Short Communication

Metabolic engineering of flavonoids with prenyltransferase and chalcone isomerase genes in tomato fruits

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Abstract   Flavonoids are phenolic secondary metabolites commonly occurring in plants. In particular, prenylated flavonoids exhibit a wide range of biological activities, including antitumor, antibacterial and antioxidant activities. In this study, we attempted to produce prenylated flavonoids in tomato as a host plant by means of metabolic engineering through the introduction of both the naringenin 8-dimethylallyltransferase (*N8DT*) gene, encoding a prenyltransferase from *Sophora flavescens*, and the chalcone isomerase (*CHI*) gene from *Nicotiana tabacum* cv. Samsun-NN. Liquid chromatography-tandem mass spectrometry analysis revealed the production of 8-dimethylallyl naringenin in the double transformants, while the production level was lower than N8DT single transformants. In addition, tomato fruits over-expressing both *N8DT* and *CHI* genes accumulated high levels of rutin compared with wild-type tomato. A possible endogenous regulation of the synthesis of flavonoid derivatives is discussed.

Key words: Prenyltransferase, chalcone isomerase, dimethylallyl naringenin, tomato.

Flavonoids are one of the major secondary metabolite groups and are represented by approximately 7,000 derivatives in plants (Arita and Suwa 2008). They occur commonly in most plant species, including in various vegetables and fruits. It is well known that flavonoids have various biological activities, including antioxidant, antitumor, antibacterial, antiasthmatic and antiallergic properties (Hertog et al. 1993; Hertog et al. 1995; Kandaswami et al. 2005; Lago et al. 2014). It is also known that these phenolic compounds exist in intact plant tissues mostly as glycosylated, methylated, prenylated or other derivatives, and only rarely occur as free forms. Approximately 1,000 prenylated phenolic compounds have been reported in various plant species, and the majority of these have a flavonoid core (Barron and Ibrahim 1996; Tahara and Ibrahim 1995). Most of these compounds were isolated from medicinal plants, and the prenylation of flavonoids often strongly enhances the biological activity of the base compounds in such species (Murakami et al. 2000).

Prenylation of flavonoids occurs by the addition of an isoprene unit, such as a dimethylallyl (C5), geranyl (C10), or farnesyl (C15) moiety, of which C5-prenylation is the predominant form in prenylated flavonoids. The occurrence of prenylated flavonoids is taxonomically limited in some plant families, where flavonoid-specific prenyltransferases of membrane-bound type are responsible for their biosynthesis (Yazaki et al. 2009). Bacterial prenyltransferases recognizing plant flavonoids are mostly soluble proteins, whose homologues are not found in plants (Sugiyama et al. 2011). We previously reported the isolation and characterization of prenyltransferase genes, including naringenin 8-dimethylallyltransferase (N8DT) from *Sophora flavescens*, which share moderate homology with homogentisate prenyltransferase involved in vitamin E biosynthesis (Sasaki et al. 2008). N8DT is a membrane-intrinsic protein and contains a transit peptide sequence at the N-terminus and is localized to the plastid even when expressed ectopically in heterologous host plants.
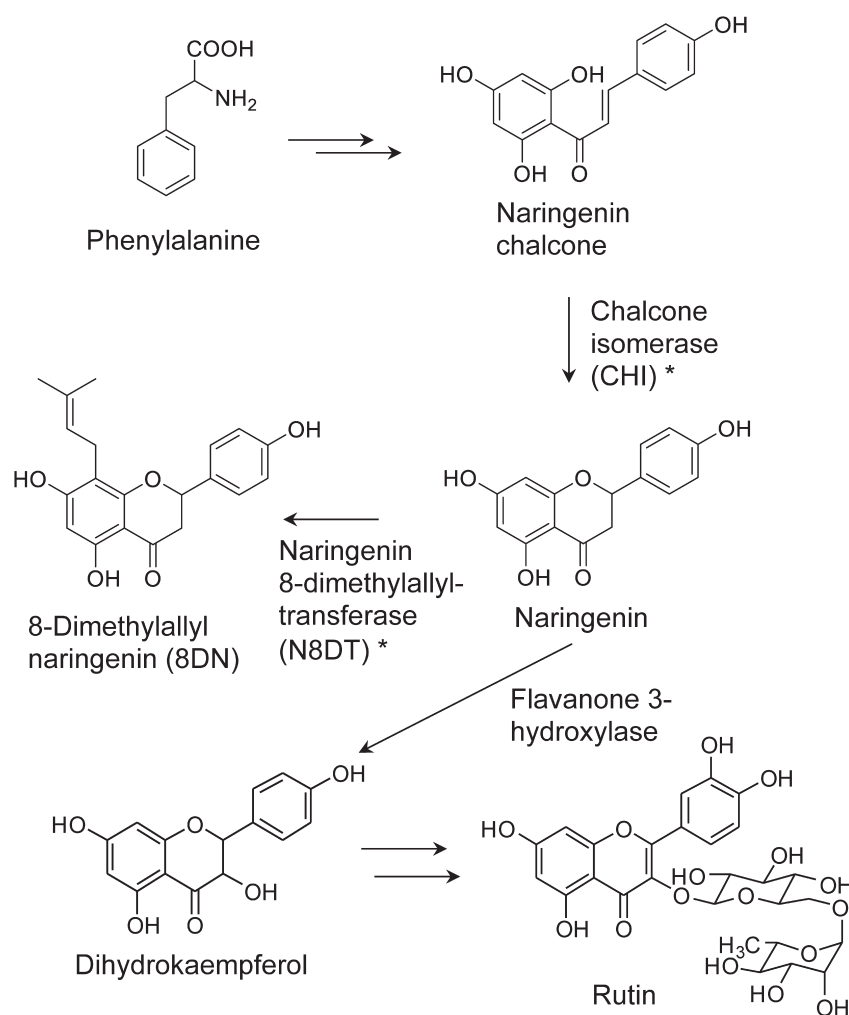
Figure 1. Biosynthetic pathway of prenylflavonoid and rutin. * Genes introduced in the present study.

N8DT is involved in the prenylation of a limited number of flavanones, including naringenin, liquiritigenin, and hesperetin, a subgroup of flavonoids, at the 8 position; and N8DT has high specificity for dimethylallyl diphosphate (DMAPP) as the prenyl-donor substrate. This means that N8DT shows high specificity for both substrates and its enzymatic reaction product. Moreover, we reported previously single transformation of the *N8DT* gene driven by fruit-specific E8 promoter in tomato fruits, and analysis of the metabolites showed that although 8-dimethylallyl naringenin was produced the amount was very low (2.8 μg/g dry weight) (Koeduka et al. 2011).

There are several strategies to improve the production of prenylated flavonoids, of which an increase *in vivo* substrate supply has yet to be investigated. In this study, chalcone isomerase (CHI) was introduced to increase the *in vivo* supply of naringenin because tomato fruits contain a high amount of chalcone, which is converted to naringenin by the enzymatic action of CHI (Figure 1). Here, we report the analysis of metabolites in tomato double transformants overexpressing both *N8DT* and

CHI, along with the transgenic tomato overexpressing *CHI* gene alone.

For the generation of double transformants, we prepared two constructs using a pRI201AN binary vector (Takara, Kyoto, Japan). In one construct, the *N8DT* gene was subcloned downstream of the cauliflower mosaic virus (CaMV) 35S promoter, which is a constitutive promoter, but the fruit-specific E8 promoter was used in the other expression vector instead of the CaMV35S promoter. The *CHI* gene was under the regulation of the fruit-specific E8 promoter in both constructs. These plasmids were designated as pRI201AN-E8::N8DT-E8::CHI and pRI201AN-35S::N8DT-E8::CHI, respectively (Figure 2). As another binary construct, the *CHI* gene was cloned into a modified pBI121 binary vector, in which the CaMV 35S promoter was replaced by the fruit-specific E8 promoter, and the resulting plasmid was designated pBI121-E8::CHI (Figure 2). As a host plant, a tomato strain Micro-Tom was obtained from the National BioResource Project (NBRP) (http://tomato.nbrp.jp/) of Japan. The plasmids were introduced into *Agrobacterium tumefaciens* (EHA105), and we
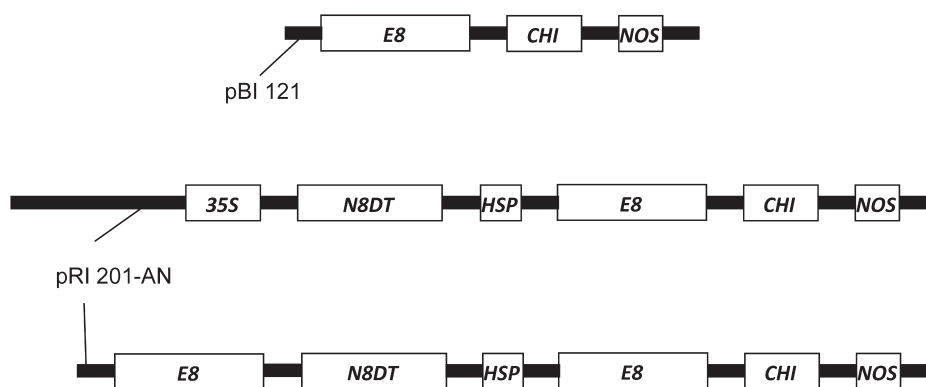
Figure 2. Expression constructs of chalcone isomerase and naringenin 8-dimethylallyltransferase used in the study. 35S, CaMV35S promoter; *CHI*, chalcone isomerase; E8, fruit-specific promoter; HSP, terminator of *Arabidopsis thaliana* heat shock protein; *N8DT*, naringenin 8-dimethylallyltransferase; NOS, terminator of *Agrobacterium tumefaciens* nopaline synthase.

generated transgenic tomato plants in accordance with a standard protocol (Sun et al. 2006). We obtained fifteen regenerated double transformant plants with E8::N8DT and E8::CHI genes, whereas transformants with the CaMV35S-driven N8DT gene could not be recovered from the transformation processes. We presumed that N8DT somehow hampers the regeneration process, and we have also observed this problem frequently with other prenyltransferase genes if they are expressed from this constitutive promoter. Thus, we only analyzed E8 promoter-driven double transformants expressing *N8DT* and *CHI*.

Total RNA was extracted from tomato fruits using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) for semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). After the treatment of RNA samples with DNase using a DNA-free kit (Ambion; Applied Biosystems Japan, Tokyo, Japan), cDNAs were synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), followed by incubation with RNase H (Invitrogen). RT-PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA) with the following gene-specific primer sets: N8DT forward 5′-CGG GAT CCA TGG GTT CTA TGC TTC TTG CAT C-3′ and reverse 5′-CGA GCT CTC CAT CTA AAC AAA GGT ATG AGG AAG T-3′; CHI forward 5′-GAT GCA GGC CAT TGA GAA GT-3′ and reverse 5′-GGA CAT GGT CTA TTT CTC TTC GAT-3′; and actin forward 5′-ATG ACT CAA ATC ATG TTT GAG ACC TTC-3′ and reverse 5′-TAC CTT AAT CTT CAT GCT GCT TGG AG-3′. The expression of both the *CHI* and *N8DT* genes in the fifteen E8::N8DT/E8::CHI transgenic plants lines was screened, out of which five exhibited positive signals for both genes. Figure 3 shows the detection of both transgenes in the five clones by semi-quantitative RT-PCR analysis, whereas these genes are undetectable in wild-type tomato fruits.

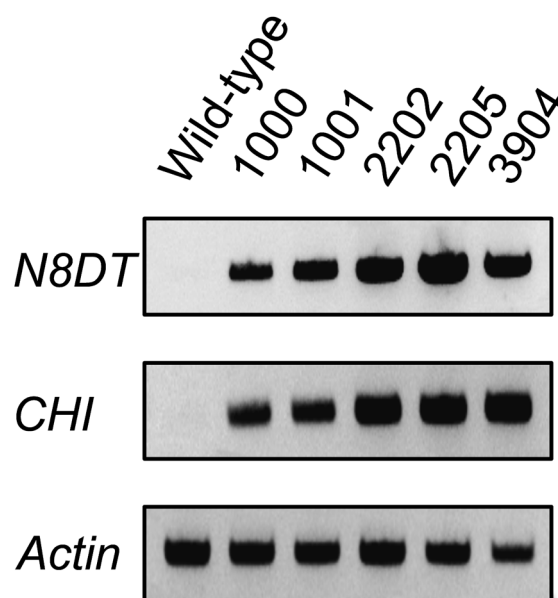The five positive E8::N8DT/E8::CHI or E8::CHI

Figure 3. Gene expression in naringenin 8-dimethylallyltransferase/chalcone isomerase transgenic tomato fruits. The expression levels were analyzed by semi-quantitative RT-PCR. Numbers represent the clone number of the tomato double transformants. *Actin* was used as the loading control. *CHI*, chalcone isomerase; *N8DT*, naringenin 8-dimethylallyltransferase.

transgenic clones were then used for further metabolite analyses. The transgenic tomato fruits were frozen in liquid nitrogen, powdered using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and lyophilized completely. The dry powder (150 mg) was extracted with methanol (3 ml) at 60°C for 1 h, and the centrifuged supernatant was passed through an Ultrafree-MC column (0.45 μm; Millipore, Billerica, MA, USA) to remove debris. Analysis of prenylated flavonoids in transgenic tomato fruits was performed by liquid chromatography. The above filtrate was injected into a high-pressure liquid chromatography (HPLC) apparatus (LC-10A, Shimadzu, Kyoto, Japan) with a TSKgel ODS-80Ts 4.6×250 mm column (TOSO, Tokyo, Japan). For the routine analysis

of prenylated flavonoids, elution was at 0.8 ml/min with solvent system A (water containing 0.3% (v/v) formic acid) and B (acetonitrile) with a linear gradient program from 15 to 22% B over 18 min, followed by a linear gradient from 22 to 35% B over 10 min and a linear gradient from 35 to 80% B over 22 min, with detection at 262 nm. For the analysis of rutin, elution was at 1.0 ml/min with solvent system A (water containing 0.3% (v/v) formic acid) and B (MeOH) with a linear gradient program from 30 to 50% B over 30 min, with detection at 355 nm. The metabolites from transgenic tomato were confirmed by direct comparison with the retention time and mass fragmentation pattern of standard 8-dimethylallyl naringenin (8-DN) using HPLC linked to an Applied Biosystems API3000 MS/MS spectrometer (ESI⁻).

We found that all the E8::N8DT/E8::CHI transgenic tomato clones produced 8-DN, whereas wild-type tomato did not produce any detectable level of prenylated naringenin. However, the production level of 8-DN in E8::N8DT/E8::CHI transgenic tomato was only 0.3–0.8 µg/g dry weight (Figure 4). In general, the product accumulation level does not seem to correlate with the gene expression level, as observed in other prenyltransferase transformants (Sugiyama et al. 2011). In addition, the HPLC analysis revealed that the E8::N8DT/E8::CHI transgenic tomato fruits produced 7- to 18-fold higher amount of rutin than in wild-type tomato (Figure 5). The accumulation level of rutin was 1,700–5,000 µg/g dry weight, which was more than 2,000 times higher than prenylated naringenin in transgenic tomato.

Many tomato cultivars, including Micro-Tom, accumulate a large quantity of naringenin chalcone in their fruits, especially in the ripening stage (Muir et al. 2001), and expression of CHI converts naringenin chalcone into naringenin (Figure 1). Therefore, expression of the *CHI* gene is expected to provide a large amount of naringenin, the *in vivo* substrate of N8DT. In the literature, the overproduction of rutin by *CHI* expression was reported in tomato fruits (Muir et al. 2001), and in the present study, the single transformant of E8::CHI also accumulated rutin (data not shown). In double transformants of N8DT and CHI, the naringenin supply *in vivo* increased, where competition takes place and either this intermediate is prenylated to provide 8-DN or it is further converted to rutin by endogenous enzymes. In fact, HPLC analysis revealed that naringenin level appears to be higher in transgenic lines (17–23 µg/g dry weight) than wild type (7.9 µg/g dry weight), but more dramatically naringenin chalcone (723 µg/g dry weight in wild type) is strongly converted to rutin, as this chalcone became undetectable level in transgenic lines.

The low productivity of 8-DN suggested several possibilities; for instance, the K_m value of N8DT for
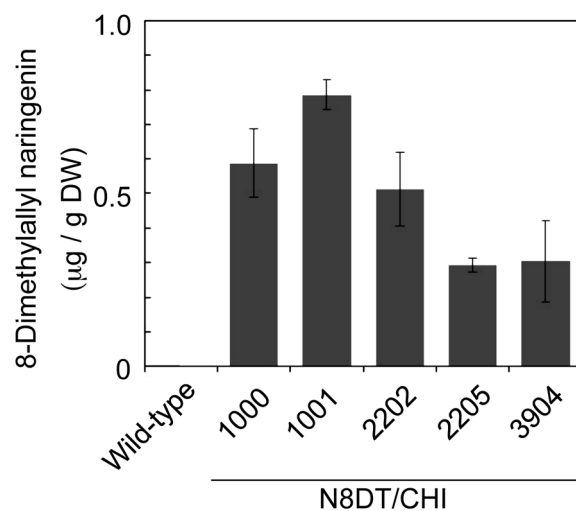

Figure 4.   Production of 8-dimethylallyl naringenin in naringenin 8-dimethylallyltransferase/chalcone isomerase transgenic tomato fruits. Numbers represent the clone number of the tomato double transformants. Bars represent means±standard deviation of triplicate samples. CHI, chalcone isomerase; DW, dry weight; N8DT, naringenin 8-dimethylallyltransferase.
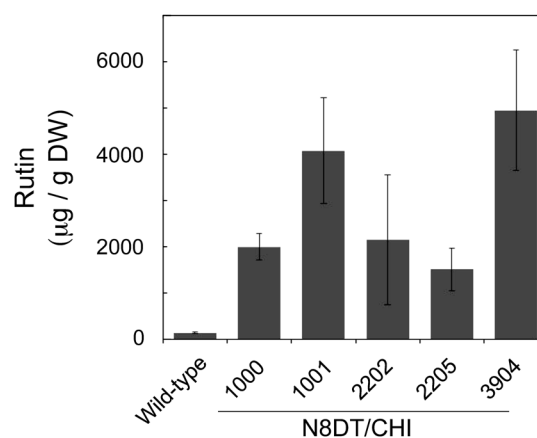

Figure 5.   Production of rutin in naringenin 8-dimethylallyltransferase/chalcone isomerase transgenic tomato fruits. Numbers represent the clone number of the tomato double transformants. Bars represent means±standard deviation of triplicate samples. CHI, chalcone isomerase; DW, dry weight; N8DT, naringenin 8-dimethylallyltransferase.

naringenin is 55 µM (Sasaki et al. 2008), while that of flavanone 3-hydroxylase (F3H) is reported to be 2.2–24 µM depending on the plant species (Lukacin and Britsch 1997; Owens et al. 2008). Considering the competitive utilization of naringenin as *in vivo* substrate, F3H is dominant over N8DT. Another possibility is that there is a species-dependent preference for which compounds accumulate, and conversion toward completely new compounds is less preferable, whereas as increase in endogenous metabolites is more effective for metabolic engineering. Disruption of the F3H gene to block rutin biosynthesis may contribute to the accumulation of higher amounts of 8-DN, which is

planned for a future study (Figure 1).

Another important point in the design of successful metabolic engineering approaches is the subcellular compartment in which metabolic enzymes and intermediates are localized. As reported, the preferred subcellular compartment for prenyltransferase is the plastid, where DMAPP is supplied via the methyl erythritol phosphate pathway, whereas flavonoid substrates are synthesized in the cytosol (Sugiyama et al. 2011). Thus, an efficient transport mechanism to provide flavonoid substrate to a plastid-localized prenyltransferase may be important, and the identification of a plastidial flavonoid transporter may aid in increasing 8-DN yield.

Acknowledgements

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