Overexpression of ginseng UGT72AL1 causes organ fusion in the axillary leaf branch of Arabidopsis
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ABSTRACT
Background: Glycosylation of natural compounds increases the diversity of secondary metabolites. Glycosylation steps are implicated not only in plant growth and development, but also in plant defense responses. Although the activities of uridine-dependent glycosyltransferases (UGTs) have long been recognized, and genes encoding them in several higher plants have been identified, the specific functions of UGTs in planta remain largely unknown.

Methods: Spatial and temporal patterns of gene expression were analyzed by quantitative reverse transcription (qRT)-polymerase chain reaction (PCR) and GUS histochemical assay. In planta transformation in heterologous Arabidopsis was generated by floral dipping using Agrobacterium tumefaciens (C58C1). Protein localization was analyzed by confocal microscopy via fluorescent protein tagging.

Results: PgUGT72AL1 was highly expressed in the rhizome, upper root, and youngest leaf compared with the other organs. GUS staining of the promoter: GUS fusion revealed high expression in different organs, including axillary leaf branch. Overexpression of PgUGT72AL1 resulted in a fused organ in the axillary leaf branch.

Conclusion: PgUGT72AL1, which is phylogenetically close to PgUGT71A27, is involved in the production of ginsenoside compound K. Considering that compound K is not reported in raw ginseng material, further characterization of this gene may shed light on the biological function of ginsenosides in ginseng plant growth and development. The organ fusion phenotype could be caused by the defective growth of cells in the boundary region, commonly regulated by phytohormones such as auxins or brassinosteroids, and requires further analysis.

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1. Introduction
To overcome their sessile lifestyle, plants produce a large amount of natural compounds that are not directly involved in plant growth and development but play crucial roles in plant defense responses to various adverse environmental conditions. These compounds contribute to plant–biosphere interactions and are referred to as plant secondary metabolites [1]. They exhibit considerable structural diversity, which is the result of various skeleton modification steps throughout their biosynthetic processes including oxidation/hydroxylation, substitution, and glycosylation. Glycosylation is considered to have a prominent role and is mainly involved in the final modification steps of secondary metabolite biosynthesis. These glycosylation processes are mediated by members of a multigene glycosyltransferase (GT) superfamily, which catalyze the transfer of single or multiple activated sugars to a wide range of substrates [2]. Although their activities have long been recognized, the specific function of GTs in planta remains largely unknown.

The system of GT classification is mainly based on amino acid sequence similarities, catalytic mechanisms, and the presence of a conserved sequence motif [3,4]. In total, 101 GT families from different species have been classified (http://www.cazy.org/GlycosylTransferases.html). Most GTs belong to family 1 of the uridine diphosphate (UDP)-glycosyltransferases, which catalyze the transfer of a UDP-activated sugar to wide-ranging acceptors, and are therefore referred to as UDP-dependent GTs (UGTs) [5,6]. Hundreds of different UGT genes exist in each plant genome, which is a reflection of the
diversity of these secondary metabolites. More than 120 UGTs have been identified from Arabidopsis thaliana even though this species does not possess a large number of secondary metabolites [7].

Triterpenoids represent a large group of natural secondary products with more than 40,000 different compounds identified [8]. Triterpenoid saponins from ginseng, called ginsenosides, may account for the well-known pharmacological efficacies of these compounds, including their antitumor, antistress, antiaging, and immune system-enhancing actions [9]. Thus, their utilization by humans has been attractive for many decades. Glycosylation plays an important role in the modification of ginsenoside skeletons leading to structural diversity [10] via the addition of monosaccharides to triterpene aglycones mainly at C-3 and/or C-20 for propanoxadiol (PPD)-type ginsenosides, and at C-6 and/or C-20 for PPT-type ginsenosides [11]. Therefore, the biological activities of ginsenosides are regulated mainly by UGTs. The removal of sugar residues from saponins, terpene glycosides, often results in the loss of antifungal properties [12]. Although sequence analysis has revealed that numerous UGTs exist in ginseng, only a few have been functionally characterized [13–16]. In the present study, molecular cloning and functional characterization of PgUGT72AL1, a UDP-dependent GT in ginseng, were performed.

2. Materials and methods

2.1. Plant materials and growth conditions

Korean ginseng (Panax ginseng Meyer) cv. Chun-Poong was used to analyze gene expression. Different ginseng organs (stem, leaf, root, petiole, and rhizome) were harvested from 2-year-old ginseng plants grown in soil at 25°C under a 16-h photoperiod. The Columbia ecotype (Col-0) of A. thaliana was used as a heterologous system for functional analysis. Seeds were sown on 1/2 MS medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 1% sucrose, 0.5 g/L MES (2-[N-morpholino] ethanesulfonic acid), pH 5.7 adjusted with 1M KOH, and 0.8% phytoagar. Seeds that were cold-treated for 2 days were germinated under long-day conditions.

2.2. DNA analysis

Polymerase chain reaction (PCR) products were checked by nucleotide sequencing. Amino acid sequences were analyzed to predict and identify conserved domains or active sites using online programs (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignment was performed using the BioEdit program (version 7.1.9, Copyright ©1997–2017 Tom Hall). A phylogenetic tree was generated by the neighbor-joining method using the MEGA6 (version 6.06, Copyright 1993–2013) program.

2.3. Abiotic stresses and hormone treatment

Three-week-old ginseng plantlets (cv. Chun-Poong) were stimulated with various abiotic treatments and two defense-modulating plant hormones. The plantlets were placed on a petri dish by dripping the root in 60 mL solution containing the following concentrations of chemicals; 5mM salicylic acid (SA), 0.2mM jasmonic acid (JA), 100μM abscisic acid (ABA), 10mM H2O2, and 100mM NaCl. Methanol, for JA, and distilled water, for SA, ABA, NaCl, and H2O2, were used as solvents. Chilling stress was induced by placing the roots in tap water at 4°C. Each treated plantlet sample, with the corresponding control sample, was collected at intervals of 1 h, 4 h, 8 h, 12 h, 24 h, and 48 h after treatment. The sampled materials were immediately frozen in liquid nitrogen and stored at −70°C.

2.4. RNA isolation and (quantitative) real-time reverse transcription-PCR

Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, with modifications. Contaminating genomic DNA was removed by treatment with DNase I (Takara, Japan) in a total 100-μL reaction volume for 1 h just prior to the washing step. The concentration of RNA was determined using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo, Wilmington, DE, USA). To synthesize first-strand cDNA, 3 μg of total RNA was reverse transcribed using RevertAid Reverse transcriptase (Thermo, USA). RT-PCR was performed in a 25-μL total reaction volume composed of 1–2 μL of cDNA, 20 pmol of each primer, and AmpIONE Taq DNA polymerase (GeneAll, Seoul, Korea) using a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) PCR machine under the following conditions: 95°C for 2 min, followed by 28 cycles of 95°C for 30 s, 60°C for 20 s, 72°C for 40 s, and extension at 72°C for 10 min. The RT-PCR products were visualized on 1.0% agarose gel. Quantitative PCR was carried out using the Thermal Cycle Dice real-time PCR system (Takara, Shiga, Japan) according to the manufacturer’s instructions. The thermal cycler conditions recommended by the manufacturer were used as follows: initial denaturation at 95°C for 30 s followed by 45 cycles of 95°C for 5 s and 56°C for 10 s, and 72°C for 20 s in a 25-μL total reaction volume. At the end of the PCR, a dissociation curve was generated to evaluate the generation of by-products. To determine the absolute fold differences in expression for each sample, the threshold cycle (Ct) value of each sample was normalized to that of β-actin and calculated relative to a calibrator using the 2−ΔΔCt method. Three independent experiments were conducted. The gene-specific primers for PgUGT72AL1 were 5'-ATG GAT ACC GAA AAG CTT-3' (forward) and 5'-CAG ATC TTC CCA CGT GTT-3' (reverse). Control primers for ginseng β-actin (DC03005B05) were 5'-AGA GAT TCC GTC GAT AA-3' (forward) and 5'-ATC AGC GAT ACC AGG GAA CA-3' (reverse).

2.5. Vector construction and in planta transformation

To characterize the function of ginseng PgUGT72AL1, genomic sequences of the N-terminal half of PgUGT72AL1 (PgUGT7202AL, 648 nucleotides) and the C-terminal half of PgUGT72AL1 (PgUGT7201, 627 nucleotides) were expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. PgUGT72AL1 genomic DNA was amplified using primers containing KpnI and AvrII sites (underlined) as follows: 5'-CTG ACC ATG ACC GAA AAC CCTG TCT-3' and 5'-CTG ATC TTC CCA CGT GTT-3' (reverse). PgUGT72AL1 genomic DNA was amplified using primers containing SalI and AvrII sites (underlined) as follows: 5'-GTA GAC ATG TAT GTA TGG TGG GGG-3' and 5'-GCA CTT GAC ATG ACC GAA AAC CCTG TCT-3'. The promoter region was amplified using primers containing PstI and SalI sites (underlined) as follows: 5'-CTG ACC ATG TAT GTA TGG TGG GGG-3' and 5'-GCA CTT GAC ATG ACC GAA AAC CCTG TCT-3'. Individual PCR products were subsequently cloned into cloning sites of pcCAMBl1300 vectors containing Pro35S and yellow fluorescent protein (YFP) fusion proteins. The promoter:GUS fusion construct was generated based on the obtained upstream intergenic region (952 bp) of PgUGT72AL1. The promoter region was amplified using primers containing PstI and SalI sites (underlined) as follows: 5'-CTG ACC ATG ACC GAA AAC CCTG TCT-3' (forward) and 5'-GTA GAC ATG TAT GTA TGG TGG GGG-3' (reverse). The amplified PCR product was subsequently cloned into a pcAM1300 vector containing a gusA reporter gene. All transgene constructs were confirmed by nucleotide sequencing prior to transformation in planta. These constructs were transformed into Arabidopsis Col-0 using Agrobacterium tumefaciens C58C1 (pMP90) [17].
Fig. 1. Phylogenetic tree and sequence alignment of PgUGT72AL1 with other characterized UGTs in plants. (A) The phylogenetic tree was constructed using the ClustalX program (neighbor-joining method). The GenBank accession numbers are: UGT74C1 (AT2G31790), UGT74D1 (AT2G31750), UGT74B1 (AT1G24100), UGT74F2 (AT2G43820), UGT74F1 (AT2G43840), UGT73C7 (AT3G53160), UGT73B4 (AT2G15490), UGT73B2 (AT4G34135), UGT73B1 (AT4G34138), UGT79B1 (AT5G54060), BpUGT94B1 (Q5NTH0), CaUGT3 (BAH80312), GmUGT73P2 (BAI99584), MtUGT73K1 (AAW56092), MtUGT73C7 (AT3G53160), MtUGT73B4 (AT2G15490), MtUGT73B2 (AT4G34135), MtUGT73B1 (AT4G34138), MtUGT79B1 (AT5G54060), BpUGT94B1 (Q5NTH0), CaUGT3 (BAH80312), GmUGT73P2 (BAI99584), MtUGT73K1 (AAW56092), MtUGT74A1 (NP_001105326), ZmUGT74A1 (NP_001105326), PgUGT4 (KM491306), PgUGT94Q2 (JX898529), and PgUGT94Q2 (JX898530). The Genbank ID abbreviations are as follows; At, Arabidopsis thaliana; Bp, Bellis perennis; Ca, Catharanthus roseus; Gm, Glycine max; Mt, Medicago truncatula; Pg, Panax ginseng; Sv, Vaccaria hispanica; Zm, Zea mays. The bar represents 0.2 substitutions per amino acid position. (B) Sequence alignment of PgUGT72AL1 with other close homologs. The underlined domain represents the PSPG consensus motif. The triangles indicate important residues for catalytic activity and substrate binding. PSPG, plant secondary product glycosyltransferase; UGTs, uridine-dependent glycosyltransferases.
Transformants were selected on hygromycin-containing plates (50 μg/mL), and more than 15 T1 independent lines for each construct were used for further analyses. Homozygous transgenic lines carrying one copy of the insertion and following a Mendelian segregation ratio were further characterized.

2.6. GUS histochemical staining

GUS staining was performed by incubating samples in staining solution containing 1mM 5-bromo-4-chloro-3-indoyl-β-D-glucoside, 0.1M of NaH₂PO₄, 0.1% (w/v) Triton-X, and 0.5mM potassium ferricyanide and ferrocyanide, respectively, at 37°C. GUS histochemical staining was performed by incubating samples in staining solution containing 1mM 5-bromo-4-chloro-3-indoyl-β-D-glucoside, 0.1M of NaH₂PO₄, 0.1% (w/v) Triton-X, and 0.5mM potassium ferricyanide and ferrocyanide, respectively, at 37°C until a blue color appeared (1–3 h). Stained samples were sequentially cleared in 70% (v/v) ethanol and then in 100% ethanol for 2 h. Samples were further cleared by incubation in 10% (v/v) glycerol/50% (v/v) ethanol and 30% (v/v) glycerol/30% (v/v) ethanol stepwise with a final dehydration step. Samples were photographed using a digital camera (Nikon D80; Nikon, Tokyo, Japan) and under a microscope (Leica EZ4HD, Ltd. Korea).

2.7. Confocal microscopy

The fluorescence from reporter proteins was viewed using an Olympus Fluoview 500 confocal laser scanning microscope (Olympus, Tokyo, Japan). YFP was detected using the 514/530 nm excitation/emission filter set. Roots of 4-day-grown seedlings were used to observe the subcellular localization of proteins. Fluorescence images were digitized with the Olympus FV10-ASW 4.0 Viewer (Copyright OLYMPUS CORPORATION).

3. Results and discussion

3.1. Isolation and identification of ginseng PgUGT72AL1 genes

Total expressed sequence tags (ESTs) from previously constructed EST libraries, from 14-year-old ginseng and hairy roots [18], were used as a starting material to obtain full-length coding sequences. Consequently, putative genes encoding UGTs were identified [19]. Thereafter, full-length genomic DNA sequences of PgUGT72AL1 were obtained by a basic local alignment search tool (BLAST) search against the ginseng genome database constructed at Seoul National University, Korea. PgUGT72AL1 has a length of 1437 bp encoding 478 amino acids with no introns. Amino acid sequences of well-characterized UGTs in plants were collected from the National Center for Biotechnology Information bank database and used to generate the phylogenetic tree (Fig. 1A). Among the identified ginseng PgUGTs, PgUGT72AL1 clustered into the same group as PgUGT71A27 (UGTpg1), which is reported to be involved in the production of ginsenoside compound K (C-K) [16]. Overall, the N-terminal part of the amino acid sequence was diverse among the selected UGTs, with the exception of the C-terminal plant secondary product GT (PSPG) box (Fig. 1B). The important active binding domain in plant UGTs, referred to as the PSPG box that is responsible for the binding activity of UGTs to donor sugar moieties nucleotide [5,20], is well conserved in PgUGT72AL1 and in other characterized UGTs in plants (Fig. 1B). Therein, several crucial residues for catalytic activity [21], including cysteine and arginine/serine/asparaginase, are depicted with opened triangles (Fig. 1B).

3.2. Organ-specific expression patterns of PgUGT72AL1

Ginseng plants display a distinct anatomical structure at different ages [11]. Ginseng plantlets show only three organs—the root, stem, and a compound leaf with three leaflets—until it reaches 1 year of age. From the 2nd year, compound leaves with five leaflets appear with the same number of petioles, which are connected to each compound leaf, with the number correlated to the year of cultivation [11]. In order to investigate the gene expression patterns of PgUGT72AL1, 2-year-old ginseng plants, which possess a full anatomical structure, were selected as the source material. In general, PgUGT72AL1 transcripts are ubiquitously expressed in all organs, with predominant expression in the roots and rhizomes rather than in the leaves (Fig. 2). However, notably, it is also highly expressed in youngest leaflets as compared with older leaflets.

3.3. Differential transcript levels of PgUGT72AL1 in response to biotic and abiotic stresses

The involvement of UGTs in the modification of secondary metabolites is associated with differences in their expression levels in response to various external conditions. Several studies have documented the involvement of UGTs in plant immune systems [22–24] as well as phytohormone production [25,26]. Thus, in order to provide basic knowledge regarding the function of PgUGT72AL1, changes in mRNA levels were analyzed during different exposure times to several abiotic stresses such as chilling stress (4°C), NaCl (100 mM), H₂O₂ (10 mM), and phytohormones such as ABA (100 μM), SA (5 mM), and JA (0.2 mM). SA and JA are key components of signal transduction pathways involved in plant defense and resistance. Following treatment with phytohormones, PgUGT72AL1 transcripts were gradually reduced to 30 and 25% by JA and ABA, respectively, and to 6% by SA after 48 h of treatment (HAT) (Fig. 3). In the case of H₂O₂ treatments, PgUGT72AL1 was initially up-regulated at 1 HAT and subsequently down-regulated from 12 to 48 HAT, in average 48%, compared to that observed at 0 HAT (Fig. 3). Cold treatment increased PgUGT72AL1 mRNA levels up to 2.4-fold at 1 HAT and maintained transcripts at higher levels up to 12 HAT,
they were then down-regulated from 24 to 48 HAT (Fig. 3). Interestingly, NaCl treatment increased gene expression by 3-fold at 4 HAT and by an average 3.5-fold from 12 to 48 HAT (Fig. 3). These data suggest that \textit{PgUGT72AL1} may play a role in salt-tolerance.

3.4. Spatial and temporal expression of \textit{PgUGT72AL1} in heterologous Arabidopsis

To further investigate the tissue- and cell-specific expression patterns of \textit{PgUGT72AL1}, \textit{Arabidopsis} was selected as the target host for further characterization. A vector harboring a ~1-kb promoter region of \textit{PgUGT72AL1} transcriptionally fused with the \textit{gusA} reporter gene (Fig. 4A) was then introduced into \textit{Arabidopsis} via the \textit{A. tumefaciens}-mediated floral dipping method. The T2 home line was histochemically analyzed for GUS activity. GUS staining was visible throughout the vasculature of the root and hypocotyl of 2-day-old seedlings, with the exception of the root tip (Fig. 4B). The expression pattern was maintained in 5-day-old seedlings and was enhanced in the cotyledon vasculature. In 12-day-old seedlings, in which the true leaf is emerged, the GUS signal in roots was weakened, and was restricted to the vasculature of cotyledons, and extended to the tip-side of leaf veins (Fig. 4B). GUS staining in trichomes and the inner membrane of stomata was also observed in 12-day-old seedlings (Figs. 4C and D). Overall, GUS staining was restricted to the vasculatures in the main roots except for the lateral roots and root hairs (Fig. 4E), and in leaf veins. In adult plants, a signal was detected in the youngest rosette leaves (Fig. 4F), sepals, stigmas, filaments in inflorescences (Fig. 4G), fully matured and young siliques (Fig. 4H), trichomes, and tips of cauoline leaves (Fig. 4I). It is noteworthy that the GUS signal could also be detected in the axillary branch organs between the main stem and the lateral stem. Scale bars are provided in each image.
3.5. Over expression of PgUGT72AL1 in Arabidopsis resulted in axillary organ fusion

Plant UGTs share structural similarity with GT1 enzymes such as GtfA and GrfB, which are involved in vancomycin biosynthesis in *Amycolatopsis orientalis*, and OleD and Ole, which are involved in the glycosylation of macrolide antibiotics in *Streptomyces*. This indicates that there is structural similarity in the overall folding and in the core structures [21]. Generally, the C-terminal domains share higher similarity than the N-terminal domains; presumably, the C-terminal domains recognize the same or similar donors, whereas the N-terminal domains recognize different acceptors [21]. In order to determine the functional differences in plant growth and development, two halves of PgUGT72AL1 (PgNΔUGT72AL1 and PgCΔUGT72AL1) were introduced in planta via *Agrobacterium*-mediated transformation. Among 15 independent transgenic lines, at least two different lines, which were confirmed by quantitative PCR (Fig. 5A), were selected for phenotyping. No phenotypic defects were observed in plants at the young stage. However, as observed in the promoter::GUS expression patterns (Fig. 4I), an organ fusion phenotype was observed between the main stem and axillary cauline leaves (Fig. 5B). Organ fusion can occur as the result of an imbalance in hormones, such as brassinosteroid. Brassinosteroid hormone is well documented that plays important role in boundary organ formation [27]. It suggests that the possible involvement of PgUGT72AL1 in hormonal changes.

3.6. Subcellular localization of PgUGT72AL1 in Arabidopsis

The subcellular location of two halves of PgUGT72AL1 was analyzed by generating C-terminal fusion with a fluorescent protein (YFP). Both the N-terminal and C-terminal halves of PgUGT72AL1

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**Fig. 5.** Overexpressing PgUGT72AL1 in Arabidopsis caused organ fusion in axillary leaves. (A) Overexpression of the N-terminal and C-terminal halves of PgUGT72AL1 resulted in organ fusion of the axillary branch in two 6-week-old transgenic lines, respectively. The scale bar represents 2 mm. (B) Overexpressed transcripts were confirmed by qPCR in two independent transgenic lines. Data represent the mean ± SE for three independent replicates. Averages for treated samples were significantly different compared to the control at *p < 0.05* and **p < 0.01. ABA, abscisic acid; JA, jasmonic acid; qPCR, quantitative polymerase chain reaction; SA, salicylic acid; SE, standard error.

**Fig. 6.** Subcellular localization of PgNΔUGT72AL1-YFP and PgCΔUGT72AL1-YFP. The N-terminal half of PgUGT72AL1 is partially merged with the Golgi marker (ST-mRFP) and the C-terminal half of PgUGT72AL1 is localized in unidentified vesicles.
were localized to Golgi-like small vesicles (Fig. 6). To confirm whether these small vesicles are merged with Golgi, transgenic lines were crossed with the Golgi marker sialyol transferase (ST)-mRFP line [27]. Some of the fluorescent signals from PgNAUGT72AL1-YFP were merged with ST-GFP, indicating that the N-terminal domain of PgUGT72AL1 plays an important role in localization to the Golgi. Thus, the diverse N-terminal domain may possess a signal peptide that directs the protein to the correct subcellular location, and the C-terminal domain is involved in the recognition of appropriate donors.

4. Conclusion

Glycosylation of low molecular weight compounds can alter the properties of aglycones by increasing their solubility and accumulation, and regulating their subcellular localization and bioactivity [28,29] The family 1 GTs, UGTs, are the most common enzymes involved in catalyzing these processes in the plant kingdom. Glycosylation by UGTs is required in various biological processes during plant growth and development, especially in the modulation of secondary metabolites and in plant hormone homeostasis. To date, only a few UGTs from plants have been characterized in detail. Members of the UGT71 family in Arabidopsis have been shown to recognize a range of substrates such as benzoates, flavonoids, terpenoids [30–32], ABA [33], cytokinins [34], and brassinosteroid [35]. The PgUGT72AL1 gene sequence is not clustered with any of the previously characterized UGTs. However, it is clustered with PgUGT71A27 (same with UGT1P1) among existing UGTs (Fig. 1), the only one UGT characterized from ginseng which has been shown to be involved in C-K production by transferring a glucosyl moiety to free C-20 OH of PPD [16]. PgUGT72AL1 shares 25% amino acid identity with UGT1P1, whereas 84% with UGT1P2 which was not proven to produce C-K [16]. This suggests that PgUGT72AL1 may also function in the production of C-K or C-K-like compounds. C-K has never been found in Panax plants [36]. Thus, further characterization may shed light on the biological functions of C-K in ginseng plant. But it also needs to be considered that it may function in other plant growth and developmental processes.

Considering the ubiquitous and enhanced gene expression of PgUGT72AL1 in roots and young leaves (Fig. 2), and spatially distributed GUS staining in roots and young leaves (Fig. 2), and spatially may function in other plant growth and developmental processes. Further characterization may shed light on the biological functions of PgUGT72AL1, which remains to be elucidated in the future.

Conflicts of interest

The authors have no conflicts of interest to declare.

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