of the genes is functional.

d TEJ, temperate japonica; IND, indica; TRJ, tropical japonica. Est. F, allele estimated to be functional; these genes have no mutations affecting amino acid sequences. A.A.S., allele with amino acid substitution(s) in comparison with the presumed functional allele. L.O.F., loss-of-function allele.

(t) and (k) indicate the alleles carrying ‘Toride 1’-type and ‘Koshihikari’-type loss-of-function mutations, respectively, in the DFR gene (Nakai et al. 1998).
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Carrying pOsLSSP03, a few accumulated anthocyanin (Fig. 4E); in regenerated plants, anthocyanin accumulation was observed only in the leaf sheath (Fig. 4F, 4G). The pOsLSSP03 vector was also introduced into six other cultivars. The resultant calluses of all cultivars showed anthocyanin accumulation. The regenerated plants accumulated anthocyanin in the leaf sheath (Supplemental Fig. 1); in comparison with ‘Nipponbare’, anthocyanin pigmentation was stronger in ‘Momiroman’ and ‘Kasalath’ and similar or weaker in the other cultivars. Anthocyanin pigmentation was very clear at the juvenile phase but weakened later (Fig. 4F, 4G, Supplemental Fig. 1). In some cultivars, anthocyanin pigmentation was also observed in the ligules, auricles, apiculi, and stigmata (Supplemental Fig. 1).

Analysis of the tissue specificity of the LSSP03 promoter in rice revealed GUS staining in a few calluses and only in the leaf sheath of regenerated plants (Fig. 5); this pattern coincided with anthocyanin pigmentation in calluses and regenerated plants carrying pOsLSSP03.

Genetic segregation and phenotypes of T1 pOsLSSP03 transgenic plants

Southern blot analysis was carried out to select regenerated plants carrying pOsLSSP03 in one locus. A single fragment was detected in the T0 plant pOsLSSP03#16 and multiple fragments were detected in the T0 plants pOsLSSP03#20 and #34 (Supplemental Fig. 2). In T1 seedlings derived from pOsLSSP03#16, segregation of leaf sheath–specific anthocyanin pigmentation was observed (Fig. 6A, 6B), and anthocyanin pigmentation coincided with the presence of the transgene detected by PCR (Fig. 6C).

Some of the T1 plants with the transgene showed poorer growth than the wild type, but others showed similar growth (Fig. 7). In T2 populations derived from T1 plants with poorer growth, all plants showed anthocyanin pigmentation. In T2 populations derived from T1 plants with wild-type growth, genetic segregation of anthocyanin pigmentation was observed (Supplemental Table 2).

Natural variations in the ORFs of enzyme genes related to anthocyanin biosynthesis

A RAP-DB search revealed 16 loci involved in anthocyanin biosynthesis, including 4 CHS loci (including CHS2), 3 CHI loci, and an F3H locus (Fig. 2, Table 2).

In all cultivars tested, anthocyanin pigmentation was induced by DFR and the two TFs; this means that the cultivars have active alleles of all genes for enzymes of the anthocyanin biosynthesis pathway except DFR. We used the NGS data from five cultivars (‘Kasalath’, ‘Koshihikari’, ‘Nipponbare’, ‘Tachiaoba’, and ‘Tachisugata’), ‘T65’, and the O. rufipogon accession ‘W0120’, which we defined as the “seven primary genotypes” having the active anthocyanin biosynthesis pathway (other than DFR). We compared the other 18 genotypes with the “seven primary genotypes” in TASUKE and detected many natural variations in the ORFs of the genes involved in anthocyanin biosynthesis.
generally localized in plastids) or type III peroxidases (POD; localized in the vacuoles and apoplasts) (Pourcel et al. 2007, Takahama 2004). Such quinones are highly reactive with phenols, amino acids, or proteins, and generate a complex mixture of brown products (Pourcel et al. 2007).

Oxidative browning in cultured cells often results in growth retardation and inhibition of plant regeneration (Dong et al. 2016, Jones and Saxena, 2013, Laukkanen et al. 2000).

All regenerated plants carrying pOsLSSP01 accumulated anthocyanin in the whole body and grew poorly (Fig. 4B). Anthocyanin biosynthesis and accumulation are increased by various environmental factors, including UV, high light intensity, low temperature, and depletion of sucrose and other nutrients (Petroni and Tonelli 2011, Pourcel et al. 2007). Anthocyanin biosynthesis is also induced by jasmonate (JA) (De Geyter et al. 2012, Qi et al. 2011, Schweizer et al. 2013), and activation of the anthocyanin biosynthesis pathway could upregulate genes for JA biosynthesis and signaling (Pourcel et al. 2013). Activation of JA biosynthesis and signaling inhibits plant growth by inhibiting cell cycle progression (Noir et al. 2013, Pauwels et al. 2008, Świątek et al. 2002, Zhang and Turner 2008) and photosynthesis (Attaran et al. 2014). Therefore, when anthocyanin pigmentation is used as a marker trait, accumulation of the pigments needs to be limited to tissue(s) where it is easily detectable other than leaf blades, which are the primary sites of photosynthesis.

**Discussion**

The cause of inferior growth and regeneration in highly pigmented rice calluses

Transgenic rice calluses constitutively overexpressing OsB2, ZmCl, and OsDFR genes accumulated high amounts of anthocyanins (Fig. 3), but they grew more slowly than wild-type calluses and hardly regenerated. Flavonoids, including anthocyanins, are synthesized via the phenylpropanoid pathway and accumulated in vacuoles. Under stress or senescence, these phenolic compounds can be oxidized into corresponding quinones by polyphenol oxidases (PPO; of 15 loci except DFR, but these variations (SNPs and indels) were included in the “seven primary genotypes”. No mutations that could be responsible for the loss of enzymatic activity, such as insertions, deletions, or frame shifts, were detected (Table 2).

**Phenotypic variation among the transformants**

The degree of anthocyanin pigmentation in the leaf sheath depended on the genotype. Pigmentation was stronger in ‘Momroman’ and ‘Kasalath’ than in ‘Nipponbare’. Depending on the cultivar, pigmentation was also observed in ligules, auricles, apiculi, and stigmata (Supplemental Fig. 1).

In rice, pigmentation of various tissues is controlled
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mainly by the C, A, and P/Pl genes (Nagao and Takahashi 1963, Takahashi 1957). Several genetic variants of the TF genes C and Pl have been found. The OsC1 (C) gene has 13 haplotypes, of which 9 are non-functional (Sun et al. 2018). Allelic variations were also found in Pl (OsB1/B2) genes (Sakamoto et al. 2001, Shih et al. 2008). Tissue and temporal specificity and the levels of expression of the genes for MYB and bHLH play important roles in anthocyanin pigmentation and its distribution (Consonni et al. 1993, Ludwig et al. 1989, Sakamoto et al. 2001, Schwinn et al. 2006). This complexity confers extensive natural variation in the pigmentation profiles to a wide range of genotypes. In maize, the regulation of the anthocyanin pathway involves a direct interaction of MYB and bHLH (Goff et al. 1992). In rice, Sun et al. (2018) showed that the S1/bHLH protein, which is also called OsB2 or Kala4 (Oikawa et al. 2015, Sakamoto et al. 2001), interacts with OsC1/MYB, and that the two factors coordinately activate the expression of the DFR/A gene through direct binding to its promoter.

Because ‘Kasalath’ has lost OsC1 (C) function (Sun et al. 2018), we assumed that its strong anthocyanin pigmentation might be caused by the interaction between its own P/Pl gene or the other endogenous genes of TFs in its genome and the introduced ZmC1 gene. Similarly, ‘Nipponbare’ has no functional OsC1 and DFR genes (Furukawa et al. 2007, Nakai et al. 1998, Sun et al. 2018), the intermediate pigmentation might be caused by the interactions between the introduced genes and endogenous TFs. Especially, there are many genes for the bHLH family in the rice genome (RAP-DB, Sakai et al. 2013; http://rapdb.dna.affrc.go.jp/) and some of them regulate the tissue specificity of anthocyanin biosynthesis in the presence of the active DFR and MYB TF(s). The differences in the degree and site of anthocyanin pigmentation among cultivars are likely due to the interaction

Fig. 6. Transgene identification by leaf sheath–specific anthocyanin pigmentation in pOsLSSP03 plants. (A, B) pOsLSSP03#16 T1 seedlings (A) with or (B) without anthocyanins in their leaf sheaths. (C) Genomic PCR analysis of T1 plants transformed with pOsLSSP03. M: 1-kbp DNA Ladder One marker (Nacalai Tesque, Kyoto, Japan); Purple LS: plants with purple leaf sheath; Green LS: plants with green leaf sheath; Vector: pOsLSSP03 vector as a positive control; Nipponbare: a negative control. The location of mHPT-specific primers is indicated in Fig. 1.

Fig. 7. Growth of a pOsLSSP03 transgenic plant (T1). Left: ‘Nipponbare’; right: pOsLSSP03#16 plant (T1). Scale bar, 10 cm.
between the introduced and these endogenous TFs.

Anthocyanin pigmentation was clearly observed at the juvenile stage, and became faint at later stages. Because the interaction between bHLH and MYB is required for anthocyanin pigmentation, this decrease might be due to the differences in expression in terms of micro-tissue specificity and timing between OsB2 driven by LSSP03 and ZmC1 and OsDFR driven by the 35SP.

**Inferior growth of plants homozygous for pOsLSSP03**

No segregation of anthocyanin pigmentation was observed in the T2 populations derived from T1 plants that showed inferior growth (Supplemental Table 2), likely because the T1 plants were homozygous for the transgenes. The segregation in the T2 populations derived from T1 plants with normal growth likely resulted from the heterozygosity of the T1 plants. Homozygosity of the transgene that inhibited growth in the T2 plants was likely caused by the negative effect of the 35SP–driven Myb gene and its dose effect. Generally, overexpression of TF genes inhibits cell and plant growth, as it disturbs the coordinated expression of many genes. Overexpression of the Myb gene in Arabidopsis thaliana and Brassica napus inhibits growth (Cominelli et al. 2008, Ren et al. 2012). Anthocyanin pigmentation may also inhibit photosynthesis and therefore plant growth by hindering absorption of light (Pietrini and Massacci 1998).

To develop useful rice lines for practical RSUTMS, it will be necessary to consider alternative promoters to replace 35SP. RSUTMS would allow allogamous-based breeding even in autogamous crops such as rice. In this system, dominant male sterility and genes for the marker trait are maintained as heterozygotes in the progeny of crosses with other materials (Tanaka 2010). Therefore, in RSUTMS, the inferior growth observed in homozygotes for the transgenes for anthocyanin pigmentation will not be a significant issue.

**Toward the application of a dominant visible marker trait in RSUTMS**

A marker trait for RSUTMS needs to be applicable to many genetically diverse genotypes. In this study, application of the three genes introduction strategy conferred leaf sheath pigmentation in all seven genotypes tested (Supplemental Fig. 1). Since anthocyanin pigmentation using LSSP03 and the three genes introduction strategy can be applied for efficiently discriminating the male sterile individuals from fertile ones at the juvenile stage before transplanting to the field. However, anthocyanin pigmentation in the leaf sheath of some indica cultivars (Reddy et al. 1995) would interfere with accurate detection. For such materials, it is necessary to reconsider the tissue specificity of the pigmentation.

By comparing the NGS data of 18 cultivars, we found only a few differences between 16 of them and the above 7 genotypes, with no mutations that could result in a loss of function. Although this result does not guarantee the function of all anthocyanin biosynthesis pathway–related genes, the upstream part of the pathway is also involved in the biosynthesis of important secondary metabolites, including lignin, catechins, and flavonoids, and a loss of function of these genes would cause serious growth defects (Huang et al. 2010) or a clear phenotype such as the golden hull trait in rice (Hong et al. 2012). Therefore, we believe that the three genes introduction strategy will be effective for widely diverse genotypes. Scrutinizing the sequences of the genes of founder materials by Sanger sequencing or NGS before starting the crossings for RSUTMS should provide practical reassurance.

The three gene introduction strategy described here can be further improved, for example by exchange improved promoter driven Myb from 35SP. Male sterility gene cassettes have been developed (Abe et al. 2018, Akasaka et al. 2018), and the development of a positive or negative selectable marker trait is the last important step toward the effective application of RSUTMS. The three genes introduction strategy will be ideal for the introduction of a dominant visible marker trait that can be used as a positive or negative selectable marker. If efficient outcrossing could be realized with easy identification of the male-sterile individuals, even autogamous crops would be able to perform outcrossing-based breeding using genomic information, as in maize, and the application of this strategy to RSUTMS will help to overcome the limitations in rice breeding.

**Acknowledgments**

We thank Prof. W. Sakamoto of Okayama University and Dr. K. Kadowaki and Dr. H. Saika of Institute of Agrobiological Sciences for providing OsB2, ZmC1, and OsDFR. We also thank Prof. M. Maekawa of Okayama University and Prof. I. Takamure of Hokkaido University for their productive advice about anthocyanin biosynthesis, and Dr. A. Miyao of Institute of Crop Science, NARO, and Dr. Y. Kawahara and Dr. H. Sakai of the Advanced Analysis Center, NARO, for their technical advice on access to and handling of NGS data. We also thank J. Shiota, Y. Iguchi, M. Yamao, I. Kawaguchi, and C. Itoh from Institute of Crop Science or Institute of Agrobiological Sciences, NARO, for their technical help. We thank Dr. N. Suzuki of Sophia University, Japan, for revision of the English in the manuscript. This work was partially supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation GMO-1001).

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