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Production of dammarane-type sapogenins in rice by expressing the dammarenediol-II synthase gene from *Panax ginseng* C.A. Mey

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ABSTRACT

Ginsenosides are the main active ingredients in Chinese medicinal *ginseng*; 2,3-oxidosqualene is a precursor metabolite to ginsenosides that is present in rice. Because rice lacks a key rate-limiting enzyme (dammarenediol-II synthase, DS), rice cannot synthesize dammarane-type ginsenosides. In this study, the *ginseng* (*Panax ginseng* CA Mey.) *DS* gene (GenBank: AB265170.1) was transformed into rice using agrobacterium, and 64 rice transgenic plants were produced. The Transfer-DNA (T-DNA) insertion sites in homozygous lines of the T_2 generation were determined by using high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) and differed in all tested lines. One to two copies of the T-DNA were present in each transformant, and real-time PCR and Western blotting showed that the transformed *DS* gene could be transcribed and highly expressed. High performance liquid chromatography (HPLC) analysis showed that the dammarane-type sapogenin 20(S)-protopanaxadiol (PPD) content was 0.35–0.59 mg/g dw and the dammarane-type sapogenin 20(S)-protopanaxatriol (PPT) content was 0.23–0.43 mg/g dw in the transgenic rice. LC/MS analysis confirmed production of PPD and PPT. These results indicate that a new "*ginseng* rice" germplasm containing dammarane-type sapogenins has been successfully developed by transforming the *ginseng DS* gene into rice.

1. Introduction

Rice is one of the world's principal food crops, and the hybridization of rice has increased rice yields dramatically. The demand for high-quality rice has also gradually gone up as the standard of living has increased. The development of transgenic technology provides a means of efficiently improving rice quality through the creation of rice germplasm that can synthesize active ingredients. For example, Ye et al., transformed key enzymes in the β-carotene biosynthesis pathway from daffodil and bacteria into rice, and by expressing the genes specifically in the endosperm, they produced "golden rice". With golden rice Vitamin A deficiencies can be prevented, as the β-carotene content reached substantial levels [1]. Paine et al., transformed the gene encoding phytoene synthase (PSY) in the pathway of β-carotene synthesis from maize instead of bacteria into rice and produced the second generation of "golden rice" with improved β-carotene content [2].

Lee et al. transformed the encoding gene of sesame methionine-rich 2S albumin into rice and produced "sesame nutrition rice", which substantially increased the levels of methionine, an essential and limiting amino acid [3].

Abbreviations: DS, Dammarenediol-II synthase; T-DNA, Transfer-DNA; HPLC, High performance liquid chromatography; PPD, 20 (S)-Protopanaxadiol; PPT, 20 (S)–Protopanaxatriol; DAD, Dammarenediol-II; ESI, Electrospray ionization; EIC, Extracted ion chromatogram; PSY, Phytoene synthase; HMG-CoA, 3-Hydroxy-3-methylglutaryl coenzyme A; MVA, Mevalonic acid; IPP, Isopentenyl pyrophosphate; DMAPP, Dimethylallyl pyrophosphate; GPP, Geranyl pyrophosphate; FPP, Farnesyl pyrophosphate; GGPP, Geranylgeranyl pyrophosphate; HMGR, 3-Hydroxy-3-methylglutaryl-CoA reductase; IPPS, Isoprenyl diphosphate synthase; MC, Monoterpene cyclases; FPPS, Famesyl diphosphate synthase; SC, Sesquiterpene cyclases; GGPPS, Geranylgeranyl diphosphate synthase; SQS, Squalene synthase; SQE, Squalene epoxidase; CS, Cycloartenol synthase; LS, Lanosterol synthase; αAS, α-Amyrin synthase; LUS, Lupeol synthase; βAS, β-Amyrin synthase; OSCs, 2,3-Oxidosqualene cyclases; *P. Quinquefolius*, *Panax quinquefolius*; *P. notoginseng*, *Panax notoginseng*; *E. Coli*, *Escherichia coli*; *A. tumefaciens*, *Agrobacterium tumefaciens*; 35S polyA, terminator of *CaMV* 35S gene; HPT, Hygromycin phosphotransferase gene; 35S P, 35S promoter; NOS T, Terminator of nopaline synthase gene; Ubi P, Ubiquitin promoter; LB, Left border; RB, Right border; Hyg, Hygromycin B; hiTAIL-PCR, High-efficiency thermal asymmetric interlaced PCR; qRT-PCR, Real-time fluorescence quantitative PCR; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; PC, Positive control; CK, compound k; PgUGT1, *P. ginseng* UDP-glycosyltransferase.

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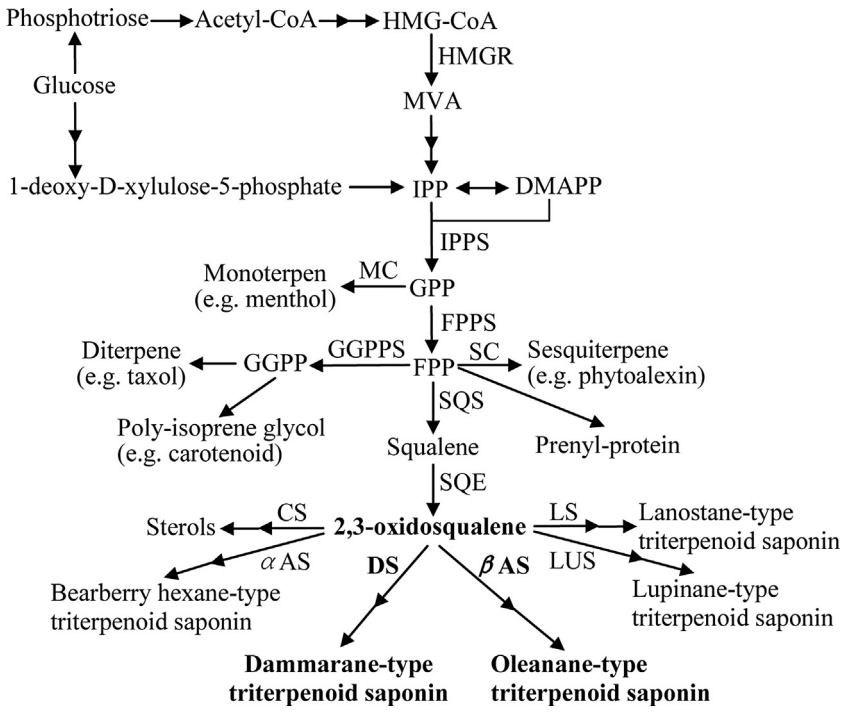

Fig. 1. The isoprenoid biosynthetic pathway in plants [6–8]. Intermediates: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate. Enzymes: HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPPS, isoprenyl diphosphate synthase; MC, monoterpene cyclases; FPPS, famesyl diphosphate synthase; SC, sesquiterpene cyclases; GGPPS, geranylgeranyl diphosphate synthase; SQS, squalene synthase; SQE, squalene epoxidase; CS, cycloartenol synthase; LS, lanosterol synthase; αAS, α-amyrin synthase; LUS, lupeol synthase; DS, dammarenediol-II synthase; βAS, β-amyrin synthase.

Panax ginseng, which belongs to *Panax* within Araliaceae family, is a rare traditional Chinese herbal medicine, and its active ingredient is ginsenosides [4]. All ginseng plants in the genus have relatively high medicinal values, and have roles in adaptation, immunity, tumor prevention, anti-atherosclerosis, and myocardial protection [4,5].

Ginsenosides are triterpenoid saponins of plant secondary metabolites, i.e. the product of triterpenoid saponins biosynthesis branch in isoprenoid pathway (Fig. 1) [6–8].

As shown in Fig. 1, triterpenoid saponins are formed by the cyclization of squalene. The synthesis of squalene is a branch point in the central isoprenoid pathway entering the triterpenoid saponins biosynthesis branch [7,9,10].

Squalene epoxidase (SQE) catalyzes squalene to produce 2,3-oxidosqualene, causing a linear shift from squalene to cyclic triterpenoids [11,12]. 2,3-oxidosqualene cyclases (OSCs) further catalyze the cyclization of 2,3-oxidosqualene to form dammarane-type tetracyclic triterpenoids, oleanane-type pentacyclic triterpenoids, or other compounds. Then the triterpenoid skeleton forms triterpenoid saponins of many different structures through oxidation, replacement, glycosylation, and other forms of chemical modification. OSCs are the rate-limiting enzymes of the triterpenoid saponin biosynthesis pathway [6,11,13–15]. Although the synthetic precursor of both ginsenoside and rice sterols is 2,3-oxidosqualene, the type and catalytic function of OSCs are different between ginseng genus and rice [7,9]. The OSCs of the ginseng genus are mainly dammarenediol-II synthase (DS) and β-amyrin synthase (βAS), which catalyze 2,3-oxidosqualene to produce dammarane-type and oleanane-type substances, respectively (specifically, dammarenediol-II and β-amyrin). Rice OSC is a cycloartenol synthase (CS), which catalyzes 2,3-oxidosqualene to produce cycloartenol [6,11,14], but due to the absence of DS and βAS, although rice contains ginsenosides precursor 2,3-oxidosqualene, it cannot synthesize ginsenosides.

The necessary knowledge base of ginsenoside synthesis is now present and provides an opportunity to improve staple rice quality using ginsenoside biosynthetic genes to allow the production of saponins. The *DS* gene has been made available through cloning in the ginseng genus, such as *P. ginseng* (GenBank: AB265170.1, GU183405.1, JN596111.1) [16,17], *P. quinquefolius* (GenBank: GU997679.1), *P. notoginseng* (GenBank: GU997680.1), etc. In the present work the ginseng *DS* gene was transformed into rice allowing the synthesis of dammarane-type sapogenins, creating a new germplasm called “ginseng rice” and providing germplasm resources for future germplasm improvements.

2. Materials and methods

2.1. Materials

Rice cultivar ‘Shuhui 527’, *Escherichia* coli (*E. coli*) strain DH5α, *Agrobacterium tumefaciens* (*A. tumefaciens*) strain LBA4404, plasmids pMD-Gt1-AmA1, pBlue-Ubi, and pCD-AMA1-hpt are kept by the Agricultural Product Quality Institute of Fujian Agriculture and Forestry University.

In order to facilitate the subsequent construction of plant expression vector without changing the amino acid sequence of ginseng *DS* gene (GenBank: AB265170.1), the recognition sites of *Bam*H I, *Sac* I, *Hind* III, *Sma* I, and *Eco*R I were removed from the coding region of the *DS* gene. The optimized *DS* gene was designated as *OPDS* (2310 bp in length). The recognition sites of *Bam*H I and *Sac* I were added to the 5′ end and 3′ end of *OPDS*, respectively. The whole *OPDS* gene was synthesized and linked to the pMD®18-T Simple Vector (Takara, D103A) by Shanghai Invitrogen Biotechnology Co., and the constructed plasmid was designated as pMD-DS.