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In Silico Analysis of a MRP Transporter Gene Reveals Its Possible Role in Anthocyanins or Flavonoids Transport in *Oryze sativa*[*]

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ABSTRACT

There are many studies on enzymatic pathways of anthocyanin biosynthesis, but little is known about the anthocyanins transport in *Oryze sativa*. *In silico* analysis, the *OsMRP*15 (LOC_Os06g06440), an orthologous gene of mazie anthocyanin transporter *ZmMRP*3, has been identified in rice. The *OsMRP*15 contained a 4425bp open reading frame (ORF) encoding a 1475 amino acid protein, belonging to a MRP subfamily of ABC transporters, and has a high sequence identity, very similar protein structure, and the same arrangement of domains to *ZmMRP*3, but the genomic structure of *OsMRP*15 was significant difference with *ZmMRP*3. The prediction promoter of *OsMRP*15 has many presumed anthocyanin regulatory sites. The phylogenetic analysis of MRPs in rice, mazie and *Arabidopsis* showed that *OsMRP*15 and *ZmMRP*3 belonged to the same subbranch. The expression pattern indicated that *OsMRP*15 was co-expression with two anthocyanin transcription factors. These analysis results implied that as an ortholog of *ZmMRP*3, the function of *OsMRP*15 was possibly as a membrane-bound transporter required for vacuolar uptake of anthocyanins in rice.

Keywords: *In Silico* Analysis; OsMRP15; a MRP Transporter; Anthocyanins or Flavonoids Transport; *Oryze sativa*

1. Introduction

The multidrug resistance-associated proteins (MRPs) belong to ATP-dependent, proton-gradient-independent transporters (ATP-Binding Cassette, ABC) superfamily [1], and the primary function of plant MRPs are involved in the vacuolar sequestration of potentially toxic metabolites, for example flavonoids and other secondary metabolites [2]. The full-size or typic MRP transporters consist of five core domains [3]: three hydrophobic transmembrane domains (TMDs) that each contains multiple transmembrane α-helices; and two soluble nucleotide binding domains (NBDs) that each is composed of the Walker A and B motives linked by an ABC signature motif. These domains are arranged with the topology: TMD0-TMD1-NBD1-TMD2-NBD2, in which the TMD0 is required for correct targeting and two (TMD-NBD)s compose a transmembrane transport channel.

Now, many MRPs have been identified in some different plants, but only several genes involved in flavonoids transport have been studied. There are 15 putative MRP genes in *Arabidopsis* genome. *AtMRP*1 and *AtMRP*2 transport anthocyanin-glutathione conjugates in vacuolar yeast [4,5]. Moreover, *AtMRP*2 is involved in chlorophyll degradation *in vivo* [6]. *AtMRP*4 and *AtMRP*5 are involved in guard cell regulation [7]. Maybe due to the function redundance in vacuolar uptake of anthocyanins, flavonoidless phenotypes have not been obatained by knockout mutants in some *AtMRPs* [2]. In mazie, *ZmMRP*3 is a membrane-bound anthocyanin transporter. The expression pattern of *ZmMRP*3 correlated with the anthocyanin accumulation pattern and is controlled by anthocyanin regulators [8]. *ZmMRP*4, the other related MRP, acts mainly as phytic acid transporter in the seed and its other possible function is partially redundant with *ZmMRP*3 in anthocyanin transport in the scutellum [9].

In rice, we identified 16 MRP genes and excluded the LOC_Os11g05700 that is a half transporter and consists of a single TMD and NBD, and originally called OsMRP16 in [3], using the latest RGAP V6.1 (Rice Genome Annotation Project) database, and only one gene was studied. *OsMRP*5 (LOC_Os03g04920, now-called *OsMRP*13 in this paper), an orthologus gene of *ZmMRP*4, is a phytic

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acid transporter in seeds [10].

Anthocyanin is an important biologically active substance in black, purple and red rice [11]. Although the regulators and key enzymes have been studied, little is known about anthocyanin transport in the final phase of anthocyanin biosynthesis in rice. In order to improve the content of anthocyanin in rice seeds, in this paper we identified a MRP gene, *OsMRP*15, and analyzed its possible role in anthocyanins or flavonoids transport.

2. Materials and Methods

2.1. Plant Materials

The Green rice varieties (ZH11, Nipponbare and T65) and red leaf variety ZY were planted in our lab.

2.2. RNA Isolation, cDNA Synthesis and RT-PCR Analysis

The total RNA was isolated by Trizol reagent kit (Invitrogen, USA) and two micrograms of total RNA were reverse-transcribed using M-MLV kit (Promega, USA), according to the manufacturer's instructions. Using *OsActin*1 as a reference gene, two primers of *OsMRP*15, F15 (5'-agatgggtcgaattggagcatgggtc-3') and R15 (5'-cgtaggtttgtcatac tccaccac-3') were designed to RT-PCR analysis, according to the procedure: predenaturation at 94˚C for 4 min, 35 cycles at 94˚C for 0.5 min, 58˚C for 0.5 min and 72˚C for 0.5 min, followed by the final extension at 72˚C for 5 min.

2.3. Bioinformatics Analysis

The latest RGAP V6.1 data was used to assay the rice MRP gene family. Gene prediction and structure analysis used a FGENESH web software. Molecular characterization and multiple sequence alignment of *OsMRP*15 was analyzed using Vector NTI 10.0 program (Invitrogen, USA). The promoter prediction and cis-acting regulatory elements analysis utilized the latest PromPredict program [12] and PLACE program [13], respectively. The conserved protein domain analysis used the SMART web tool, and protein subcellular localization prediction adopted WolF PSORT program. The peptide sequences of MRPs were aligned with Clustal W and subsequently a phylogenetic tree was constructed by the neighbor-joining (NJ) method with MEGA 5.0 by bootstrap with 1000 replicates [14]. Our Rice GeneChip Database was used to analyze gene expression patterns.

3. Results

3.1. Molecular Characteristics of *OsMRP*15

Using *ZmMRP*3 as a probe, the orthologus gene *OsMRP*15 was identified by BLAST search in RGAP V6.1 database. The *OsMRP*15 was located in Chr.6, and the TIGR number is LOC_Os06g06400. The genome length of *OsMRP*15 was 8445bp including 299bp 5'-UTR, 4425bp ORF and 389bp 3'-UTR. Although the gene CDS structure of *OsMRP*15 including 11 exons and 10 introns is very different with *ZmMRP*3 CDS structure that only contained 4 exons and 3 introns, the two genes have very similar phase of introns which implied that the introns loss maybe happened in *ZmMRP*3 (**Figure 1**). In addition, there were two introns in 5'-UTR of *ZmMRP*3, but no introns in prediction 5'-UTR of *OsMRP*15.

The *OsMRP*15 encoded a 1475 amino acid protein with a molecular weight of 164.51 kDa and a pI value of 7.69, belonged to a MRP subfamily of ABC transporters, and shared the high identity of 84.3% with *ZmMRP*3 protein. Multi-alignment of *OsMRP*15 with other plant MRPs showed high similarities. Like *ZmMRP*3, *OsMRP*15 was a full-size MRP transporter that has conserved TMD and NBD domains, and the domains arrangement were TMD0-TMD1-NBD1-TMD2-NBD2. The NBD domains are also characterized by Walker A motif (G-X(4)-G-K-[ST]), Walker B motif ([RK]-X(3)-G-X(3)-L-[hydrophobic]) and linked by an ABC signature motif ([LIVMFY]-S-[SG]-G-X(3)-[RKA]-[LIVMYA]-X-[LIVMF]-[AG]) [7] (**Figure 2**).

The subcellular localization prediction of *OsMRP*15 showed its presence in the plasma membrane and was an integral membrane protein, which is similar with *ZmMRP*3 that located in the tonoplast [8]. And 48 conserved phosphorylation sites were predicted by NetPhos 2.0. These sites suggested that posttranslational modification may play an important role for *OsMRP*15 normal function. The protein structure analysis displayed that *OsMRP*15 and *ZmMRP*3 have similar second structure and almost exactly same of conserved structures except for lacking two transmembrane-spanning alpha-helices of TMD2 in *ZmMRP*3 (**Figure 3**).

3.2. The Analysis of Prediction Promoter Region of *OsMRP*15

In the 1.5 kb upstream of 5'-UTR of *OsMRP*15, a prediction core promoter region from −443bp to −503bp, many

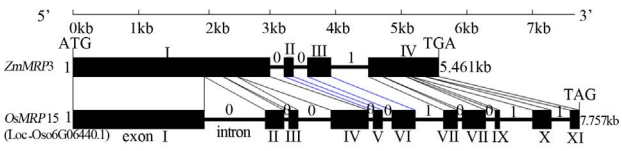

Figure 1. The genome structure of *OsMRP*15 compared with *ZmMRP*3. The number of 0 and 1 indicated the phase of introns: phase-0 introns (intron located between two codons), phase-1 introns (intron located between first and second nucleotide of a codon), respectively.

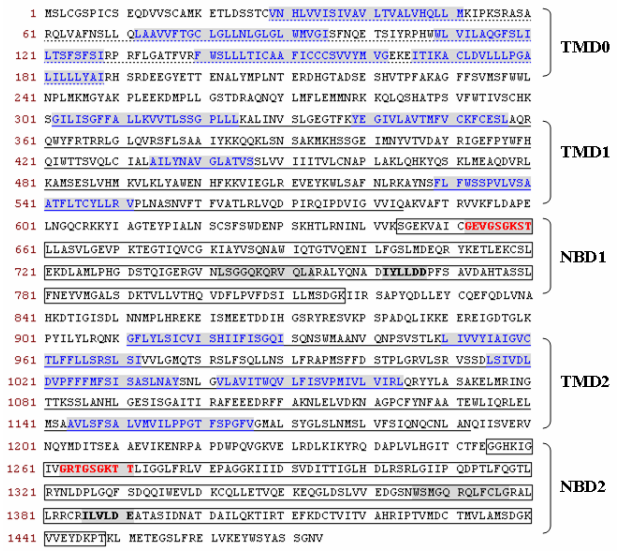

Figure 2. The deduced protein sequence and conserved structure of *OsMRP*15. The TMD0 was indicated by a dotted underline. The TMD1/TMD2 and NBD1/NBD2 were indicated by underlines and the boxes, respectively. The putative transmembrane-spanning alpha-helices were blue letter and shown in shadow. The Walker A and B motifs were indicated by red letter and bold letter in a grey background respectively, and the ABC signature motifs were shown in grey background.

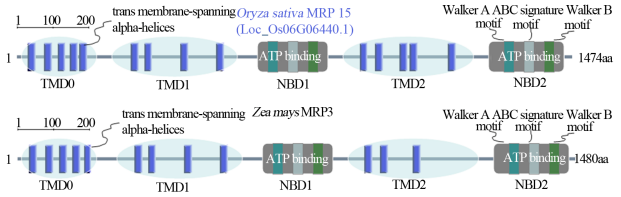

Figure 3. The protein structure comparison between *OsMRP*15 and *ZmMRP*3.

cis-active elements and many myb or myc cis-regulatory elements were found by using PromPredict and PLACE program, respectively (**Figure 4**). Moreover, a cis-sequence of *OsMRP*15 promoter similar to an anthocyanin regulatory element (ARE) [8], is present at −570 position (**Figure 4**). These promoter characteristics of *OsMRP*15 conformed to the *ZmMRP*3 promoter and implied that *OsMRP*15 expression was controlled by similar antho-cyanin regulatory genes.

3.3. The Phylogenetic Tree Analysis of *OsMRP*15

Using the latest RGAP V6.1 data, all 16 rice MRP genes were identified by BlASTP searches. The nomenclature for *OsMRP*1 to *OsMRP*7 and *OsMRP*9 to *OsMRP*15 were in accordance with [3]. The *OsMRP*8 was only one new locus not two in [3], and old *OsMRP*16 in [3] is a single TMD and NBD structure which was excluded in the rice MRP family. So, the new named *OsMRP*16 in this paper was the old *OsMRP*17.

In the phylogenetic tree (**Figure 5**), there were two clades: the clade I including two rice MRPs in which the possible function of *OsMRP*1 maybe was related with transport of anthocyanin-glutathione conjugates like *AtMRP*1 and *AtMRP*2 in yeast [4,5]; the clade II containing many subcludes in which *OsMRP*15 and *ZmMRP*3, and *OsMRP*13 and *ZmMRP*4 all belonged to the same subbranch with 100 bootstrap value, respectively. Therefore, it can be postulated that OsMRP15 was an anthocyanin transport similar with *ZmMRP*3, and *OsMRP*13 has possible redundant function in anthocyanin transport like *ZmMRP*4.

3.4. The Expression Pattern of *OsMRP*15

In our Rice GeneChip Database, *OsMRP*15 expression was higher in stigma than embryo, and very low in other tissues and organs. Furthermore, *OsMRP*15 was co-expression with two anthocyanin transcription factors, *OsC*1 and *OsB*1, in all tissues and organs, except for embryo (**Figure 6**). These expression patterns resembled *ZmMRP*3 [3] that expressed in all anthocyanin assembled and colored tissues and organs, and was controlled by the anthocyanin regulators *B* (myc type) and *Pl* (myb type).

In red leaf rice, the anthocyanin biosyntheses is controlled by *OsC*1 and *OsB* (*OsB*1 and *OsB*2) [15]. But in green leaf, the *OsC*1 is induced by UV-B and very low expression, and the *OsB*2 is non-function. Moreover, the anthocyanin key structure gene *DFR* is mutant and non-function in many japonica rice varieties, for example Nipponbare [15].

In order to detect the co-expression pattern of *OsMRP*15, the RT-PCR was analyzed in a red leaf variety ZY and 3 green leaf varieties ZH11, T65, and Nipponbare. The RT-PCR result indicated that *OsMRP*15

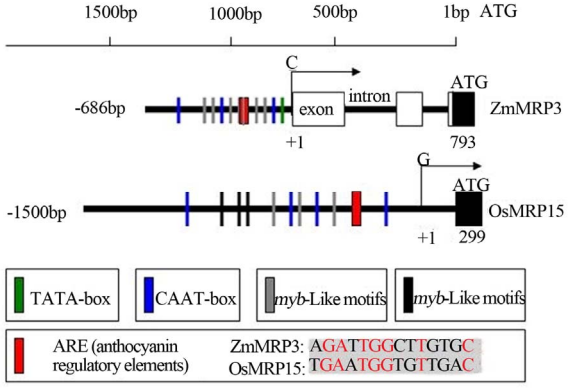

Figure 4. The promoter region comparison between *OsMRP*15 and *ZmMRP*3.

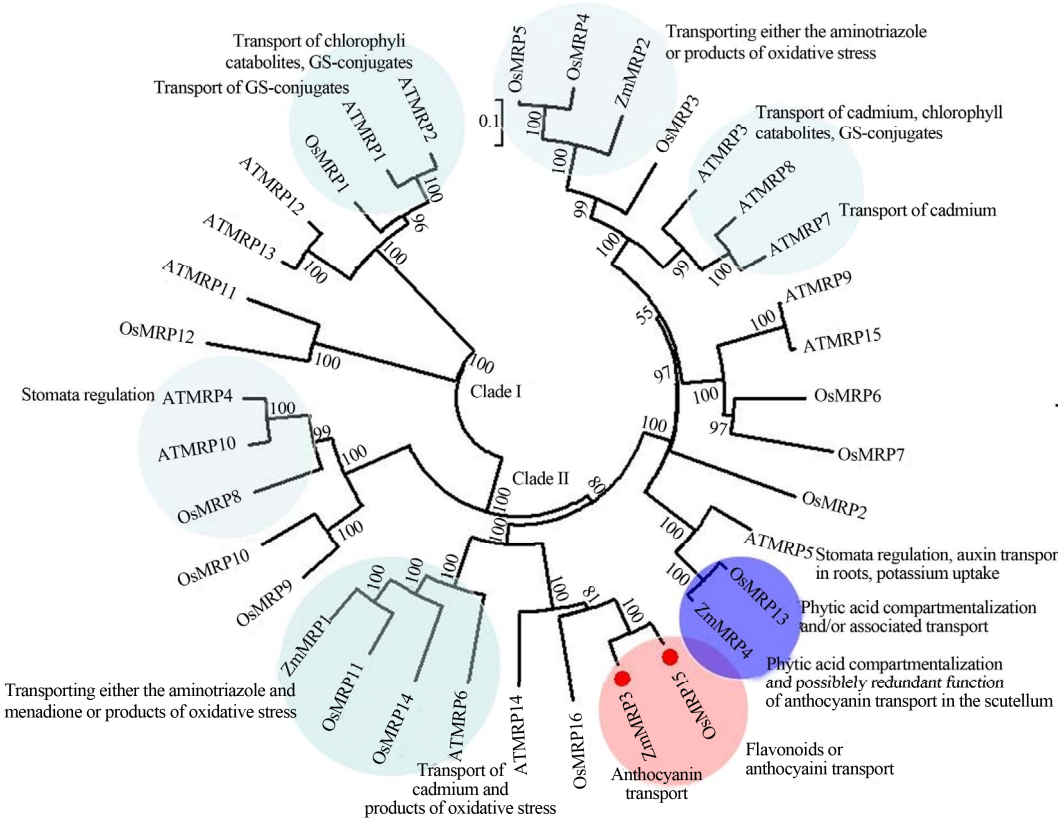

Figure 5. Phylogenetic tree of the MRP gene family in Arabidopsis, maize and rice. The OsMRP15 and ZmMRP3 were in the same subbranch indicated in pink background and others similar function genes were claded into different subclades in different colored background. The accession numbers of MRPs are: Arabidopsis and rice (OsMRP1 to OsMRP7 and OsMRP9 to OsMRP15) in accordance with [3]; OsMRP8: LOC_Os01g25386; OsMRP16: LOC_Os12g37580; ZmMRP1: AY186245; ZmMRP2: AY186247; ZmMRP3: AY609318.1; ZmMRP4: EF586878.

expression was co-expression with anthocyanin regulators in red leaf of ZY, but no expression in green leaf varieties (**Figure 7**). The integrative expression pattern suggested that *OsMRP*15 played a possibly important role in rice anthocyanin synthesis.

4. Discussion

As an ortholog of *ZmMRP*3 [8], a mazie anthocyanin transport, *OsMRP*15 was identified *in silicon* analysis using latest rice genome data. At the level of genome structure (**Figure 1**), it was very different that *OsMRP*15 has many exons in CDS and no introns in prediction 5'-UTR, but *ZmMRP*3 was only 4 exons in CDS and two introns in 5'-UTR. Compared with *ZmMRP*3, other orthologus structure were very similar with *OsMRP*15, which implied that introns have lost in *ZmMRP*3. On the other hand, at the level of protein, *OsMRP*15 has highest identity, the same protein structure, conserved domains and similar sublocation with *ZmMRP*3 (**Figures 2** and **3**). In prediction promoter of *OsMRP*15, many *cis*-active elements and *cis*-regulatory elements were very similar with *ZmMRP*3 promoter, specially the similar ARE [8].

The result of *OsMRP*15 and *ZmMRP*3 with high bootstrap value in the same subbranch of phylogenetic tree strongly suggested that the two genes have similar function. Interestingly, *OsMRP*16, the same subclade with *OsMRP*15, was no or very low expression in all tissue and organs (**Figure 6**), which implied that *OsMRP*16 maybe was a non-function gene in rice. The expression pattern of *OsMRP*15 co-expression with *OsC*1 and *OsB*1 was strongly supported by RT-PCR between red and green leaf rice varieties.

In green stigma, the function of *OsMRP*15 maybe was a flavonoids transport, in which transported some flavonoids, e.g. flavonols. Maybe because some key structure genes in anthocyanin biosynthesis were non-function or mutant, the pathway was incomplete though *OsC*1 and *OsB*1 were high expression. In addition, in embryo, *OsMRP*15 was expression but *OsC*1 and *OsB*1 were no expression. These results suggested that maybe *OsMRP*15 has other regulatory factors and played an unknown role in embryo, which was different with *ZmMRP*3.

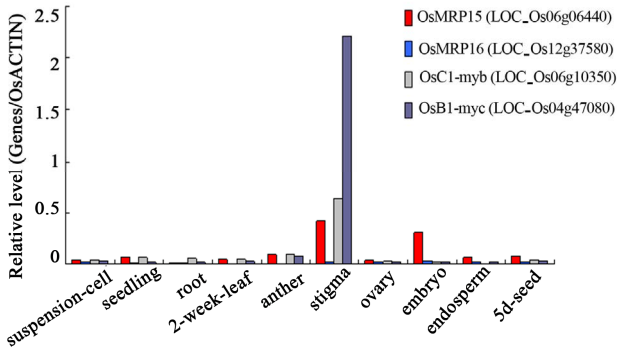

Figure 6. The expression patterns of *OsMRP*15 in different tissues and organs of rice. The relative level is the ratio of the signal intensity values between genes and *OsACTIN*1.

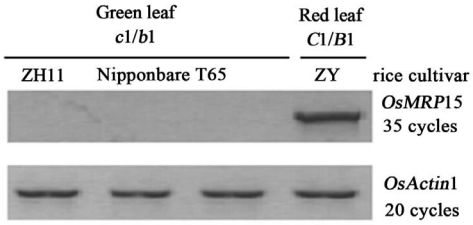

Figure 7. The RT-PCR analysis of *OsMRP*15 in green and red leaf rice cultivars.

Based on *in silicon* analysis and RT-PCR result, it can be deduced that *OsMRP*15 was a membrane-bound transporter that is required for vacuolar uptake of anthocyanins or flavonoids in rice, like *ZmMRP*3 in maize. Moreover, there was a possible GST gene presumptively forming anthocyanin-glutathione conjugates by *OsMRP*15 transporting into vacuolar in rice.

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