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Genistein production in rice seed *via* transformation with soybean *IFS* genes

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ABSTRACT

To produce genistein in rice, the isoflavone synthase (*IFS*) genes, *SpdIFS1* and *SpdIFS2* were cloned from the Korean soybean cultivar, Sinpaldalkong II as it has a higher genistein content than other soybean varieties. *SpdIFS1* and *SpdIFS2* show a 99.6% and 98.2% identity at the nucleotide level and 99.4% and 97.9% identity at the amino acid level, respectively, with *IFS1* and *IFS2* from soybean (GenBank accession Nos. AF195798 and AF195819). Plant expression vectors were constructed harboring *SpdIFS1* or *SpdIFS2* under the control of a rice globulin promoter that directs seed specific expression, and used to transform two rice varieties, Heugnam, a black rice, and Nakdong, a normal rice cultivar without anthocyanin pigment. Because naringenin, the substrate of SpdIFS1 and SpdIFS2, is on the anthocyanin biosynthesis pathway, the relative production rate of genistein was compared between *SpdIFS*-expressing transgenic Heugnam and Nakdong. Southern blot analysis of eight of the resulting transgenic rice plants revealed that the T_0 plants had one to three copies of the *SpdIFS1* or *SpdIFS2* gene. The highest level of genistein content found in rice seeds was 103 μg/g. These levels were about 30-fold higher in our transgenic rice lines than the genistein aglycon content of a non-leguminous *IFS*-expressing transgenic tobacco petal, equaling about 12% of total genistein content of Sinpaldalkong II. There were no significant differences found between the genistein content in Heugnam and Nakdong transgenic rice plants.

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1. Introduction

Isoflavones, which are typical polyphenolic compounds found in leguminous plants, are called phytoestrogens because they have similar structures and functions to human estrogen [1–3]. The beneficial effects of isoflavones on osteoporosis [4], lowering cholesterol [5], hormone-related cancers [6], and cardiac disorders [7] have been reported in a number of clinical trials. The introduction of isoflavones into more widely consumed cereal crops such as rice, maize or wheat that do not naturally produce these compounds has thus been of great interests [8].

Isoflavones are synthesized from an intermediate substrate of the phenylpropanoid pathway, naringenin, which is commonly found in most plants, or from liquiritigenin, which is produced by the legume-specific chalcone reductase (CHR) (Fig. 1) [9,10]. Naringenin and liquiritigenin are converted into genistein and daidzein, respectively, by isoflavone synthase (IFS) [11]. The biosynthesis of isoflavones from these substrates consists of two steps. The oxidative aryl migration of naringenin or liquiritigenin by IFS generates 2-hydroxyisoflavanone, which can then be converted spontaneously to isoflavone or be dehydrated by dehydratase [2].

There have been several studies reporting the successful engineering of the isoflavone pathway in non-legumes, including rice, *Arabidopsis*, tobacco, maize, lettuce and petunia, using soybean-derived *IFS* genes *IFS1* or *IFS2* [2,8,9,12]. However, the resulting isoflavone levels in those transgenic plants have always been extremely low compared with soybean seeds. In rice transgenic plants expressing the *IFS* gene from soybean under the control of 35S promoter, isoflavone can be produced in the leaf and root. Rice is a staple food of over half the world's population and roughly 90% of all the rice is consumed in Asia, usually polished rice. To produce isoflavone in the rice endosperm, the choice of promoter is important. Previously, the 26 kDa globulin gene (*Glb-1*) promoter has been used to direct GUS expression in inner starchy endosperm tissue [13].

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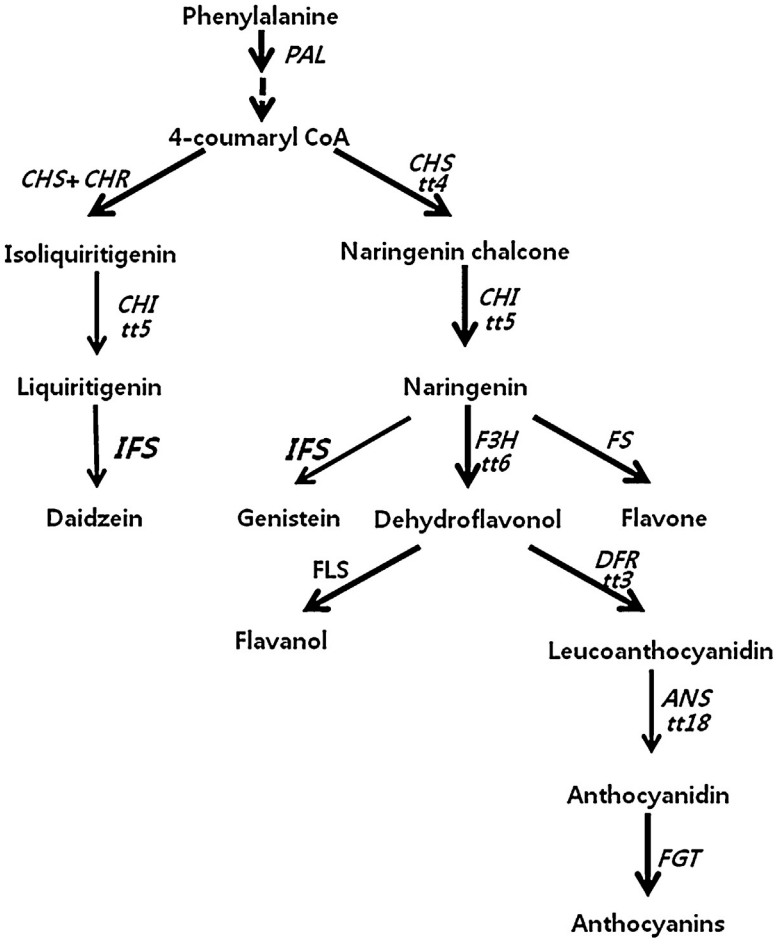

Fig. 1. Schematic representation of the phenylpropanoid pathway. Dotted arrows represent multiple steps. *PAL*, phenylalanine ammonia-lyase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *IFS*, isoflavone synthase; *F3H*, flavanone-3-hydroxylase; *FS*, flavone synthase; *FLS*, flavonol synthase; *DFR*, dihydroflavonol reductase; *ANS*, anthocyanidin synthase; *FGT*, flavonoid-3-*O*-glycosyltransferase. *Arabidopsis tt* mutations in each steps are indicated.

The aim of our present study was to generate transgenic rice lines that stably express *IFS* genes in the endosperm. For this purpose, we cloned the *SpdIFS1* and *SpdIFS2* genes from *Glycine max* cv. Sinpaldalkong II, a soybean variety which contains particularly high levels of isoflavones [14]. We then constructed plant expression vectors under the control of rice 26 kDa *Glb-1* promoter and expressed *SpdIFS1* and *SpdIFS2* in the japonica rice variety, Nakdong, and also the black rice variety, Heugnam. The expression patterns of the anthocyanin biosynthetic genes, *OsPAL* (phenylalanine ammonia-lyase), *OsCHS* (chalcone synthase), *OsCHI* (chalcone isomerase), *OsF3H* (flavanone-3-hydroxylase), *OsDFR* (dehydroflavonol reductase), and *OsANS* (anthocyanidin synthase) were subsequently analyzed in both transgenic and non-transgenic rice plants. Our results demonstrate that transgenic rice expressing heterologous *IFS* genes originating from soybean produce isoflavones. Our experiments also facilitated the molecular dissection of the anthocyanin biosynthetic pathway in non-transgenic and *SpdIFS* transgenic rice.

2. Materials and methods

2.1. Plant materials

The rice cultivars, Nakdong and Heugnam (provided by the National Institute of Crop Science, RDA) were used in the transformation experiments (Fig. S1a and f).

2.2. Isolation of isoflavone synthase genes from soybean

Soybean genomic DNA was prepared from the *G. max* cv. Sinpaldalkong II variety following the method of Dellaporta et al. [15]. From this DNA as the template, a fragment harboring the *SpdIFS1* gene was produced by PCR using the primers, SpdIFS1FOR: 5′-GTAATTAACCTCACTCAA ACTCGG-3′ and SpdIFS1REV: 5′-CAACTGCGATGGCAAGACACTACTATTGTAT-3′. A DNA fragment containing *SpdIFS2* was also produced with the primers, SpdIFS2FOR: 5′-AAAATTAGCCTC ACAAAAGCAAAG-3′ and SpdIFS2REV: 5′-GCAAACGAAGACAAATGGGAGATGATA-3′. The PCR reactions contained 10 ng of genomic DNA, 10 pmol of each primer, 4 μl of 2.5 mM dNTP mix and 1 unit of ExTaq polymerase in 1× ExTaq buffer supplied by the manufacturer (Takara Shuzo, Shiga, Japan) in a total volume of 50 μl. The amplification conditions comprised an initial denaturation step at 94 °C for 15 s followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. A final extension step at 72 °C for 7 min completed the program. Amplifications were performed in a PTC-100 thermal cycler (Bio-Rad, Hercules, CA).

2.3. Phylogenetic analysis of IFS proteins

To obtain phylogenetic data, the deduced amino acid sequences of the *IFS* genes were aligned using Clustal W software [16]. Neighbor-joining analysis [17] was employed to reconstruct phylogenetic trees using DNASTAR 7.0 (DNASTAR Inc., Madison, WI).

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