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ORIGINAL PAPER

Genetic and molecular analysis of a purple sheath somaclonal mutant in *japonica* rice

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Abstract Natural and artificially induced mutants have provided valuable resources for plant genetic studies and crop improvement. In this study, we investigated the genetic and molecular basis of the purple sheath trait in a somaclonal mutant Z418, which was regenerated from a green sheath rice variety C418 through tissue culture. The purple sheath trait in Z418 was heritable and stable based on our 10 years of evaluation. Genetic analysis revealed that the purple sheath trait of the mutant was controlled by a single dominant gene. To map the gene, we scored 89 polymorphic SSRs markers in a F_2 population of 232 plants derived from a cross between Z418 and HX-3, an *indica* variety with green sheath trait. The gene was initially mapped to the short arm of chromosome 6 between two SSR markers, RPM5 and RM402, with a genetic distance of 1.1 and 10.3 cM, respectively. Thirty-one SSR and indel markers located within the target region were further used to fine-map the gene to a 153-kb interval between two SSR markers (RPM8 and RPM11). The *OsC1* gene, which locates within the region and encodes a MYB family transcription factor, was chosen as the candidate gene controlling the purple sheath trait in Z418. Sequencing analysis revealed that *OsC1* gene and its transcript in Z418 was 34 bp longer than that in C418. The possible mechanisms for the gene mutation, the developmental and tissue-specific expression of purple anthocyanin pigmentation in Z418, were finally discussed.

Keywords Somaclonal mutant · Purple sheath · Rice · Regulatory gene

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Introduction

Somaclonal variation induced by in vitro culture has often been observed in cultured cells and regenerated plants (Larkin and Scocroft 1981; Evans et al. 1984; Jain 2001). Such variation may present as a problem in the process of plant transformation and micropropagation of true-to-type clones, as undesirable mutations may reduce the agronomic value of the cultivars or hinder the incorporation of transgenes into plant genomes (Bao et al. 1996; Bregitzer et al. 1998; Martins et al. 2004; Toki et al. 2006). On the other hand, somaclonal variation leads to the creation of genetic variability, and can generate valuable genotypes to plant breeders. Under the continued pressure to develop sustainable food production, somaclonal mutants have provided valuable genetic

resources for crop breeding (Larkin and Scocroft 1981; Evans et al. 1984; Breiman et al. 1987; Rodríguez López et al. 2004). The occurrence, principle and application of somaclonal variation have become the important topics in plant breeding, plant micropropagation as well as theoretical genetic studies.

Somaclonal variation in rice was firstly reported by Oono (1978). Since then, a large variety of somaclonal mutants have been identified, many of them show heritable variation in traits of agronomic importance, including grain quality (Shen et al. 1995; Yang et al. 1996), plant height (Sun et al. 1994; Yamagishi et al. 1996), disease resistance (Ling et al. 1985; Araijo et al. 1998; Hernalatha et al. 1999; Gao et al. 2002), abiotic stress tolerance (Adkins et al. 1995; Bertin and Bouharmont 1997; Sint Jan et al. 1997; Lutts et al. 1999) and other traits (Xie et al. 1995). In rice plants, it has been reported that some endogenous transposons, including *Tos17* and *mPing*, can be activated by tissue culture (Hirochika et al. 1996; Jiang et al. 2003), these elements can be used as insertional mutagens to generate somaclonal mutants. Due to the economic importance and ease of regeneration, rice has become a model system to study the genetic mechanisms of somaclonal variation (Muller et al. 1990; Harada et al. 1991; Hirochika et al. 1996; Oono et al. 1999; Yang et al. 1999; Liu et al. 2004). The recent completion of rice whole genome sequence (International Rice Genome Sequencing Project 2005) and the genome annotation database (Ouyang et al. 2007) have provided unprecedented opportunities for researchers to explore the molecular mechanisms of somaclonal mutation at the genome level.

Utilization of heterosis has been a major strategy for increasing rice productivity. In China, hybrid rice cultivars can yield 10–20% more than conventional ones (Yuan 1998; Cheng et al. 2007). C418 is an elite restorer line with wide compatibility. It can overcome fertility barrier between *indica* and *japonica* subspecies and has been widely used to develop *indica–japonica* hybrid rice (Yang et al. 1998). However, C418 is susceptible to bacterial leaf blight disease. To improve the agronomic performance of C418 and create novel mutants for our rice breeding program, we carried out in vitro tissue culture using C418 as the parental plant. C418 has green leaf sheaths; however, among the regenerated plants, we found one somaclonal mutant (Z418) with purple colored leaf sheaths. The anthocyanin pigmentation in Z418 had a tissue- and developmental time-specific pattern. Genetic analysis demonstrated that the trait was controlled by a single dominant gene. Further mapping and sequencing analysis revealed that a Colorless1/Purple leaf (*C1/Pl*) like regulatory gene (*OsC1*) was likely responsible for the purple sheath trait in Z418. The purple sheath trait in Z418 can be used as a morphological marker for hybrid rice breeding; the somaclonal mutant also provides valuable material for future studies of anthocyanin pigmentation regulation in rice.

Materials and methods

Obtaining the somaclonal mutant Z418 by tissue culture

Young spikes (3–5 cm) of a green sheath cultivar C418 (*japonica*) were inoculated on a nutrient medium comprising Murashige and Skoog (MS) basal medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 5% sucrose and 7 g/L agarose. Explants were cultured at 25°C in darkness. Four weeks later, induced calli were transferred to a shoot regeneration medium comprising MS medium with 2 mg/L 6-benzylaminopurine (6-BA), 1 mg/L 6-furfurylamino-purine (KT), 3% sucrose and 7 g/L agarose. Cultures were incubated at 25°C under constant white light. The regenerated plants were grown in soil in the greenhouse at the Jiangsu Academy of Agricultural Sciences (JAAS) to harvest seeds. During 1999–2008, seeds from the regenerated plants and their progenies were planted in the experimental field at JAAS for phenotype evaluation. The purple leaf-sheath trait was examined at both seedling and adult stages.

Crossing experiments

To determine the inheritance pattern and genetic basis of the purple sheath trait in Z418, we crossed Z418 with C418 and two *indica* varieties (93-11 and HX-3) with green leaf sheath. Z418 were used as the pollen parent in all crosses. F_1 plants were self-pollinated to produce F_2 seeds. F_1 and F_2 plants were grown in a greenhouse or growth chambers at Michigan State University, with the temperatures at 30°C/25°C (day/night) and a photoperiod at 12:12 (L:D). Since normally the leaf sheath of Z418 begin to turn purple at 6-leaf stage, we evaluated phenotypes of the F_2 plants after that stage (at seedling or tiller stage).

Screening and development of SSR markers

A total of 256 simple sequence repeats (SSRs) covering all the 12 rice chromosomes were screened to identify polymorphic makers between the mutant Z418 (*japonica*) and a green sheath variety HX-3 (*indica*). These SSR markers were developed by Temnykh et al. (2001) and McCouch et al. (2002), and the sequences are available at http://www.gramene.org. Genomic DNA was extracted from freshly harvested young leaves of Z418 and HX-3

using CTAB method. PCR amplification was performed in a 25 μl volume containing 20 ng of genomic DNA, 1× PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM each of forward and reverse primers and 1.0 unit *Taq* DNA polymerase. Thermal cycling consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s; 55°C or 60°C (depending on the melting temperature of the primers) for 45 s; 72°C for 50 s, and a final extension at 72°C for 5 min. Microsatellite alleles were separated by running the reactions on 3% agarose gels containing 0.5 μg/ml ethidium bromide, and/or on 6% denaturing polyacrylamide gels as described by Gao et al. (2009). To obtain more polymorphic molecular markers for genetic linkage mapping, a total of 29 new SSR and indel (insertion/deletion) markers were developed based on the orthologous sequence differences between Nipponbare (*japonica*) and 93-11 (*indica*). These newly developed markers are listed in Supplementary Table 1.

Genetic linkage analysis

Two hundred and thirty-two F_2 plants derived from the F_1 hybrids of Z418 and HX-3 were used as the mapping population for genetic linkage analysis. Genomic DNA was extracted from the leaves of parental and F_2 plants using the CTAB method. Twenty nanogram DNA from each plant was used for the amplification of polymorphic markers in a 25 μl PCR reaction. Linkage analysis and map construction were performed using Mapmaker 3.0 software (Lander et al. 1987). The maximum-likelihood map order for markers was determined with a log of odds (LOD) score threshold of 3.0. The map distances were calculated in centiMorgan (cM) using Kosambi function.

Southern hybridization

Genomic DNA (6 μg each) from Z418, C418, Nipponbare and HX-3 was digested by *Xba*I or *Hin*dIII(New England, Ipswich, MA)at 37°C for 8 h. The digested DNA fragments were separated by electrophoresis on a 1.0% (w/v) agarose gel at 70 V for 5 h, then transferred onto a Hybond N^+ membrane (Amersham Biosciences, Piscataway, NJ). A 567-bp fragment (AP005652: 67511–68077) of *OsC1* gene was used as probe for the hybridization reaction. The probe was amplified from Z184 genomic DNA (forward primer: 5′-ATCGCTCAGTCTCACACCG-3′; reverse primer: 5′-CGACAGTACCAAATAGCTCC-3′), and labeled with digoxigenin-labeling mix (Roche, Penzberg, Germany). Hybridization was performed using a digoxigenin detection system (Roche, Mannheim, Germany) according to the manufacture's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

To investigate the expression profiles of the candidate gene *OsC1*, we collected leaves at 4 and 6 leaf stages, sheaths at 5 and 6 leaf stages from both Z418 and C418 for RT-PCR analysis (additional sheath tissues were collected for Z418 at 7-leaf stage). Total RNA was isolated using the TRIZOL Reagent (Invitrogen, Carlsbad, CA). Four microgram total RNA from each sample was converted into single-strand cDNA with reverse transcriptase (Invitrogen, Carlsbad, CA). Reactions were diluted 4- to 5-fold, and 2 μl of the diluted cDNA were used as templates for PCR amplifications. RT-PCR primers were designed based on the full cDNA sequence of *OsC1* gene (GenBank accession number: Y15219. Forward primer: 5′-ATCGCTCAG TCTCACACCG-3′, reverse primer: 5′-CGACGAACTA ATGTCACG CAC-3′). The rice actin gene was amplified in parallel with the target gene as an internal standard for quantitative comparison of mRNA levels, using actinF (5′-CAAGGCCAATCGTGAGAA-3′) and actinR (5′-AG CAATGCCAGGGAACATAGT-3′) primers.

Comparison of cDNA and genomic DNA sequences of the *OsC1* gene between Z418 and the wild-type C418

The same RT-PCR primer pair described above was used to amplify cDNA and the corresponding genomic DNA sequences of the *OsC1* gene in both Z418 and C418. PCR products were recovered and purified from 1% agarose gel using the QIA quick Gel Extraction kit (Qiagen, Chatsworth, CA). PCR products were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Sequencing reactions were carried out by the Genomics Technology Supporting Facility at Michigan State University. Multiple randomly selected recombinant clones were sequenced for each sample to verify the result.

Phylogenetic analysis of MYB-related genes

R2R3 conserved domains of 121 MYB-related genes, which include 91 MYB genes from *O. sativa* and 30 MYB-related sequences from other species, were used for the phylogenetic analysis. The 91 *O. sativa* MYB genes used in this study are described in Supplementary Table 2; other 30 MYB sequences from wild rice species as well as other plants and animals are listed in Supplementary Table 3. Multiple pairwise alignments of the 121 conserved R2R3 sequences were generated using ClustalW program (Larkin et al. 2007) under the default settings. The phylogenetic tree was constructed using neighbor-joining method in MEGA4 (Tamura et al. 2007). The analysis was based on

1,000 bootstrap replicates with complete deletion parameters using the nucleotide: maximum composite likelihood model.

Results

Inheritance and expression pattern of purple pigmentation in the leaf sheath of Z418

In 1998, young spikes of C418 were cultured to induce somaclonal mutation. Among the 51 regenerated plants, a mutant Z418 displayed purple leaf sheath and good agronomic characters. To evaluate the genetic stability of the purple sheath trait, we planted Z418 and its self-fertilized offspring for 10 successive years (1998–2008). During the period of time, no phenotypic segregation was observed in the plants of the later generations, indicating that the purple sheath trait in Z418 is heritable, and Z418 is homozygous for the trait. The purple pigmentation in Z418 shows a tissue-specific and developmental stage-specific pattern. Z418 has green leaf sheath before 5-leaf stage (Fig. 1a); its sheaths begin to turn purple at around 6-leaf stage (Fig. 1b), and the same color remains thereafter (Fig. 1c). Z418 also displays purple color in stigma (Fig. 1f, g), but not in its spikes and seeds (Fig. 1h–j). In contrast, the original plant C418 has green sheaths and stigmata throughout its life cycle.

The purple sheath in Z418 is controlled by a single dominant gene

All F_1 plants from crosses between Z418 and the three green sheath cultivars uniformly exhibited purple color in leaf sheath, indicating dominant inheritance of the trait. In F_2 populations, plants from the three crosses segregated for purple and green sheath traits, and the segregation showed a good fit to the 3:1 ratio (Table 1). All these results demonstrate that the purple sheath trait in Z418 is controlled by a single dominant gene.

Molecular mapping of the purple sheath gene in Z418

Among the 256 SSR markers screened for polymorphism between the parental varieties Z418 and HX-3, 89 (34.8%) of the SSR loci were polymorphic. The polymorphic rate of SSR markers on different chromosomes ranged from 19.4 to 60%, with the highest rate on chromosome 9 (60.0%), and the lowest on chromosome 2 (19.4%).

A total of 232 F_2 plants derived from the cross Z418/HX-3, including 171 purple sheath and 61 green sheath plants, were used as the mapping population. Bulked segregating analysis was used to map the purple sheath gene in Z418. First, DNAs from 20 green sheath and 20 purple sheath individuals were bulked into two DNA pools and then genotyped with 89 polymorphic SSR markers, 2 SSRs on chromosome 6, RM204 and RM3, did not fit 1:2:1

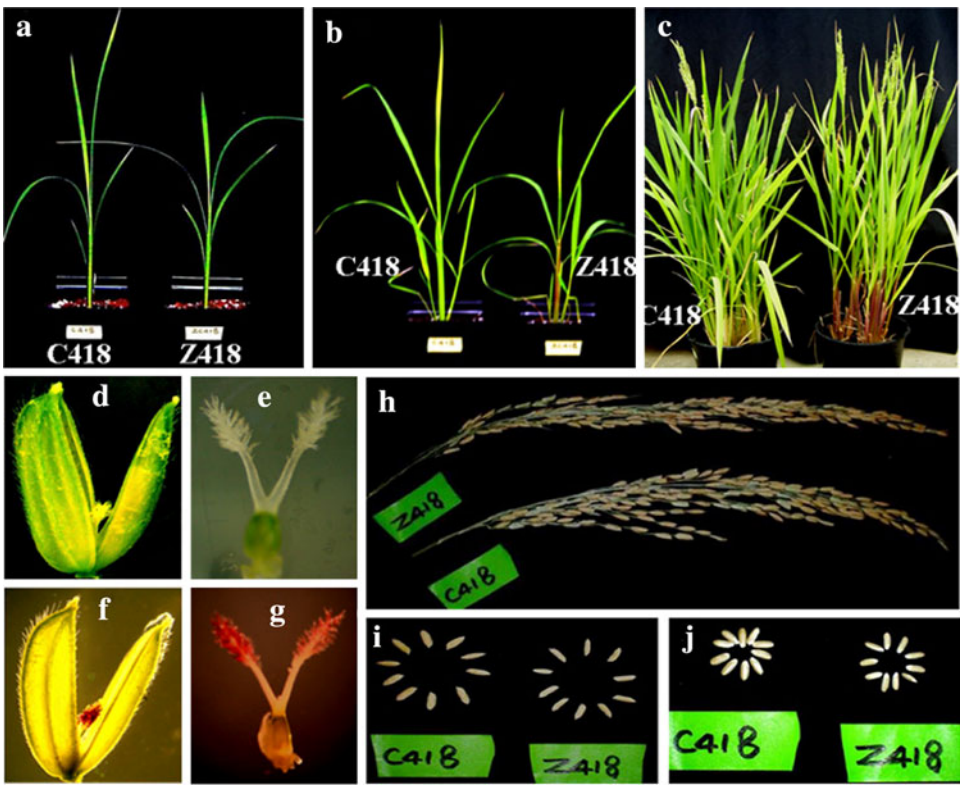

Fig. 1 Phenotypes of the somaclonal mutant Z418 and its wild-type C418. Seedling at 5-leaf stage (**a**) and 6-leaf stage (**b**). Rice plant at adult stage (**c**). Flower (**d**) and stigma (**e**) of C418, Flower (**f**) and stigma (**g**) of Z418, Spikes of Z418 and C418 (**h**), Hulled (**i**) and dehulled (**j**) seeds of Z218 and C418

Table 1 Segregation of F_2 populations from crosses between Z418 and 3 green sheath cultivars

Cross	Total plants	Plants with purple sheath	Plants with green sheath	Purple/green	*P* value
Z418/HX-3	1,228	909	319	2.85	0.3–0.5
Z418/9311	403	293	110	2.66	0.2–0.3
C418/Z418	613	453	160	2.83	0.5–0.7

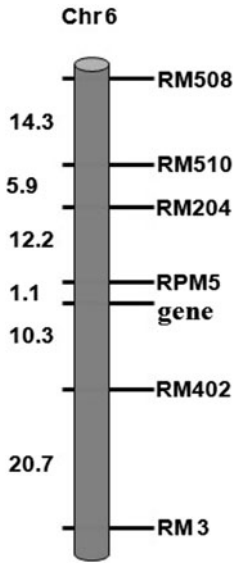

Fig. 2 The gene controlling purple sheath trait in Z418 is mapped to the short arm of rice chromosome 6 between two SSR markers, RPM5 and RM402, with a genetic distance of 1.1 and 10.3 cM, respectively

(P1:F1:P2) segregation pattern, suggesting that the target gene is located on chromosome 6. Subsequently, all 232 F2 individuals were genotyped with 6 polymorphic SSR markers on chromosome 6. Our linkage analysis initially mapped the purple sheath gene on the short arm of rice chromosome 6 between two SSR markers, RPM5 and RM402, with a genetic distance of 1.1 and 10.3 cM, respectively (Fig. 2).

Fine mapping of the target gene

To refine the localization of the purple sheath gene in Z418, we selected 319 F_2 green sheath plants derived from Z418/HX-3 cross, and screened for recombinants among these plants using RM204 and RM402 markers. We identified 82 and 65 recombinants based on the genotypes at RM204 and RM402 locus, respectively. These 147 recombinant plants were further used as the material for the fine mapping of the target gene.

Thirty-one SSR markers located between RM204 and RM402 were further genotyped and screened for polymorphism between HX-3 and Z418. These markers include two (RM11 and RM253) of the SSRs reported early and 29 additional SSR or indel markers newly developed in this study (Supplementary Table 1). Among these markers, RM253, RPM8 and RPM11 were polymorphic between the parents. These 3 markers were subsequently used to genotype the 147 F_2 recombinant plants which were polymorphic either at RM204 or RM402 locus (described above). Among the 82 F_2 recombinants polymorphic at RM204 locus, 4 plants were also polymorphic at RPM8 locus. On the other hand, all the 65 plants polymorphic at RM402 showed homogenous genotype at RPM8. The results indicate that RM204 and RPM8 are located on the same side of the candidate gene. Among the 65 plants polymorphic at RM402 locus, 6 and 3 individuals were also polymorphic at RM253 and RPM11 locus, respectively. Therefore, along with RM402, these two markers were located on the other side of the target gene. As the result of our linkage analysis, the target gene was finally anchored to a 153-kb interval between RPM8 and RPM11 markers (Fig. 3). Based on the Rice Genome Annotation Project Data (http://rice.plantbiology.msu.edu), this region contains a total of 18 non-transposon-related genes (Supplementary Table 4). An expressed gene (Os06g10350) encoding a MYB family transcription factor was chosen as the candidate gene controlling the purple leaf sheath in Z418. Further Blastn searches revealed that this candidate gene has been reported as the rice homolog (*OsC1*) of maize *C1* gene (Reddy et al. 1998, Saitoh et al. 2004), which is a basic regulatory gene required for pigmentation pathway in maize.

Expression analysis and sequence comparison of *OsC1* gene

RT-PCR analysis showed that the *OsC1* gene expressed in both leaf and sheath tissues at all the developmental stages tested (leaves at 4 and 6-leaf stages, sheathes at 5 and 6-leaf stages) in both Z418 and C418. However, while all RT-PCR products from C418 appeared as a single band on the 1% agarose gel, all RT-PCR products from Z418 apparently showed two bands (Fig. 4). Further sequencing and alignment analysis showed that the bigger transcript of *OsC1* gene in Z418 (GenBank accession number: HQ379703) was 34 bp longer than that in C418 (GenBank accession number: HQ379701). The smaller-sized transcript detected from Z418 contained a 161-bp deletion within the third exon of the *OsC1* gene (GenBank accession number: HQ379704), possibly resulted from alternative splicing of *OsC1* mRNA. To verify the homozygosity of the *OsC1* gene in Z418, three recombinant clones of the

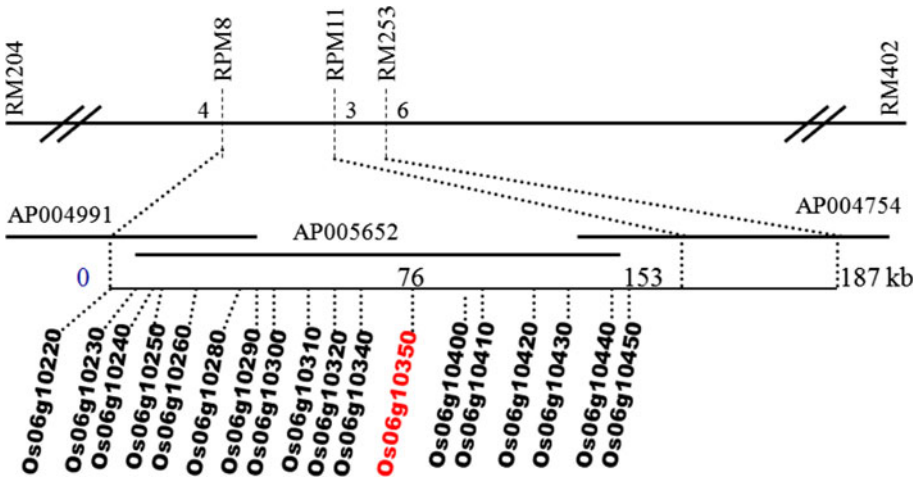

Fig. 3 Fine mapping of the purple sheath gene. The location of the target gene was narrowed down to a region of approximately 153 kb (spanning 3 BACs: AP004991, AP005652, AP004754) between two SSR markers, RPM8 and RPM11. Eighteen non-transposon-related genes located within the region were shown. The candidate gene Os06g10350 encodes a MYB family transcription factor *OsC1*. The numbers of recombinant were indicated near the SSR markers

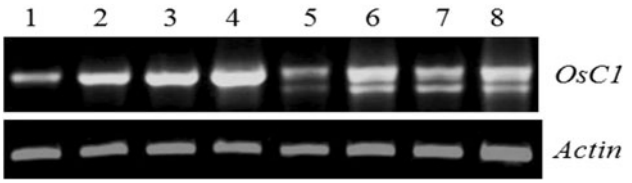

Fig. 4 RT-PCR analysis on the expression of *OsC1* gene in leaf and sheath tissues collected at different developmental stages of Z418 and C418. 1. Leaf of C418, 4-leaf stage; 2. sheath of C418, 4-leaf stage; 3-4. leaf and sheath of C418, 6-leaf stage; 5. Leaf of Z418, 4-leaf stage; 6. sheath of Z418, 4-leaf stage; 7-8. leaf and sheath of Z418, 6-leaf stage. The rice actin gene was amplified as an internal standard for quantitative comparison of mRNA levels

PCR amplified gene product were randomly selected for sequencing. These three clones contain a uniform gene sequence, which has a 34-bp indel at the position between 973 and 1006 (GenBank accession number: HQ379705) when compared with the sequence of the same gene from C418 (GenBank accession number: HQ379702). Additionally, nucleotide difference was found in the small flanking region (5 and 6 bp in length) of the indel (Supplementary Fig. 1). The cDNA sequence of *OsC1* from Z418 is identical to that has been reported by Saitoh et al. (2004), GenBank accession number: Y15219).

Amino acid sequences analysis showed that the *OsC1* gene in Z418 encodes a 272-amino acid sequence, which is 13 amino acids longer than that in C418 due to the 34-bp indel in the gene sequence (Fig. 5). The amino acid sequence *of OsC1* in Z418 shows high similarity to the orthologous gene from Nipponbare and two wild rice species, *O. rufipogon* and *O. glaberrima*. Further comparison indicates that *OsC1* protein in Z418 shares the conserved R2R3 MYB domain in the N-terminal region with other 8 MYB-related proteins, suggesting the functional importance of these motifs. In contrast, the C-terminal region of these proteins is highly variable in sequence and in size. Surprisingly, a high sequence divergence in the C-terminal region is also present between Z418 and its wild-type C418, which is probably caused by the frame-shift of the amino acid sequence due to the indel in the gene (Fig. 5).

Detection of *OsC1* and other MYB-related genes in the rice genome

To examine the copy number of *OsC1* gene in the genome of Z418 and other rice plants, a fragment of *OsC1* gene was used as the probe to hybrid with either *Eco*RI or *Hin*dIII digested total DNA from Z418 and plants of three rice varieties with green leaf sheath. In all instances a strong hybridization band was detected, which most likely represented the *OsC1* gene (Fig. 6). Meanwhile, multiple weak hybridization signals were also detected on the blots, which might represent other MYB-related genes sharing low homology with *OsC1*.

To investigate the distribution of MYB-related genes in rice genome, R2R3 domain sequences of *OsC1* gene in rice and *C1* gene in maize were used as queries to search against Genbank and the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu). A total of 91 MYB-related genes were identified. These genes are distributed across all the 12 rice chromosomes; all but 2 of them contain the conserved and intact R2R3 domains (Supplementary Table 2). These MYB-related genes appear to be clustered on the rice chromosomes. For example, the chromosome 1 contains 19 MYB-related genes, while the chromosome 10 only has 2 MYB genes and both are located on the long arm.

To provide insight into the evolutionary relationship of rice *OsC1* gene and other MYB-related genes, the conserved R2R3 protein sequences of 89 MYB-related genes from *Oryza sativa* ssp. *japonica,* and 30 MYB-related genes from other species were used to build a phylogenetic tree (Fig. 7). All MYB-related sequences from different

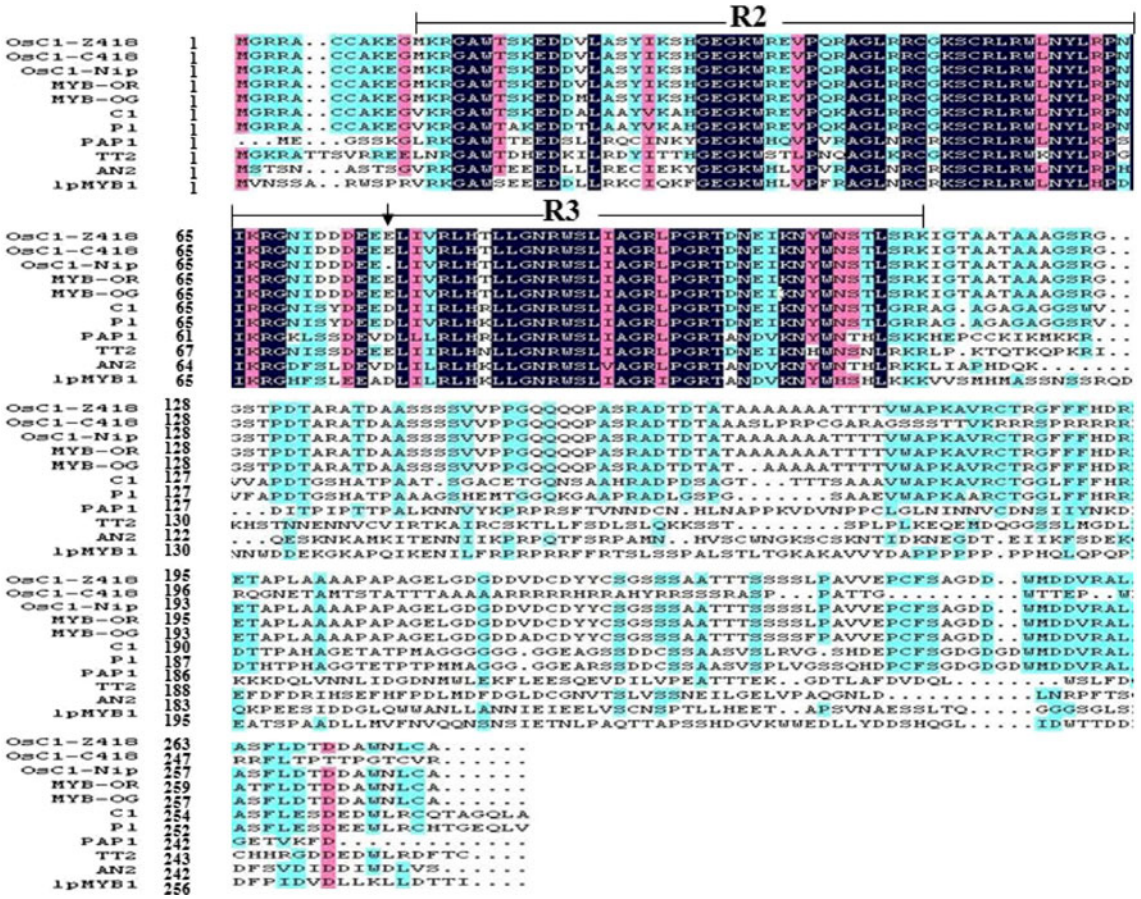

Fig. 5 Amino acid sequence comparison of *OsC1* from Z418, C418 and Nipponbare (rice cultivars) and other MYB protein from different plant species, including MYB-OR from *O. rufipogan*, MYB-OG from *O. glaberrima*, C1 and Pl from maize, PAP1 and TT2 from arabidopsis, AN2 from petunia and IpMYB1 from japanese morning glory. Highlighted are the conserved amino acid residues. R2 and R3 repeats of MYB-DNA binding motif were marked. The *arrow* indicates the location of the lost amino acid residue in Nipponbare

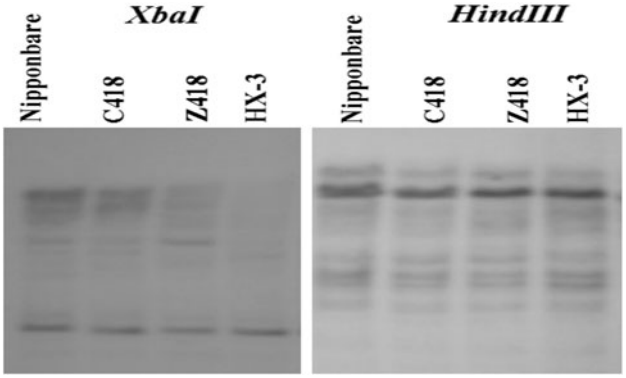

Fig. 6 Southern blot analysis of *OsC1* gene from Z418 and 3 rice cultivars with green leaf sheath. Genomic DNA from Z418, C418, Nipponbare and HX-3 was digested by *Xba*I and *Hin*dIII and separated in 1.0% (w/v) agarose gel, then transferred onto a Hybond N^+ membrane. A 567-bp digoxigenin-labeled DNA probe, which was prepared by PCR amplification of the coding region of *OsC1* gene from genomic DNA of Z184, was used to hybrid with the digested DNA fragments

animals were grouped into a major family, while all those from plant species were grouped into another big family consisting of five subfamilies. The *OsC1* gene of Z418 and two wild rice species, the *C1* gene of maize were grouped into to the same clade (II-5), suggesting the relatively recent divergence of these genes.

Discussion

The hypothesis that *OsC1* is the homolog of maize *C1* gene in rice (*OsC1*) was first proposed by Saitoh et al. (2004) based on a comparative map between maize and rice. In the same study, it was found that rice lines with colored and colorless apiculus had different alleles of *OsC1* gene. Two colorless lines had a 10-bp deletion in the *OsC1* gene compared with the colored rice line T65, suggesting a loss-of-function mutation in their *OsC1* allele due to the deletion. In our current study, the gene regulating the purple

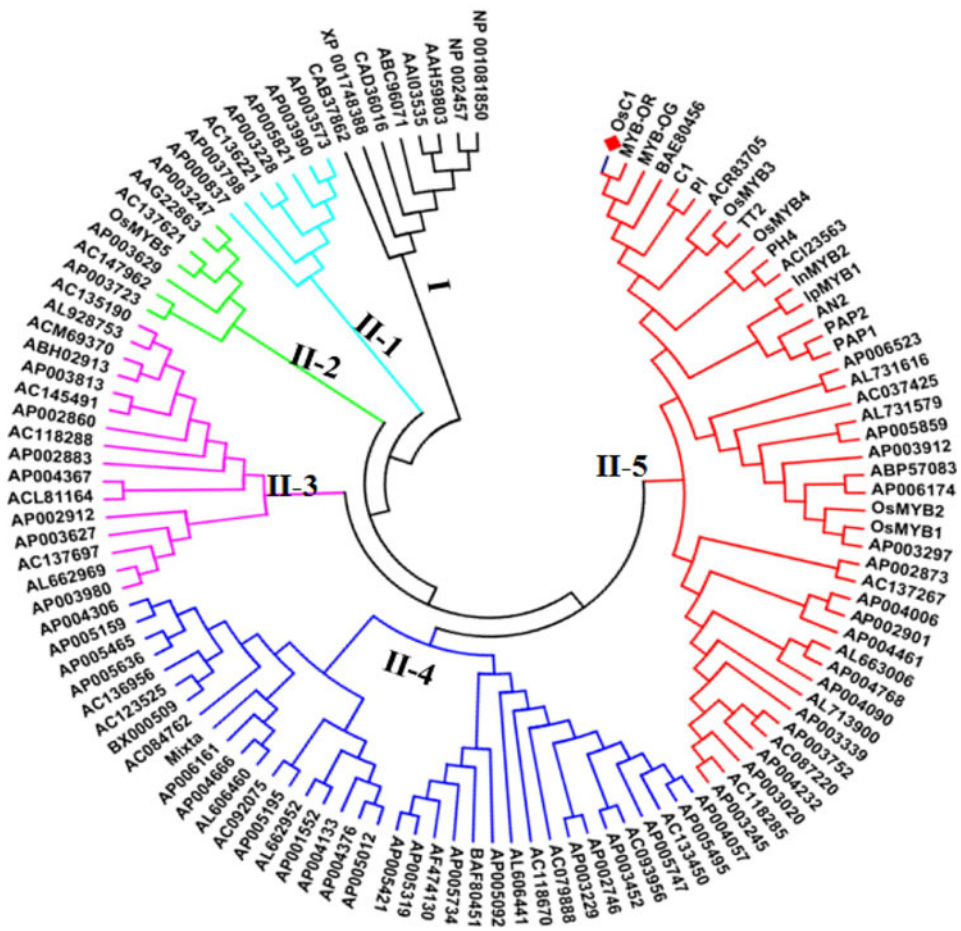

Fig. 7 Phylogenetic analysis of R2R3 conserved domains of MYB-related genes in different plants and animals. All MYB-related genes were grouped into two big families: family I and family II. The family II can be grouped into 5 subfamilies: II-1, II-2, II-3, II-4 and II-5

leaf-sheath trait in the somaclonal mutant Z418 is mapped to a 153-kb interval on the short arm of chromosome 6, where *OsC1* is located. Sequence analysis reveals that Z418 had the same *OsC1* sequence as that in T65, while the original plant C418 and other 3 green sheath varieties have a 34-bp deletion in *OsC1* gene compared to Z418. Therefore, our data strongly support the previous hypothesis that *OsC1* is the rice homolog of Maize C1, which is the basic regulatory factor required for anthocyanin pigmentation in rice plants. Z418 and C418 have a functional and non-functional (due to the 34-bp deletion) alleles for *OsC1* gene, respectively. How did the gain-of-function mutation occur in the *OsC1* gene of the original plant (C418) during tissue culture? Further investigation into this question would increase our understanding of the genetic basis for somaclonal variation in rice plants.

Possible mechanisms for the mutation in the *OsC1* gene of C418

The genetic mechanisms of somaclonal variation in plants are still not well understood. A variety of genetic changes have been reported to be associated with culture-induced phenotypic variation in plants, including polyploidy, aneuploidy, chromosome rearrangement (Larkin and Scocroft 1981; Lee and Phillips 1988; Jain 2001), point mutation (Dennis et al. 1987; Yang et al. 1996), DNA deletion (Harada et al. 1991), epigenetic modifications (Muller et al. 1990; Kubis et al. 2003), and transposon insertion (Hirochika et al. 1996; Courtial et al. 2001; Jiang et al. 2003; Liu et al. 2004). Our previous data suggests that multiple molecular mechanisms are responsible for somaclonal variation in rice (Gao et al. 2009). In this study, it is unclear how tissue culture has induced a 34-bp insertion in the *OsC1* gene of Z418. Here, we propose three possible mechanisms for future investigations.

First, the mutation in *OsC1* gene may result from homologous recombination mediated by a transposon. In this study, we found 91 MYB-related genes in the genome of Nipponbare (*Oryza sativa* L. ssp. *japonica*), and among them 6 genes were on the same chromosome where *OsC1* gene is located (chromosome 6). Therefore, it is possible that Z418 has obtained the 34-bp sequence from another MYB gene via homologous recombination during tissue culture. It has been well documented in maize that transposon *Ac* can induce homologous recombination of

pericarp color1 (*P1*) gene (Athma and Peterson 1991). Transposons contribute 35% of the rice genome (International Rice Genome Sequencing Project 2005), and several rice transposons have been reported to be active (Hirochika et al. 1996; Jiang et al. 2003; Fujino et al. 2005; Tsugane et al. 2006). It is likely that a transposon in rice was activated by tissue culture, and the activated transposon mediated the homologous recombination between genes of MYB family. A second possibility is that the insertion was caused by a transposition event. Although in many cases mutation in regulatory genes was caused by deletions (Nesi et al. 2001; Sweeney et al. 2006; Furukawa et al. 2006), it is also reported to be associated with insertions of transposons. For example, the insertion of a 7-bp nucleotide sequence has caused the mutation of the regulatory gene *InWDR1* in Japanese morning glory (Morita et al. 2006). Also, there are evidences that transposons can capture gene fragments (Jiang et al. 2004). Since transposon insertion has been considered as an important cause of genetic mutations, we cannot rule out the possibility that it may play a role in the mutation of *OsC1* gene. As our last hypothesis, the 34-bp sequence insertion might belong to extra-genomic DNA sequences (Lolle et al. 2005) and has been integrated into the *OsC1* locus during tissue culture.

Developmental time- and tissue-specific pattern of purple pigmentation in Z418

Spatial and temporal expression of regulatory genes has been reported to be associated with the anthocyanin deposition in plant tissues (Procissi et al. 1997). For instance, the expression of the transparent test 2 (*TT2*) gene in Arabidopsis, which is responsible for proanthocyanidin accumulation in the endothelium, is restricted to the seed at early stage of embryogenesis (Nesi et al. 2001). In maize, different alleles of the B gene cause tissue- and developmental time-specific patterns of anthocyanin synthesis in plant and seed tissues, and the level of B gene expression correlates with the accumulation of anthocyanins in different tissues (Radicella et al. 1992). Transcriptional control has been suggested to responsible for the expression of the regulatory genes in specific tissues (Damiani and Wessler 1993; Mol et al. 1998; Selinger and Chandler 1999; Zhang and Peterson 2005). In addition, expression of regulatory genes can also subject to post-transcriptional control (Pairoba and Walbot 2003) or epigenetic modification (Cocciolone et al. 2001; Rhee et al. 2010).

The developmental time- and tissue-specific pattern of purple coloration in Z418 suggests that a complex mechanism is involved in the regulation of the pigmentation pathway. Some recent data has indicated that besides *OsC1* gene, one or more genes may involved in the regulation of the purple pigmentation in rice plants. Yue et al. (2006) have mapped three main-effect QTLs and a pair of epistatic QTL for the purple leaf sheath coloration in rice plants. In another study, Wang et al. (2009) identified a major dominant gene *PSH1* (t) controlling the purple leaf sheath in a RIL line, and mapped it to a 23.5-kb interval on chromosome 1. Further cloning and functional analysis of these QTLs and candidate genes will enhance our understanding of regulation in pigmentation pathway, and how *OsC1* gene has interacted with other regulatory gene(s) to control the time- and tissue-specific pigmentation in rice.

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