Phytochemistry 102 (2014) 55–63

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Enzymatic and metabolic engineering for efficient production of syringin, sinapyl alcohol 4-*O*-glucoside, in *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 22 May 2013
Received in revised form 21 February 2014
Available online 22 March 2014

Keywords:
Arabidopsis thaliana
Cruciferae
Syringin
Sinapyl alcohol 4-*O*-glucoside
Coniferin
Coniferyl alcohol 4-*O*-glucoside
Monolignol biosynthesis
Glucosyltransferase 72E family
Phenylpropanoid pathway
Gene stacking

ABSTRACT

To promote efficient production of syringin, a plant-derived bioactive monolignol glucoside, synergistic effects of enzymatic and metabolic engineering were combined. Recombinant *UGT72E3/E2* chimeras, generated by exchanging parts of the C-terminal domain including the Putative Secondary Plant Glycosyltransferase (PSPG) motif of UGT72E3 and UGT72E2, were expressed in leaves of transgenic *Arabidopsis* plants; syringin production was measured *in vivo* and by enzymatic assays *in vitro*. In both tests, UGT72E3/2 displayed substrate specificity for sinapyl alcohol like the parental enzyme UGT72E3, and the syringin production was significantly increased compared to UGT72E3. In particular, in the *in vitro* assay, which was performed in the presence of a high concentration of sinapyl alcohol, the production of syringin by UGT72E3/2 was 4-fold higher than by UGT72E3. Furthermore, to enhance metabolic flow through the phenylpropanoid pathway and maintain a high basal concentration of sinapyl alcohol in the leaves, *UGT72E3/2* was combined with the sinapyl alcohol synthesis pathway gene *F5H* encoding ferulate 5-hydroxylase and the lignin biosynthesis transcriptional activator *MYB58*. The resulting UGT72E3/2+F5H+MYB58 OE plants, which simultaneously overexpress these three genes, accumulated a 56-fold higher level of syringin in their leaves than wild-type plants.

Introduction

Syringin (sinapyl alcohol 4-*O*-glucoside) (**2**) (Fig. 1) is a natural product distributed widely throughout many types of plants, with massive accumulation restricted to some medicinal plants. It has been reported that it has various pharmacological effects with little toxicity, including anti-inflammatory, anti-nociceptive, immune-modulatory, and anti-diabetic effects (Cho et al., 2001; Choi et al., 2004; Liu et al., 2008; Niu et al., 2008). These health-promoting effects of syringin (**2**) attract considerable interest for novel applications. Although its biosynthesis pathway is well-characterized, many questions need to be elucidated to improve syringin (**2**) production and accumulation in plants.

Syringin (**2**) biosynthesis branches from the phenylpropanoid pathway, which is largely a highly conserved means of lignin production in the plant kingdom (Anterola and Lewis, 2002). Sinapyl alcohol (**1**), a monolignol synthesized via the phenylpropanoid pathway, is converted to syringin (**2**) by plant family 1 UDP-dependent glucosyltransferases (UGTs). In *Arabidopsis thaliana*, 120 *UGT* genes have been identified. Among them, a small cluster of three closely related genes (At3g50740, At5g66690, At5g26310) encode the UGT72E family, which are responsible for monolignol 4-*O*-glucoside production (Lanot et al., 2006; Lim et al., 2001) (Fig. 1). UGT72E2 preferentially glycosylates coniferyl alcohol (**3**) and has a high catalytic activity, which results in efficient production of coniferin (**4**). In contrast, UGT72E3 has high specificity for the glycosylation of sinapyl alcohol (**1**), but its low catalytic activity results in inefficient production of syringin (**2**) (Lanot et al., 2006, 2008; Lim et al., 2001). An improvement of UGT72E3 catalytic activity is thus a prerequisite for the application of this enzyme for syringin (**2**) production in plants.

Low availability of the substrate for UGT is another obstacle that must be surmounted to achieve efficient syringin (**2**) production in plants. There are three enzymatic branching points in the

Abbreviations: OE, overexpression; HPLC, high performance liquid chromatography; PSPG, Putative Secondary Plant Glycosyltransferase; UGT, UDP-glucose:alcohol glucosyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-(hydroxy) cinnamoyl CoA ligase; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinammoyltransferase; CHS, chalcone synthase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase.

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http://dx.doi.org/10.1016/j.phytochem.2014.03.003
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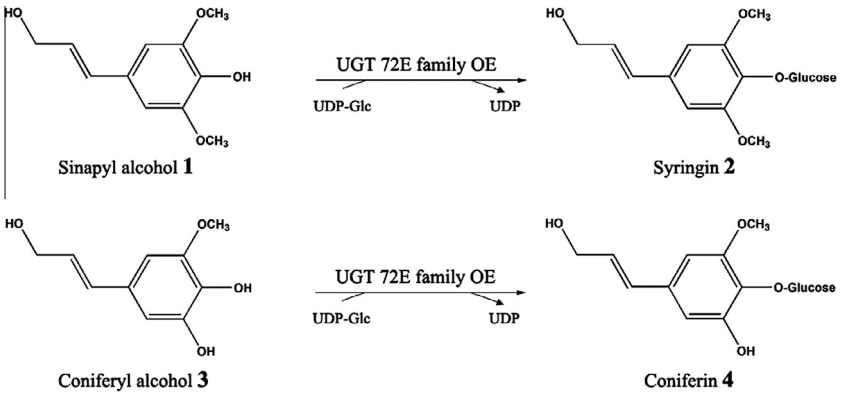

Fig. 1. Chemical structures of monolignols and their glucosides.

phenylpropanoid pathway that control the flux of carbon towards sinapyl alcohol (**1**): chalcone synthase (CHS), hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxylcinnamoyl transferase (HCT) and ferulate 5-hydroxylase (F5H). At these critical enzymatic reaction points, CHS and HCT direct *p*-coumaroyl CoA, a versatile metabolite generated by the phenylpropanoid pathway, into flavonoid synthesis and guaiacyl (G)-type monolignol synthesis, respectively (Anterola and Lewis, 2002; Whetten and Sederoff, 1995). F5H further catalyzes the conversion of G-type monolignol to syringyl (S)-type monolignol (**1**), which is a direct precursor for syringin (**2**) production (Vanholme et al., 2008). The details of the transcriptional network of monolignol biosynthesis in *Arabidopsis* have been elucidated (Zhou et al., 2009; Zhao et al., 2010). The lignin-specific transcriptional factor MYB58 directly activates most monolignol biosynthesis genes except *F5H*, which is regulated by a secondary cell wall master regulator SND1.

Light exposure induces accumulation of the monolignol glucosides, coniferin (**4**) and syringin (**2**), in *Arabidopsis* roots. This accumulation is a result of both increased production of these compounds by the phenylpropanoid pathway and altered utilization of these compounds by the lignin synthesis pathway. However, light-induced accumulation of monolignol glucosides does not occur in aerial tissues (Hemm et al., 2004).

In this study, the synergistic effects of enzymatic and metabolic engineering methods for the production of syringin (**2**) in *Arabidopsis* leaves was demonstrated. Transgenic plants overexpressing a novel recombinant *UGT72E3/2*, generated by domain swapping between UGT72E3 and UGT72E2, improved syringin (**2**) production compared to transgenic plants overexpressing *UGT72E3* or *UGT72E2*. Further, metabolic activation for S-type monolignol biosynthesis increased metabolic flux towards sinapyl alcohol (**1**). Consequently, a combination of these effects via gene stacking resulted in a drastic increase of syringin (**2**) production in the leaves of transgenic *Arabidopsis* plants when compared to wild-type plants.

Results

Generation of chimeric UGT72E2/3 and UGT72E3/2 by domain exchanging between parental UGT72E2 and UGT72E3

Although the UGT72E2 and UGT72E3 of *A. thaliana* display 85% sequence identity and 91% similarity at the amino acid level, they exhibit distinct enzymatic characteristics (Lim et al., 2001). UGT72E2 displays both high specificity for its coniferyl alcohol (**3**) substrate and a high catalytic activity, while UGT72E3 displays high specificity for its sinapyl alcohol (**1**) substrate and a low catalytic activity. To generate a novel chimeric UGT for the efficient production of syringin (**2**) in plants, a partial domain swapping strategy was used. Initially, predicted secondary structures for these enzymes with the SWISS-MODEL workspace were obtained (Schwede et al., 2003; http://swissmodel.expasy.org) and these were compared (Fig. 2A), and found to be very similar. The main differences were observed in the Nβ3–Nα3, Cβ3a–Cβ3b, and Cα8 regions. Focus was next on a portion of the C-terminal domain (amino acid residues 340–481) that contains the PSPG motif responsible for stabilization of UDP-glucose in the donor binding pocket. The exchange of this sequence between UGT72E2 and UGT72E3 resulted in the chimeric enzymes UGT72E2/3 and UGT72E3/2 (Fig. 2A). Using the SWISS-MODEL Workspace, the predicted three-dimensional (3D) protein structures of four different UGTs: 72E2, 72E3, 72E2/3, and 72E3/2 (Fig. 2B) were compared. The template used in the 3D structure modeling process, *Arabidopsis* UGT72B1 (PDB ID code: 2VCE; Brazier-Hicks et al., 2007), is expected to provide reliable predicted 3D structures, because it belongs to the same phylogenetic group (Group E) as the UGT72E family with 40% amino acid sequence identity to both UGT72E2 and UGT72E3 (Li et al., 2001). Slight differences in the predicted secondary structures of UGT72E2 and UGT72E3 were reflected in the predicted 3D structures, and the chimeric enzymes UGT72E2/3 and UGT72E3/2 exhibited similar structures to the parental enzymes UGT72E2 and UGT72E3 in both N- and C-terminal domains (Fig. 2B).

Enzymatic activities of parental UGT72E2 and UGT72E3 and chimeric UGT72E2/3and UGT72E3/2 in transgenic Arabidopsis plants

Full length ORFs encoding *UGT72E2*, *UGT72E3*, *UGT72E2/3*, and *UGT72E3/2* were individually overexpressed in *Arabidopsis* under the control of a super promoter (Lee et al., 2007). Independent transgenic plants were subjected to RT-PCR analysis to investigate the steady-state transcript levels for *UGT* transgenes. Representative transgenic plants with equivalent expression levels were selected for each construct and used for further experiments (Fig. 3A).

For qualitative analysis of the enzymatic activities encoded by each *UGT* transgene, leaf color of the transgenic plants under long wavelength (366 nm) UV light was examined (Fig. 3B). The leaves of the transgenic plants were redder than that of wild-type plants, because overexpression of *UGT*s leads to a depletion of sinapoyl malate that absorbs UV light in wild-type plants (Lanot et al., 2008). This result suggests that, like the parental UGT72E2 and UGT72E3 enzymes, the chimeric UGT72E2/3 and UGT72E3/2 enzymes may change the profiles of soluble phenolic metabolites in leaves.

High performance liquid chromatography (HPLC) was used to perform a quantitative analysis of monolignol glycosylation reactions in the leaves of the transgenic plants. In accordance with previous reports, the parental-type UGT72E2 and UGT72E3 OE plants showed a different substrate specificity (Lanot et al., 2006, 2008).