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Riboflavin Accumulation and Characterization of cDNAs Encoding Lumazine Synthase and Riboflavin Synthase in Bitter Melon (*Momordica charantia*)

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Supporting Information

ABSTRACT: Riboflavin (vitamin B_2) is the universal precursor of the coenzymes flavin mononucleotide and flavin adenine dinucleotide—cofactors that are essential for the activity of a wide variety of metabolic enzymes in animals, plants, and microbes. Using the RACE PCR approach, cDNAs encoding lumazine synthase (McLS) and riboflavin synthase (McRS), which catalyze the last two steps in the riboflavin biosynthetic pathway, were cloned from bitter melon (*Momordica charantia*), a popular vegetable crop in Asia. Amino acid sequence alignments indicated that *McLS* and *McRS* share high sequence identity with other orthologous genes and carry an N-terminal extension, which is reported to be a plastid-targeting sequence. Organ expression analysis using quantitative real-time RT PCR showed that *McLS* and *McRS* were constitutively expressed in *M. charantia*, with the strongest expression levels observed during the last stage of fruit ripening (stage 6). This correlated with the highest level of riboflavin content, which was detected during ripening stage 6 by HPLC analysis. *McLS* and *McRS* were highly expressed in the young leaves and flowers, whereas roots exhibited the highest accumulation of riboflavin. The cloning and characterization of McLS and McRS from *M. charantia* may aid the metabolic engineering of vitamin B_2 in crops.

KEYWORDS: *vitamin B_2, lumazine synthase, riboflavin synthase, cloning, characterization, Momordica charantia*

INTRODUCTION

Riboflavin (vitamin B_2) is the universal precursor of the coenzymes flavin mononucleotide and flavin adenine dinucleotide—cofactors that are essential for the activity of a wide variety of metabolic enzymes in animals, plants, and microbes.[1,2] Moreover, riboflavin is involved in numerous physiological processes involving light sensing, bioluminescence, and DNA repair.[3] Riboflavin is an indispensable vitamin, and current research has focused on the role that riboflavin plays in protecting against cancer and cardiovascular disease.[4,5] In plants, applied riboflavin enhances plant growth and resistance to diseases by triggering distinct signaling processes, including the ethylene signal transduction pathway and the systemic acquired resistance pathway.[6−9] Although riboflavin is biosynthesized by plants and many microorganisms, it must be obtained from dietary sources by animals and humans. As a consequence, riboflavin is considered to be a commercially important vitamin in food industries.

Riboflavin is synthesized from one molecule of GTP and two molecules of ribulose 5-phosphate through a nine-step pathway that is similar in all organisms.[10,11] The last two enzymes of the riboflavin biosynthetic pathway, lumazine synthase (LS) and riboflavin synthase (RS), are the most well characterized.[11] LS catalyzes the condensation of 5-amino-6-ribitylamino-2,4-(1*H*,3*H*)-pyrimidinedione with 3,4-dihydroxy-2-butanone 4-phosphate, yielding 6,7-dimethyl-8-ribityllumazine at the penultimate step (Figure 1).[12] The final step of the riboflavin biosynthetic pathway is mediated by RS, which catalyzes the disproportionation of 6,7-dimethyl-8-ribityllumazine, affording riboflavin. The second product of the disproportionation is 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, which is a substrate of LS and is recycled in the biosynthetic pathway.[13] Although LS and RS have been studied extensively in many microbial species, only a few LS and RS enzymes have been characterized in plants, including spinach, tobacco, and *Arabidopsis*.[14,15]

Bitter melon, *Momordica charantia*, is a member of the Cucurbitaceae family and is related to squash, melons, and cucumbers. It is a creeping plant that is native of Asia and found throughout the world. In folk medicine, *M. charantia* has been used to treat inflammation, diabetes, and stomach problems.[16] In recent years, phytochemists have isolated several compounds from *M. charantia* with great medicinal potential, including triterpenes, proteins, and steroids.[17,18] Moreover, the extracts of *M. charantia* have been reported to display wide medicinal

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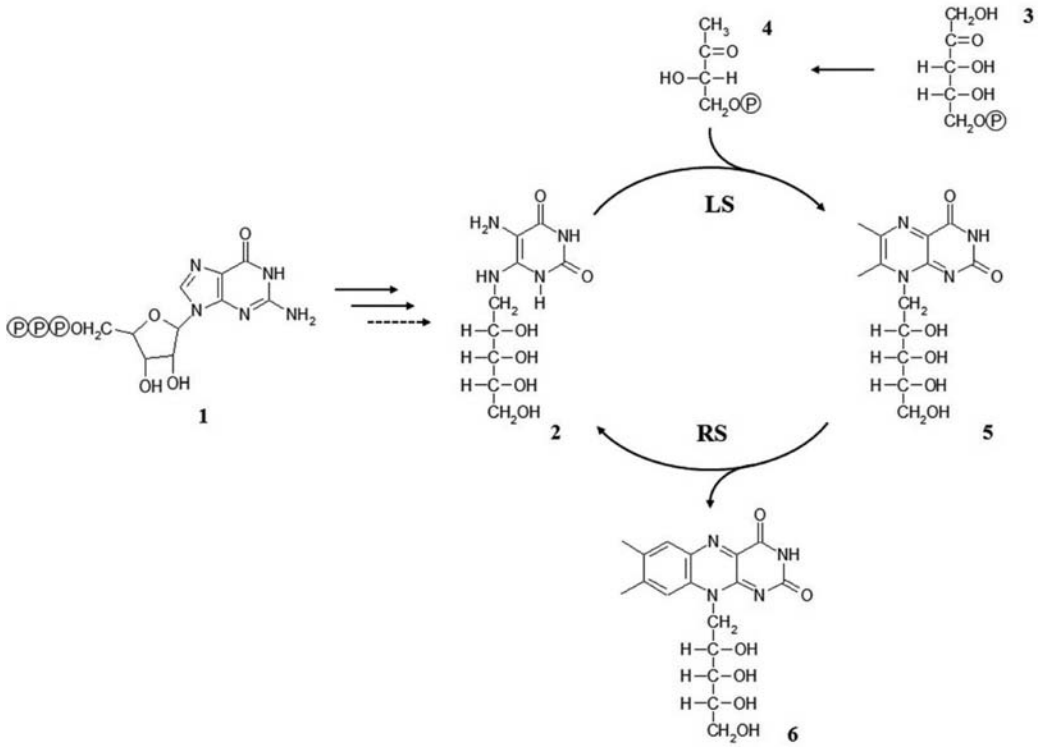

Figure 1. Terminal reactions in the riboflavin biosynthetic pathway. **1**, GTP; **2**, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione; **3**, ribulose 5-phosphate; **4**, 3,4-dihydroxy-2-butanone 4-phosphate; **5**, 6,7-dimethyl-8-ribityllumazine; **6**, riboflavin; **LS**, lumazine synthase; **RS**, riboflavin synthase.

Table 1. Primers Used To Amplify *M. charantia* Genes

primer	sequence (5′−3′)	amplicon (base pairs)
	Use: Partial Sequencing	
RS F	GGV CAY TTN GTD CAG GGH CAC GT	300
RS R	AGN ARC YTC TCR ACV TAC TTB CC	
	Use: RACE PCR	
McLS_3	TCC GAG GAA CGA TCA CCG TTC CTC	650
McRS_3	ACC TGA GGA GGA CTC TTT GTG GAT	528
McRS_5	GCA GAT CCT CTG ATG TTC TCA CCT	800
	Use: Real-Time RT PCR	
McLS_RT F	CAT TGG AGC TGT GAT TAA AGG TGA T	151
McLS_RT R	TCG ATT GAA AGC CTG ATC TAA GTT G	
McRS_RT F	GAT AAT TTC AAT GGA ACC TGA GGA	152
McRS_RT R	TAA AGG CTT TTT CCT CAT CAA AAA CA	
McCYP_RT F	GGC AAA CCC TAA AGT TTT CTT CG	174
McCYP_RT R	GAT GAG CCC TTG TAA TGA AGT GG	

properties, such as anti-HIV, antiviral, antitumor, anti-inflammatory, antiseptic, and antidiabetic effects.[19−21]

To investigate the biosynthetic mechanisms of riboflavin in *M. charantia*, full-length cDNAs encoding LS and RS were isolated using a RACE PCR method in the present study. In addition, to clarify the transcriptional regulation of riboflavin biosynthetic genes and the nutrient value of *M. charantia*, the relationship between gene expression of LS and RS and riboflavin accumulation was investigated in different organs and during fruit maturation in *M. charantia* using real-time PCR and high-performance liquid chromatography (HPLC), respectively. The cloning and characterization of *M. charantia* LS and RS (McLS and McRS) will provide a foundation to elucidate the factors controlling riboflavin synthesis as well as the contribution of riboflavin to the great medicinal properties of *M. charantia*.

MATERIALS AND METHODS

Plant Materials. *M. charantia* seeds were purchased from the Asian Seed Co. (Seoul, Korea), and *M. charantia* plants were grown at the experimental farm of Chungnam National University (Daejeon, Korea). After 5 months, the roots, stems, old leaves, young leaves, male flowers, and female flowers were excised from several plants. The fruits at six different developmental stages—ranging from 8.03 to 25.37 cm in length and from 1.27 to 5.04 cm in width—were harvested (Supporting Information, Supplementary Table 1). During maturation, the color of bitter melon fruits changed from green to yellowish. The ripening fruits at stage 6 turned orange, with the seeds covered in red pulp. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C and/or freeze-dried for RNA isolation and/or HPLC analysis.

RNA Isolation and cDNA Synthesis. The samples were ground into powder in a mortar with liquid nitrogen, and total RNA was isolated separately using a Plant Total RNA Mini Kit (Geneaid,

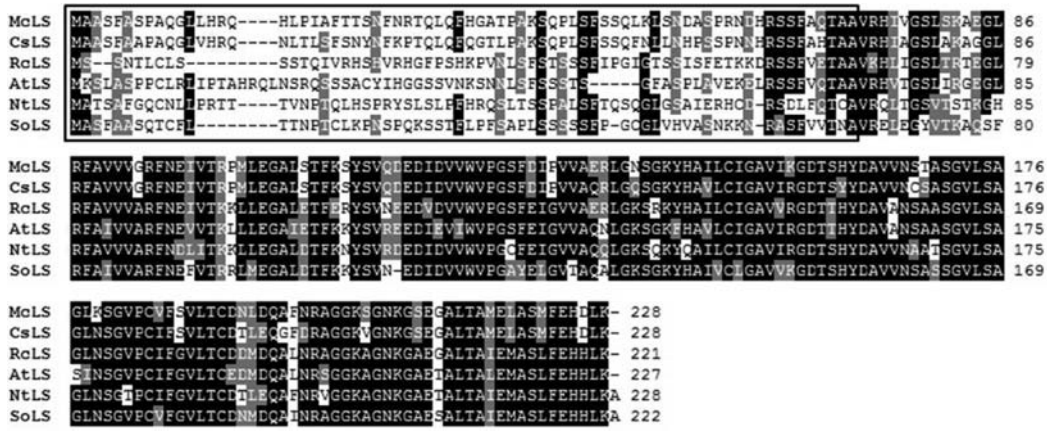

Figure 2. Multiple alignments of the amino acid sequences of McLS with other LSs. The black box shows the N-terminal extension found in plant riboflavin biosynthetic genes as compared with the homologous microbial enzymes. Identical residues are indicated by a black background, and similar residues are shaded with a gray background. CsLS, *Cucumis sativus* (EU301691); RcLS, *Ricinus communis* (XM_002531475); AtLS, *Arabidopsis thaliana* (AY091343); NtLS, *Nicotiana tabacum* (AF148648); SoLS, *Spinacia oleracea* (AF147203).

Taiwan) according to the manufacturer's instructions. The quality and concentration of total extracted RNA were determined by 1% agarose gel electrophoresis and spectrophotometer analysis, respectively. For RACE PCR, 3 μg of high-quality total RNA was used to synthesize first-strand cDNA using a GeneRacer Kit (Invitrogen, Carlsbad, CA, USA), and a 10-fold dilution of the 20 μL of resulting cDNA was used as template. For quantitative real-time PCR, 1 μg of total RNA was used for reverse transcription using the ReverTra Ace-R kit (Toyobo, Osaka, Japan), and a 20-fold dilution of the 20 μL resulting cDNA was used as template.

Cloning of cDNA Encoding Lumazine Synthase and Riboflavin Synthase. Using 5′-end information from the *M. charantia* EST library (http://pestas.kribb.re.kr/), a primer (Table 1) was designed to amplify the 3′-end of the *LS* gene from *M. charantia*. To clone the *RS* gene, degenerate primers for RS were designed on the bais of the conserved regions of *RS* genes from other higher plants. The amplified products were purified and cloned into a T-Blunt vector (SolGent, Daejeon, Korea) and subsequently sequenced. BLAST search results confirmed that these fragments were partial sequences of RS from *M. charantia*. Using the partial sequences obtained, gene-specific primers were designed to amplify the 5′- and 3′-ends of RS, and 5′- and 3′-RACE PCR was performed by following the manufacturer's protocol. The resulting PCR product was purified and cloned into a T-Blunt vector for sequencing.

Sequence Analysis. The deduced amino acid sequences of McLS and McRS were analyzed for homology by using the BLAST program at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were carried out using BioEdit Sequence Alignment Editor, version 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh, NC, USA).

Real-Time PCR. On the basis of the sequences of McLS and McRS (GenBank accession no. JX500426 and JX500427, respectively), real-time RT PCR primers were designed using the Primer3 Web site (http://frodo.wi.mit.edu/primer3/) (Table 1). The expression of these genes was calculated according to the method of relative quantification using the *M. charantia* cyclophilin *housekeeping gene* (*HQ171897*) as the reference. For quantification of standard, the PCR products amplified from cDNA were purified, and the concentration of the products was measured to calculate the number of cDNA copies. Real-time PCR reactions were carried out in a 20 μL reaction mix containing 5 μL of template cDNA, 10 μL of 1× SYBR Green Realtime PCR Master Mix (Toyobo), 0.5 μL of each primer (10 μM), and DEPC water. Thermal cycling conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 56 °C for 15 s, 72 °C for 20 s. PCR products were analyzed using Bio-Rad CFX Manager 2.0 software. Three replications for each sample were used for real-time analysis.

Riboflavin Extraction and Analysis. Extraction of riboflavin was performed according to the method of Esteve et al. with a slight modification.[22] Briefly, vitamin B_2 was released from the samples (0.1 g) by adding 0.5 mL of 0.1 N hydrochloric acid, vortex mixing for 20 s, and placing in a water bath at 80 °C for 30 min. After cooling, the pH was adjusted to between 4 and 4.5 with 2 M sodium acetate, and 0.1 mL of a freshly prepared 10% (w/v) takadiastase solution in water was added. The mixtures were placed in a water bath at 50 °C for 3 h and then heated at 80 °C for 5 min. After cooling, 0.3 mL of water was added and the extracts were filtered. Riboflavin was separated on a C18 column (250 × 4.6 mm, 5 μm, Symmetry RP18; Waters) by HPLC (Shimadzu, Kyoto, Japan) equipped with a fluorometric detector (RF-10A; Shimadzu). The compound was detected on the basis of the $\lambda_{ex}/\lambda_{em}$ at 422/515 nm. Elution was performed using a binary gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) according to the following program: 0 min, 95% A/5% B; 25 min, 65% A/35% B; 27 min, 5% A/95% B; 37 min, 5% A/95% B; 41 min, 95% A/5% B; and 51 min, 95% A/5% B. The flow rate was 1.0 mL/min, and the column temperature was 40 °C.

RESULTS AND DISCUSSION

Cloning of LS and RS from *M. charantia*. The McLS_3 primer was used for 3′-RACE PCR to amplify a 650 bp fragment, which showed sequence similarities to other LSs according to a BLAST search. Subsequently, the overlapping sequences between the 3′- and 5′-end fragments of McLS were analyzed to provide details about full-length McLS. The complete length of McLS was 851 bp, including a 35 bp 5′-untranslated region (UTR), a 687 bp open reading frame (ORF), and a 129 bp 3′-UTR with a poly-A tail. The ORF of McLS encodes a protein of 228 amino acids with a predicted molecular mass of 24.23 kDa.

To clone RS from *M. charantia*, degenerate primers (RS F and RS R) were used to amplify a 300 bp fragment, which displayed sequence similarity to other RSs according to a BLAST search. Subsequently, the 5′- and 3′-ends of McRS were isolated using RACE PCR primers. By aligning and assembling the sequences of 5′-RACE, 3′-RACE, and the partial fragment, the 1176 bp, full-length cDNA of McRS was obtained. McRS contains an 83 bp 5′-UTR and an 849 bp OFR, encoding a protein of 282 amino acids (predicted molecular mass of 30.67 kDa), as well as a 244 bp 3′-UTR with a poly-A tail.

Sequence Analyses of McLS and McRS. A BLAST search at the amino acid level showed that McLS exhibited high

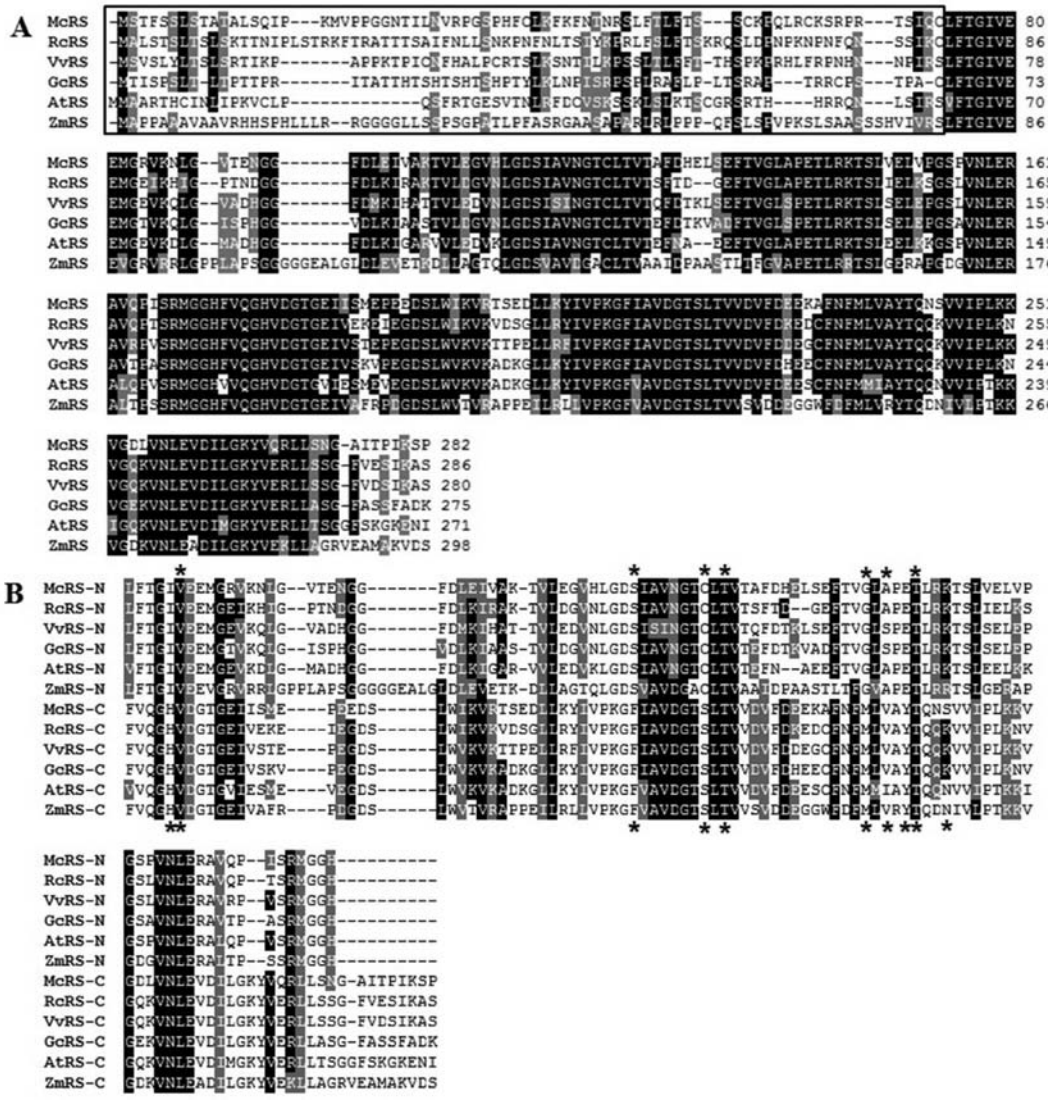

Figure 3. Multiple alignments of amino acid sequences (A) or the N- and C-terminal domains (B) of McRS with other RSs. The black box shows the N-terminal extension found in the plant riboflavin biosynthetic genes as compared with the homologous microbial enzymes. Residues that interact with the substrate of RS are marked by asterisks. Identical residues are indicated by a black background, and similar residues are shaded with a gray background. N, N-terminal domain; C, C-terminal domain. RcRS, *Ricinus communis* (XM_002517874); VvRS, *Vitis vinifera* (XM_002273993); GcRS, *Glycine max* (XM_003539872); AtRS, *Arabidopsis thaliana* (NM_127633); ZmRS, *Zea mays* (NM_001155882).

homology to other LSs (Figure 2). Specifically, McLS shared 82% identity and 89% similarity with *Cucumis sativus* LS, 67% identity and 80% similarity with *Ricinus communis* LS, 66% identity and 82% similarity with *Arabidopsis thaliana* LS, 60% identity and 73% similarity with *Nicotiana tabacum* LS, and 70% identity and 85% similarity with *Spinacia oleracea* LS. All genes described to date that are related to the biosynthesis of riboflavin in plants contain N-terminal extensions when compared with the homologous microbial enzymes.[15,23] As shown by the black box in Figure 2, McLS also carries an N-terminal extension with a length of 72 amino acids.

The alignment of the deduced amino acid sequences of McRS with its orthologs is shown in Figure 3A. McRS shares 68% identity and 78% similarity with *R. communis* RS, 72% identity and 84% similarity with *Vitis vinifera* RS, 74% identity and 84% similarity with *Glycine max* RS, 63% identity and 74% similarity with *A. thaliana* RS, and 57% identity and 70% similarity with *Zea mays* RS. The deduced amino acid sequence of McRS comprises a 73 amino acid N-terminal extension—found in plant riboflavin biosynthetic genes—compared with the homologous microbial enzymes (black box; Figure 3A). Moreover, Figure 3B reveals that McRS exhibits internal sequence homology at its N- and C-terminal domains, which is also observed for other RSs.[10] Residues that interact with the substrate of RS are marked by asterisks in Figure 3B.[24]

Expression Levels of *McLS* and *McRS* in Different Organs of *M. charantia*. Quantitative real-time PCR analysis was used to investigate the expression patterns of *McLS* and *McRS* in the roots, stems, old leaves, young leaves, male flowers, and female flowers of *M. charantia*. Organ expression analysis showed that *McLS* and *McRS* were constitutively expressed in *M. charantia* (Figure 4A,B). The expression level of *McLS* was highest in the female flowers and generally similar in other organs. The most abundant level of transcripts of *McRS* was also detected in the female flowers. The male flowers exhibited a high level of *McRS* expression, whereas the mRNA level of *McRS* was comparatively lower in the roots, stems, and young

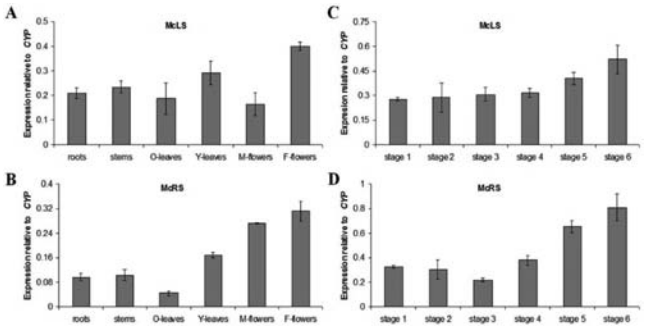

Figure 4. Expression levels of *McLS* and *McRS* in different organs (A, B) and during fruit maturation (C, D) in *M. charantia*. The height of each bar and the error bars show the mean and standard error, respectively, from three independent measurements. O-leaves, old leaves; Y-leaves, young leaves; M-flowers, male flowers; F-flowers, female flowers; CYP, *M. charantia* cyclophilin.

leaves. Only a weak expression level of *McRS* was observed in the old leaves of *M. charantia*.

Expression Levels of *McLS* and *McRS* during Fruit Maturation in *M. charantia*. As shown in Figure 4C,D, *McLS* and *McLS* were expressed throughout the maturation of *M. charantia* fruit. The expression level of *McLS* was nearly constant from stage 1 to stage 4 and increased slightly during stages 5 and 6. The expression pattern of *McRS* was fairly similar to that of *McLS* during fruit maturation. The expression of *McRS* remained moderate during the initial stages, increased strongly in the late stages, and reached its highest level during the ripening stage (stage 6).

Analysis of Riboflavin Content in Different Organs of *M. charantia*. The same plant materials as those used for quantitative real-time PCR were used for HPLC analysis of riboflavin in *M. charantia*. The accumulation of riboflavin was the highest in the roots, where its concentration was 195.4 μg/g of dry weight (Figure 5A). In comparison with the roots, other organs of *M. charantia* contained a significantly lower amount of riboflavin. The content of riboflavin in the young leaves was

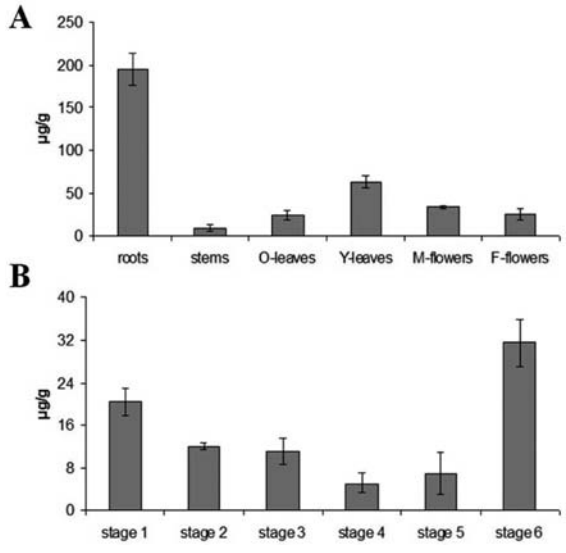

Figure 5. Riboflavin content in different organs (A) and during fruit maturation (B) in *M. charantia*. The values and error bars represent the mean and standard error of three independent measurements, respectively.

63.5 μg/g, which was higher than that of male flowers, female flowers, and old leaves (34.5, 25.8, and 24.1 μg/g, respectively. The lowest accumulation of riboflavin was detected in the stems of *M. charantia* (9.3 μg/g).

Analysis of Riboflavin Content during Fruit Maturation in *M. charantia*. The amount of riboflavin detected in the *M. charantia* fruit also varied during its maturation (Figure 5B). The content of riboflavin decreased strongly from 20.4 μg/g in stage 1 to 5.2 μg/g in stage 4, and it slightly recovered during stage 5 (7 μg/g). When the fruit turned orange and ripened at stage 6, the accumulation of riboflavin dramatically increased to 31.4 μg/g, which represents a 4.5-fold increase compared with that in stage 5.

Despite the fact that plants are the major source of vitamin B_2 in human and animal nutrition, the biosynthesis of vitamin B_2 has been less extensively studied in plants than in microorganisms. To the best of our knowledge, this study is the first report on the characterization of *LS* and *RS*, and it is the first study to examine riboflavin content in different organs and during distinct stages of fruit maturation in plants. We cloned cDNAs encoding LS and RS from *M. charantia*, which showed high identity to other orthologous genes. Similar to other genes related to the biosynthesis of riboflavin in plants, McLS and McRS carry an N-terminal extension, which displays low identity to corresponding genes and was believed to be a plastid-targeting sequence.[11,25] According to this hypothesis, *LS* and *RS* are synthesized in the cytosol with the N-terminal plastid-targeting sequences and then imported into plastids, where the N-terminal extension is removed to form the mature proteins.[11,15,26] In addition, the N- and C-terminal domains of McRS share 22.7% identical amino acid residues and were shown to have important implications for the disproportionation function.[15]

The expression patterns of *McLS* and *McRS* were analyzed in different organs and during the distinct stages of maturation of *M. charantia* fruit. Both *McLS* and *McRS* were expressed constitutively in all organs examined, with the highest expression levels found during stage 6 of fruit maturation. This correlates with the most abundant content of riboflavin, which was also detected during stage 6 of fruit maturation. Take together with the terminal functions of *LS* and *RS* in the riboflavin biosynthetic pathway, these results suggest that *McLS* and *McRS* are key enzymes involved in the regulation of riboflavin biosynthesis in *M. charantia* fruit. Analysis of expression in different organs revealed that whereas *McLS* and *McRS* were expressed actively in the young leaves and flowers, the highest accumulation of riboflavin was seen in the roots. Riboflavin is the precursor of flavin mononucleotide and flavin adenine dinucleotide, the latter of which is a cofactor for the root plasma membrane.[27,28] The high amount of riboflavin observed in the roots may result from a combination of endogenous biosynthesis and transport from the stems, where only a small amount was detected. Further research is needed to explain the abundant accumulation of riboflavin in the roots of *M. charantia*. The levels of riboflavin varied widely in different organs and during fruit maturation, suggesting that changes in riboflavin levels may exert physiological effects on *M. charantia*.[7,29]

A significant amount of riboflavin (31.4 μg/g) was found in the ripening fruits of *M. charantia*. The ripening fruits of *M. charantia* are also rich in β-carotene, which is the precursor of vitamin A,[30] and have been used in folk medicine for a long time. *M. charantia* fruit is a good source of provitamin A and

vitamin B_2, and its consumption may reduce the risk of some diseases such as stroke, heart disease, and cancer.[5,31,32] Thus, *M. charantia* fruit, which is a popular vegetable crop in Asia, may represent a valuable medicinal plant.

Riboflavin—an indispensable vitamin for animals and humans—is mainly produced by biotechnological fermentation using bacteria or yeasts for the overproduction.[33,34] However, to our knowledge, there has been no report on the increase of vitamin B_2 biosynthesis in crops. Thus, the molecular characterization of LS and RS and analysis of vitamin B_2 accumulation performed in the present study may not only broaden our understanding of the molecular mechanisms involved in the riboflavin biosynthetic pathway in *M. charantia* but also aid the metabolic engineering of vitamin B_2 in crops.

■ ASSOCIATED CONTENT

Supporting Information

Dimensions of *M. charantia* fruit during development. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

CYP, *M. charantia* cyclophilin; LS, lumazine synthase; RS, riboflavin synthase; DEPC, diethylpyrocarbonate; RACE, rapid amplification of cDNA ends; HPLC, high-performance liquid chromatography; F-flowers, female flowers; M-flowers, male flowers; O-leaves, old leaves; Y-leaves, young leaves

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