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Brief Communication

Riboflavin fortification of rice endosperm by metabolic engineering

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Riboflavin (vitamin B_2) is an essential nutrient for human health and body development. Riboflavin deficiency may increase the risk of some diseases (Thakur et al., 2017). Humans must regularly consume a sufficient amount of riboflavin, as this vitamin cannot be stored in the body. Data from the 2010–2012 China National Nutrition and Health Surveillance indicated that the riboflavin dietary intake of 85.9% of 14- to 17-year-old Chinese participants was below the estimated average requirement (Wang et al., 2017). Cereal grains are one of the most important sources of riboflavin. Many people prefer to consume processed cereal products rather than whole brown cereals that are comparatively rough tasting. Cereal processing results in a considerable loss of riboflavin, as it is mainly found in the pericarp. The finer the processed cereal, the more riboflavin is lost. For example, the riboflavin content is decreased by 38% in milled rice (0.019 mg/100 g DW) (Hegedüs et al., 1985). An alternative approach to solving this problem is the use of genetically modified cereal with fortified riboflavin levels so that sufficient riboflavin remains in products after processing.

Recently, biofortification of rice endosperm with micronutrients or phytonutrients via genetic engineering has been proposed as a potential strategy to promote human health and nutrition (Strobbe et al., 2021; Tian et al., 2020). Theoretically, riboflavin content in rice endosperm also can be enhanced via the metabolic engineering of enzymes involved in riboflavin biosynthetic pathways. First, the suitable metabolic genes must be sourced. The riboflavin biosynthesis pathway has been well established in *Saccharomyces cerevisiae*. Guanosine 5′-triphosphate and D-ribulose-5-phosphate are transformed into riboflavin via a 7-reaction pathway that is controlled by six riboflavin biosynthesis (*RIB*) genes. A recent study showed that maximal synthesis of riboflavin in the host depends on the expression of all six genes and the strain containing six genes accumulates 20 mg/L of riboflavin in shake flask cultures (Marx et al., 2008). Therefore, the six *S. cerevisiae* genes [*ScRIB1* (GenBank No. NP_009520.1), *ScRIB7* (GenBank No. NP_009711.3), *ScRIB2* (GenBank No. NP_014575.1), *ScRIB3* (GenBank No. NP_010775.1), *ScRIB4* (GenBank No. NP_014498.1) and *ScRIB5* (GenBank No. NP_009815.1)] were chosen as metabolic genes for riboflavin biofortification in this study.

In order to verify whether heterologous expression of these enzymes could produce riboflavin, we constructed the riboflavin biosynthetic pathway in *Escherichia coli* (Figure 1a). Each of the six genes was chemically synthesized in accordance with *E. coli* codon use. Each gene expression cassette was constructed by connecting its ORF between a T7 promoter and a T7 terminator through the PAGE-mediated overlap extension PCR (PTDS) method (Xiong et al., 2004) and insertion into a pET28a vector in the proper order, yielding pET28a-RB. The transformation of *E. coli* DE3 with the pET28a-RB plasmid led to yellow colonies and yellow agar around the colonies, indicating possible riboflavin accumulation (Figure 1b). Furthermore, high-performance liquid chromatography (HPLC)/mass spectrometry analyses of the liquid supernatant were performed to confirm the riboflavin biosynthesis. Riboflavin (*m/z* 377) was detected after transformation with pET28a-RB (Figure 1c). The results indicated that the expression of the six genes can produce riboflavin in a heterologous expression system.

The main objective of this study was to fortify rice endosperm to improve the riboflavin content via genetic engineering. Therefore, the six genes were chemically synthesized based on rice codon usage, and each gene expression cassette was constructed by connecting its ORF between an endosperm-specific rice glutelin-1 promoter and glutelin-1 terminator through PTDS method (Xiong et al., 2004). The PCR fragment was excised using restriction endonucleases and inserted into a pCAMBIA1301 vector, yielding pCAMBIA1301-RB (Figure 1d). Thus, we transformed seven-day-old immature zygotic rice (*Oryza sativa* L.ssp japonica, Zhonghua 11) embryos with the recombinant vector pCAMBIA1301-RB. The colours of the produced rice endosperm were varying degrees of yellow. T_1 seeds with the most intense yellow endosperm were planted in the field to generate T_2 seeds. This process was repeated to obtain T_4 seeds with the most intense yellow.

The transgenic plants with multiple genes driven by the same promoter may trigger transgene silencing (Mourrain et al., 2007). Then, Western blot analysis using T_4 transgenic seed proteins and antibodies was performed to assess whether the proteins produced by the six genes were correctly expressed. A cleaved protein of rScRIB1S, rScRIB7S, rScRIB2S, rScRIB3S, rScRIB4S and rScRIB5S was clearly detected in T_4 transgenic lines, but none of these proteins were detected in wild type (WT) (Figure 1e). The results suggested that the six genes were not silenced, and the resulting individual protein components were correctly expressed in transgenic rice.

The rice endosperm with an intense yellow colour indicated a large accumulation of riboflavin in the endosperm (Figure 1f). A previous study demonstrated that high transgene copy numbers may lead to transgenic rice with more intense yellow endosperm (Bai et al., 2016). Thus, genomic Southern blot analysis was

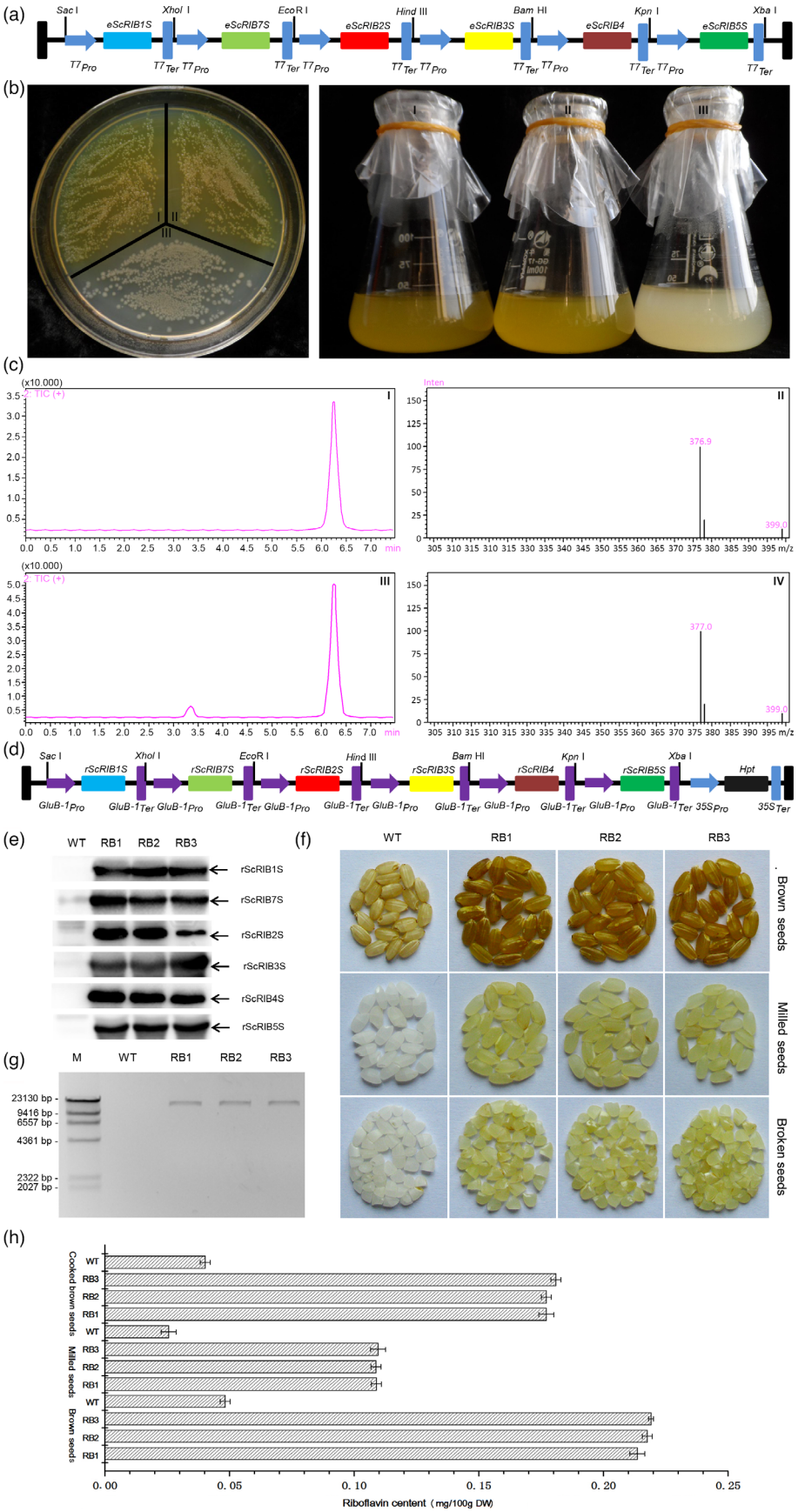

Figure 1 (a) Schematic representation of recombinant vectors used for riboflavin biosynthesis in *E. coli*. (b) Yellow colonies and yellow agar around the colonies in solid (left) and liquid (right) LB medium. (I) and (II), the transformed clone with recombinant vectors; (III), the non-transformed clone (CK). (c) Mass spectrometry analysis of riboflavin in standard and sample. (II) and (IV), the ion chromatograms of standard (II) and sample (IV); (I) and (III), the mass spectrometry data of standard (I) and sample (III). (d) Schematic representation of recombinant vectors used for riboflavin engineering in rice. (e) Western blot using the polyclonal rabbit antibodies. (f) Comparison of representative seed phenotypes between WT and RB at the T_4 generation. (g) Genomic Southern blot. The probe was amplified by PCR using the primers SNB322-F(5′- TCGTGCCATTCGTGACTCT −3′) and SNB322 -R(5′ - CACCACCTTCAACCATCACA −3′). (h) The riboflavin content in T_4 seeds and cooked rice. WT, wild type; RB, different lines of transgenic rice.

performed to determine transgene copy numbers in the transgenic rice. The results indicated that three transgenic lines contained a single copy of the transgenes using the same probe (specific to *rScRIB7S*) (Figure 1g). Subsequently, the riboflavin content in the same T_4 seeds of three transgenic lines was determined by HPLC according to National Standards of the People's Republic of China (GB5009.85-2016). An average of 0.2168 mg/100 g dry weight (DW) riboflavin had accumulated in brown seeds of transgenic lines (Figure 1h). This value was approximately 4.56-fold higher than the average riboflavin levels in WT rice (0.0475 mg/100 g DW). Also, the content of riboflavin in milled seeds of transgenic rice (0.1091 mg/100 g DW) was 4.24-fold higher than the riboflavin levels in milled seeds of WT rice (0.0257 mg/100 g DW) (Figure 1h). Compared with brown seeds, the polishing process results in approximately 50% loss of riboflavin no matter whether in transgenic seeds or WT seeds. In order to investigate whether the riboflavin fortification in transgenic seeds could increase the daily dietary supplement of riboflavin, a cooking experiment was performed. Although approximately 17% of the riboflavin was lost after cooking, 0.1784 mg/100 g DW riboflavin remained in the cooked rice from brown seeds of transgenic (Figure 1h). The estimated average requirement of riboflavin for 14- to 17-year-old Chinese children is approximately 1 mg/day (Wang et al., 2017). Taking these children as an example, the amount of rice consumed per person per day is approximately 200 g. Therefore, children can obtain more than 36% of the daily recommended amount of riboflavin just by eating this cooked rice. This contrasts with the less 10% of the riboflavin requirement they can obtain from cooked rice of WT seeds.

Starch, protein and lipids are the main rice components that affect cooking and nutritional quality (Zhou et al., 2010). These were quantified to determine the effect of riboflavin increase in transgenic seeds on the nutritional quality of the rice. The contents of amylose, amylopectin and protein in the transgenic seeds were in the same ranges as in WT seeds. The level of crude lipid was on average 1.65 times higher in the transgenic seeds (5.51%) compared with WT seeds (2.07%). The results indicated that the riboflavin fortification in transgenic seeds at least did not decrease its nutritional quality. Also, the riboflavin fortification did not affect the normal growth and grain yield of transgenic rice based on the results of agronomic traits in the field.

In conclusion, the expression of the six genes in rice endosperm significantly enhanced the riboflavin content by creating a metabolic sink towards *de novo* riboflavin biosynthesis. Riboflavin-fortified rice produced in this study can be used as a health-promoting cereal grain to decrease the prevalence of riboflavin deficiency.

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Conflict of interest

All authors declare no conflict of interest.

Author Contributions

Yao QH and Peng RH designed this research. Tian YS, Fu XY, Wang B, Deng YD, Zhang FJ, Zhang WH and Wang Y performed these experiments. Xu J, Wang LJ, Gao JJ, Han HJ and Li ZJ analysed these data. Tian YS wrote the manuscript.

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