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ARTICLE

Molecular cloning and characterization of a flavonoid glucosyltransferase from Byungkyool (*Citrus platymamma* hort. ex Tanaka)

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Abstract Uridine diphosphate glucosyltransferase (UGT) attaches glucoside to proteins, various flavonoids, and phenolic compounds. The modification of flavonoid affects its water solubility, stability, and bioavailability of flavonoids. In this study, citrus genomic sequence database was searched for UGTs of citrus, and a UDP-glucosyltransferase (*bGT173*) was isolated from Byungkyool (*Citrus platymamma* hort. ex Tanaka). The cloned cDNA gene was 1365 bp in length and encoded 456 amino acids. Phylogenetic analysis suggested that *bGT173* was a member of the flavonoid 3-*O*-glucosyltransferase group. mRNA expression of *bGT173* was higher in leaves compared to flowers, stems, and fruits. The recombinant protein of *bGT173* was expressed in *Escherichia coli*, and tested for its activity on seven flavonoids (apigenin, eriodictyol, hesperetin, kaempferol, luteolin, naringenin, and quercetin). Both kaempferol and quercetin were good substrates for *bGT173*, demonstrating that *bGT173* preferentially glucosylated the 3-hydroxyl group of flavonols. Furthermore, quercetin 3-*O*-glucoside produced in *E. coli* showed the same anti-migration activity on pancreatic cancer cells similar to the standard chemical, suggesting that *bGT173* is a good candidate for bioconversion of quercetin to quercetin 3-*O*-glucoside.

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Keywords *Citrus platymamma* · Flavonoid · Glucosyltransferase · Migration · Pancreatic cancer · Substrate specificity

Introduction

Flavonoids perform many biological functions such as oxidative stress defense, auxin movement regulation, and antimicrobial action in plants (Winkel-Shirley 2001; Iwashina 2003; Buer et al. 2010; Kumar and Pandey 2013). Various modifications including hydroxylation, methylation, and glycosylation can occur in flavonoids (Kim et al. 2013). Uridine diphosphate glucosyltransferases (UGTs) catalyze glucosylation and produce glucoside. Glucosylation increases solubility, absorption, stability, and bioavailability of flavonoid, and renders flavonoid harmless to plants (Vogt and Jones 2000; Ross et al. 2001; Noguchi et al. 2009; Ono et al. 2010). UGTs have a conserved plant secondary product glucosyltransferase (PSPG) box that consists of 44 amino acids in the C-terminal region. The PSPG box is thought to include the UDP-glucose binding site, playing an important role in activity of UGTs (Hughes and Hughes 1994; Gachon et al. 2005; Lim 2005; Lairson et al. 2008). UGTs have favored hydroxyl groups for glucosylate: the 3-hydroxyls in flavonols and the 7-hydroxyl group in flavones and flavanones (Lim et al. 2004; Ono et al. 2010; Li et al. 2014). Despite the conserved PSPG box, the overall sequence homology among UGT is low. Therefore, it is difficult to accurately predict the reaction product. Biochemical assay of each UGT is necessary to understand the function of UGTs.

Citrus has been cultivated worldwide for long time. It is enriched in flavonoids, such as kaempferol, quercetin, rutin, naringin, and hesperetin. Of these flavonoids,

kaempferol has been implicated in anticancer activity and can affect cellular processes (Chen and Chen 2013). Kaempferol regulates apoptosis, cell cycles, and inflammation in various cancer cells. Quercetin has also a wide range of biological effects that include antioxidative effect, antitumor effects, cardiovascular protection, and prevention of obesity-related diseases in animal models and humans (Bischoff 2009). Recently, quercetin 3-*O*-glucoside, which has a glucosyl residue at 3-OH position of quercetin, was shown to suppress epidermal growth factor (EGF)-induced migration on human pancreatic cancer cells by inhibiting EGF receptor (EGFR) singling (Lee et al. 2015a). The Korea native citrus species, Byungkyool (*Citrus platymamma* hort. ex Tanaka), resists low temperatures and is disease tolerant. Byungkyool peel extracts showed highest radical scavenging activity among Korean native species (Kim et al. 2009). Studies of citrus UGT-related gene functions will help increase the understanding of flavonoid biosynthesis and catabolism research in citrus plants.

Here, we report the cloning and characterization of a glucosyltransferase (*bGT173*) from Byungkyool. Phylogenic tree analysis and substrate specificity study identified it to be a flavonoid 3-*O*-glucosyltransferase. This identified gene can be used in the biotransformation-mediated production of high-quality flavonoid.

Materials and methods

Plant materials and chemicals

Various citrus tissues (leaf, flower, fruit, and stem) of Byungkyool (*Citrus platymamma* hort. ex Tanaka) were obtained from the Citrus Research Station, the National Institute of Horticultural & Herbal Science, Rural Development Administration, Korea. The flavonoids (apigenin, eriodictyol, hesperetin, kaempferol, luteolin, naringenin, and quercetin) and flavonoid glucosides (apigenin 7-*O*-glucoside, eriodictyol 7-*O*-glucoside, kaempferol 3-*O*-glucoside, luteolin 7-*O*-glucoside, naringenin 7-*O*-glucoside, and quercetin 3-*O*-glucoside) were purchased from Sigma-Aldrich (St. Louis, Mo, USA) and INDOFINE Chemicals (Hillsborough Township, NJ, USA).

RNA extraction, cloning, and sequence analysis

Total RNA was extracted from leaves using PureLink RNA mini kit (Invitrogen, Carlsbad, CA). A novel full-length cDNA sequence of *bGT173* was obtained from the citrus genomic DNA library database. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a commercial RNA PCR kit [AMV] Ver. 3.0 (Takara, Shiga, Japan). The forward primer was 5′-GCGCGGA TCCATGTCAGAAGC-AGCCGGAAGCACC-3′ and the reverse primer was 5′-CGCCGCCTCGAGTCAAGTCC TGT-TGACAACTTCA-3′. The PCR product was cloned into pGEX 4T-1 plasmid. Alignments of *bGT173* amino acid sequence were performed by BioEdit soft, CLUSTAL W, and MEGA 6.0.

Real-time quantitative RT-PCR

Total RNA was extracted from tissues, including the leaves, stems, fruits, and flowers of Byungkyool using TRIzol Reagent (Ambion, Austin, TX, USA). First-strand cDNA synthesis was performed with 1 μg of total RNA using a kit (Takara) and amplified using SYBR Green® PCR Kit (Bio-Rad, Hercules, CA, USA). mRNA levels of *bGT173* were quantified by quantitative real-time PCR with the specific primers: forward primer 5′-GCGCGGAT CCATGTCAGAAGCAGCCGGAAGCACC-3′ and reverse primer 5′-TCCCG-AATAATATCCGAGTCAACTG-3′. Real-time PCR was performed using a MiniOpticon™ Real-Time PCR Detection System (Bio-Rad).

Expression and biotransformation of bGT173

Rosetta 2 *Escherichia coli* (DE3) cells harboring the *bGT173* gene were grown overnight in 5 ml LB medium at 37 °C. One milliliter of the cultured cell was used to inoculate 20 ml of LB–AMP medium at 37 °C. The cultured cells were induced by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 2% ethanol for 5 h at 25 °C. To confirm the expression of *bGT173*, Western blotting was done. Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked in TBS-T (0.5 M Tris, 1.5 M NaCl, containing 0.05% Tween-20, pH 7.4) and 1% skim milk overnight. Protein bands were detected with anti-glutathione-*S*-transferase (GST)–peroxidase-conjugated antibody produced in rabbit (Sigma-Aldrich). Biotransformation assays were performed as described (Kim et al. 2006a, 2007; Ko et al. 2006). Reaction samples containing 50 μM of each flavonoid (apigenin, eriodictyol, hesperetin, kaempferol, luteolin, naringenin, and quercetin) were incubated at 28 °C for 18 h.

HPLC and LC/MS analysis

Reaction products were analyzed using a HPLC system (SHIMAZU, Tokyo, Japan) equipped with a photodiode array (PDA) detector and a Luna C18 (2) column (5-μm particle size, 4.6 mm × 250 nm; Phenomenex, Torrance, CA, USA) at a column flow rate of 1 ml/min and detected

at A_{270}. Product separation was performed as follows: 20% acetonitrile at 0 min, 40% acetonitrile at 10 min, 70% acetonitrile at 20 min, 70% acetonitrile at 25 min, 20% acetonitrile at 27 min, and 20% acetonitrile at 30 min. The LC–MS/MS analysis of reaction products were performed using an Accela HPLC system coupled to a Thermo LXQ ion trap mass spectrometer with ESI-negative interface (Thermo Scientific Waltham, MA, USA).

Migration assay

Cell migration assays were performed using 8.0-μm pore-sized Transwell permeable supports (Corning Costar, Lowell, MA, USA), as described previously (Lee et al. 2015a).

Results

Molecular cloning and sequence analysis of *bGT173*

The citrus genomic sequence database was searched for UGTs of citrus. We isolated a putative UGT with a high degree of homology to the PSPG region and designated it as *bGT173*. The open reading frame of *bGT173* consisted of 1365 bp and was predicted to encode a protein of 454 amino acids. Alignment of *bGT173* with 85 *Arabidopsis* UGT protein sequences (http://www.p450.kvl.dk/UGT.) (BIO-EDIT, MEGA 6.0) (Li et al. 2001; Ross et al. 2001; Hong et al. 2007) revealed *bGT173* had greatest similarity to *Arabidopsis* UGT78D1 (a member of group F) that was reported as a flavonol-specific glycosyltransferase (Li et al. 2001; Ren et al. 2012) (Fig. 1(A)). Next, we aligned the deduced amino acid sequences and additional phylogenetic tree with several verified plant glucosyltransferases (Fig. 1(B)). The phylogenetic tree of flavonoid UGTs consisted of three clusters (Cluster I, flavonoid 3-*O*-glucosyltransferase; Cluster II, flavonoid 5-*O*-glucosyltransferase; Cluster III, flavonoid 7-*O*-glucosyltransferase). *bGT173* was grouped with Cluster I, suggesting *bGT173* may glucosylate at the 3-OH of acceptor substrates.

To assess tissue-specific expression, mRNA level of *bGT173* was analyzed by real-time qPCR (Fig. 2). Expression level of *bGT173* was highest in leaf and was moderate in stem. In flower and fruit, *bGT173* was very weakly expressed compared with in leaf.

Biochemical characterization of *bGT173*

To understand *bGT173* protein function, an expression plasmid was transferred into *rosetta 2* (DE3) *E. coli*. The expressed protein band of *bGT173* was detected at about 78 kDa by SDS-PAGE (Fig. 3). This fusion protein accorded with molecular mass of *bGT173* (52 kDa) with GST (26 kDa). To verify production of the fusion protein, Western blotting was done. A clear single band was clearly observed and corresponded to the molecular mass of the *bGT173*–GST fusion protein (78 kDa). To assess the specific catalytic activity of *bGT173*, we performed a biotransformation assay with seven substrates (apigenin, eriodictyol, hesperetin, kaempferol, luteolin, naringenin, and quercetin) (Fig. 4). HPLC and LC/MS analysis of reaction products detected new peaks in kaempferol and quercetin. The retention times of the reaction products (P1 and P2) from kaempferol and quercetin were 8.6 min and 7.26 min, respectively (Fig. 4(A, B)). The retention times coincided with those of authentic kaempferol 3-*O*-glucoside (S1) and quercetin 3-*O*-glucoside (S2). Four other flavonoids (apigenin, eriodictyol, luteolin, and naringenin) displayed very weak transfer of glucose to the 7-OH position of aglycon, and any glucosylated form of hesperetin was not detected. These results demonstrated that flavonols (kaempferol and quercetin) were glucosylated at the C-3 hydroxyl group and flavanones (eriodictyol, hesperetin, and naringenin) and flavones (apigenin and luteolin) were glucosylated at the C-7 hydroxyl group.

To determine the favored substrates of *bGT173*, relative conversion rates of flavonoids were determined. Quercetin was most effectively changed to quercetin-3-*O*-glucoside by *E. coli* expressing *bGT173*. Kaempferol showed a similar relative conversion rate to quercetin (Table 1). Next, we assessed the most favorite substrate using medium containing the same concentration of quercetin and kaempferol (dual biotransformation). *E. coli* expressing *bGT173* almost completely transformed quercetin (96%) and kaempferol (97%) to their 3-*O*-glucosides within 3 h (Fig. 5(A)). Further incubation resulted in decrease of 3-*O*-glucosides (Fig. 5(B)). These results demonstrated that *bGT173* is a flavonol-specific glucosyltransferase.

Cell migration of pancreatic cancer cell

We reported that quercetin and quercetin 3-*O*-glucoside inhibit EGF-induced migration compared to quercetin 7-*O*-glucoside. Quercetin 3-*O*-glucoside (Q3G) was significantly effective (Lee et al. 2015a). Therefore, we presently examined the anti-migratory effect of biotransformation system-produced quercetin-3-*O*-glucoside (*bGT173-Q3G*) in EFG-induced migration of SNU-213 human pancreatic cancer cells (Fig. 6). We treated SNU-213 cells with different doses (50, 100 nM). All treatments similarly decreased EGF-induced migration. These results demonstrated that *E. coli*-produced quercetin 3-*O*-glucoside could be used as an effective anti-migration reagent in pancreatic cancer cells.

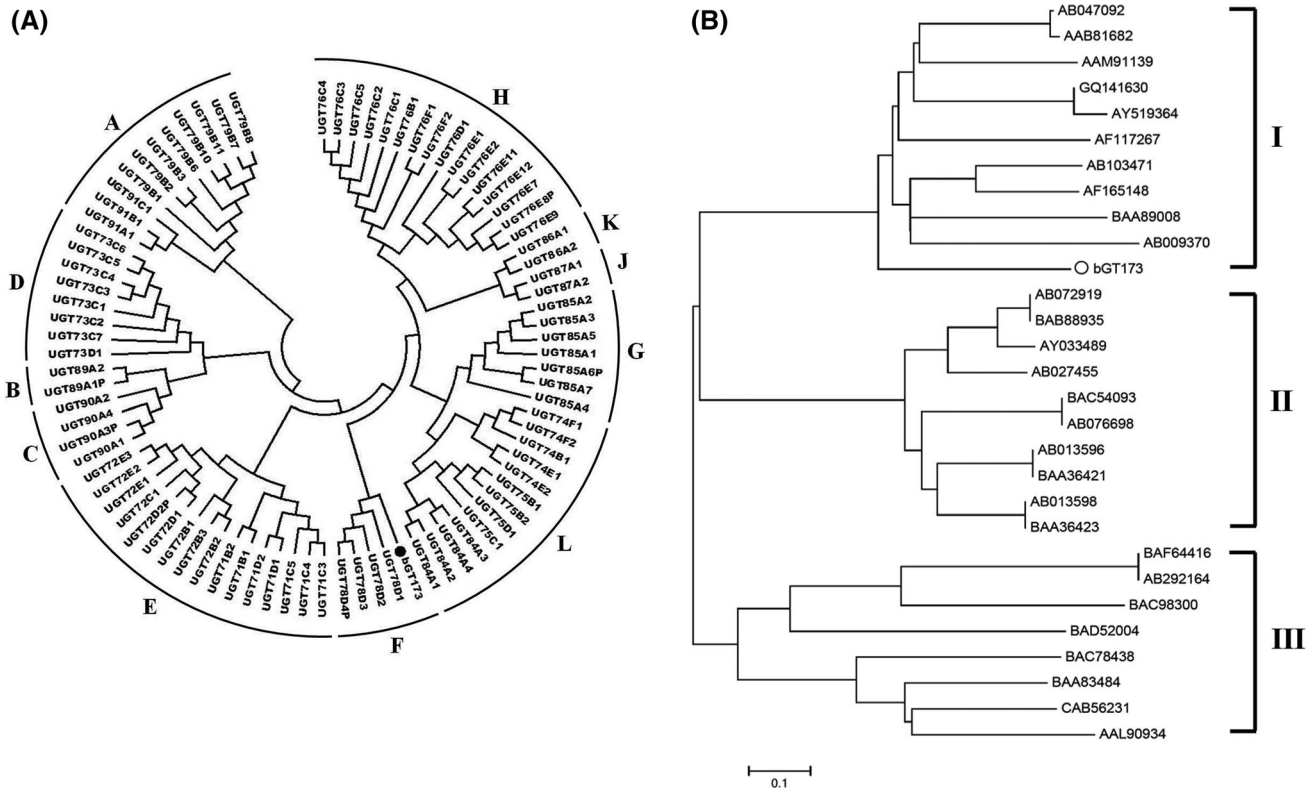

Fig. 1 (**A**) Phylogenetic tree of *bGT173* with other GTs from *Arabidopsis thaliana* with MEGA 6.0 using Neighbor-Joining method. *bGT173* is marked with an *dot*. (**B**) Phylogenetic tree of *bGT173* with other flavonoid GTase using Mega 6.0. Flavonoid 3-*O*-glucosyltransferase (AB047092); UDP-glucose: flavonoid 3-*O*-glucosyltransferase (AAB81682); flavonoid 3-*O*-glucosyltransferase-like protein (AAM91139); *Citrus* × *paradisi* flavonol 3-*O*-glucosyltransferase (GQ141630); *Citrus sinensis* UDP-glucose-flavonoid-3-*O*-glucosyltransferase (AY519364); *Malus domestica* UDP-glucose: flavonoid 3-*O*-glucosyl-transferase (AF117267); *Aralia cordata* ACGaT mRNA for anthocyanin 3-*O*-galactosyltransferase (AB103471); *Petunia* × hybrid UDP-galactose: flavonol 3-*O*-galactosyltransferase (AF165148); anthocyanidin 3-*O*-glucosyltransferase (BAA89008); *Vigna mungo* UF3GaT mRNA for flavonoid 3-*O*-galactosyltransferase (AB009370); *Nicotiana tabacum* NtGT2 mRNA for glucosyltransferase (AB072919); glucosyltransferase (BAB88935); *Solanum sogarandinum* cold-induced glucosyltransferase (Ssci17) mRNA (AY033489); *Petunia* × hybrid PH1 mRNA for anthocyanin-5-*O*-glucosyltransferase (AB027455); anthocyanin-5-*O*-glucosyltransferase (BAC54093); *Torenia* hybrid cultivar 5GT mRNA for anthocyanin 5-glucosyltransferase (AB076698); *Perilla frutescens* var. *crispa* PF3R4 mRNA for UDP-glucose: anthocyanin 5-*O*-glucosyltransferase (AB013596); UDP-glucose: anthocyanin 5-*O*-glucosyltransferase (BAA36421); anthocyanin 5-*O*-glucosyltransferase (AB013598); anthocyanin 5-*O*-glucosyltransferase (BAA36423); isoflavone 7-*O*-glucosyltransferase (BAF64416); isoflavone 7-*O*-glucosyltransferase (AB292164); baicalein 7-*O*-glucuronosyltransferase (BAC98300); flavonol 3-*O*-glucosyltransferase (BAD52004); isoflavonoid glucosyltransferase (BAC78438); flavonoid 7-*O*-glucosyltransferase (BAA83484); betanidin-5-*O*-glucosyltransferase (CAB56231); AT4g34130/F28A23_110 (AAL90934)

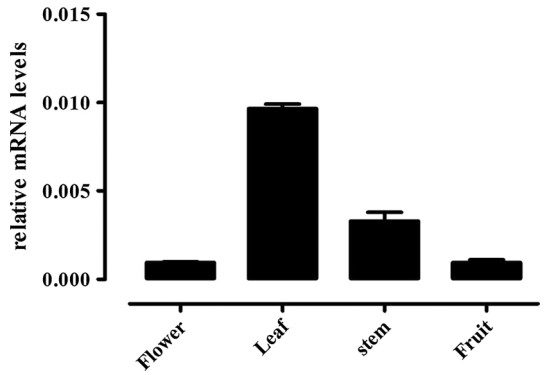

Fig. 2 Real-time PCR analysis of *bGT173* in different tissues. qRT-PCR was performed with *bGT173* specific primers and 1 μg of total RNA extracted from each sample

Discussion

Many plant UGTs have recently been identified with various catalytic functions. This is related to the regiospecificity of UGTs, which determine which OH group of flavonoid will be modified. In this study, an UDP-glucosyltransferase *bGT173* was identified in Byungkyool based on the conserved domain (PSPG box) using the citrus genomic database. Based on the phylogenetic tree of *bGT173* with *Arabidopsis thaliana* and 28 other plants, *bGT173* consisted of group F that has activity on flavonol 3-OH group. We demonstrated that *bGT173* was regiospecific, producing a single product that was glucosylated at the 3-OH group (flavonol) using HPLC and LC/

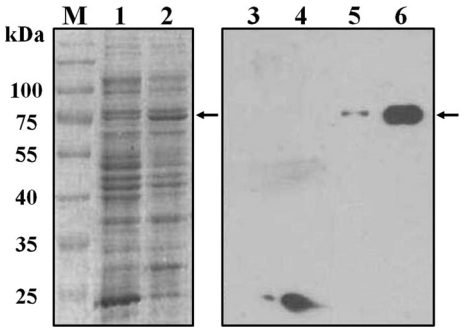

Fig. 3 SDS-PAGE and Western blotting analysis of the recombinant *bGT173*. *M*, molecular weight marker; *1*, soluble protein before induction; *2*, soluble protein after induction; *3*, pGEX vector before induction; *4*, pGEX vector after induction; *5*, soluble protein before induction; *6*, soluble protein after induction

MS analysis (Fig. 4). UGTs from diverse plants, which glucosylates the 3-OH group of flavonoid, have been reported, but their specific activity for substrate is subtly different. For example, a flavonol 3-*O*-glucoside (GQ141630) isolated from *Citrus paradise* showed 47% amino acid identity with *bGT173*. GQ141630 has approximately twofold higher activity on quercetin than

Table 1 Relative conversion rates of *bGT173* with flavonoids

Flavonoid	Conversion rate (%)
Flavonols	
Kaempferol: R1 = H, R2 = H	94
Quercetin: R1 = OH, R2 = H	93

kaempferol (Owens and McIntosh 2009). UGT78D1 from *Arabidopsis thaliana* highly converted quercetin to its 3-*O*-glucoside (100%) compared to kaempferol (69%) (Ren et al. 2012). In case of RF5 from *Oryza sativa*, relative conversion rates of quercetin and kaempferol were similar. However, RF5 showed comparatively low activity, transforming below 50% of flavonol to their glucosides (Kim et al. 2006a). Another glucosyltransferase from *Arabidopsis thaliana*, AtGT-1 (group D, Fig. 1(A)) had similar activity on kaempferol (100%) than quercetin (87%), but this enzyme showed relatively high activity on the flavanones naringenin and eriodictyol (Kim et al. 2006b). Different from previously reported UGTs, *bGT173* had similar regiospecific conversion rates of the 3-OH group of kaempferol and quercetin. Among the substrates tested,

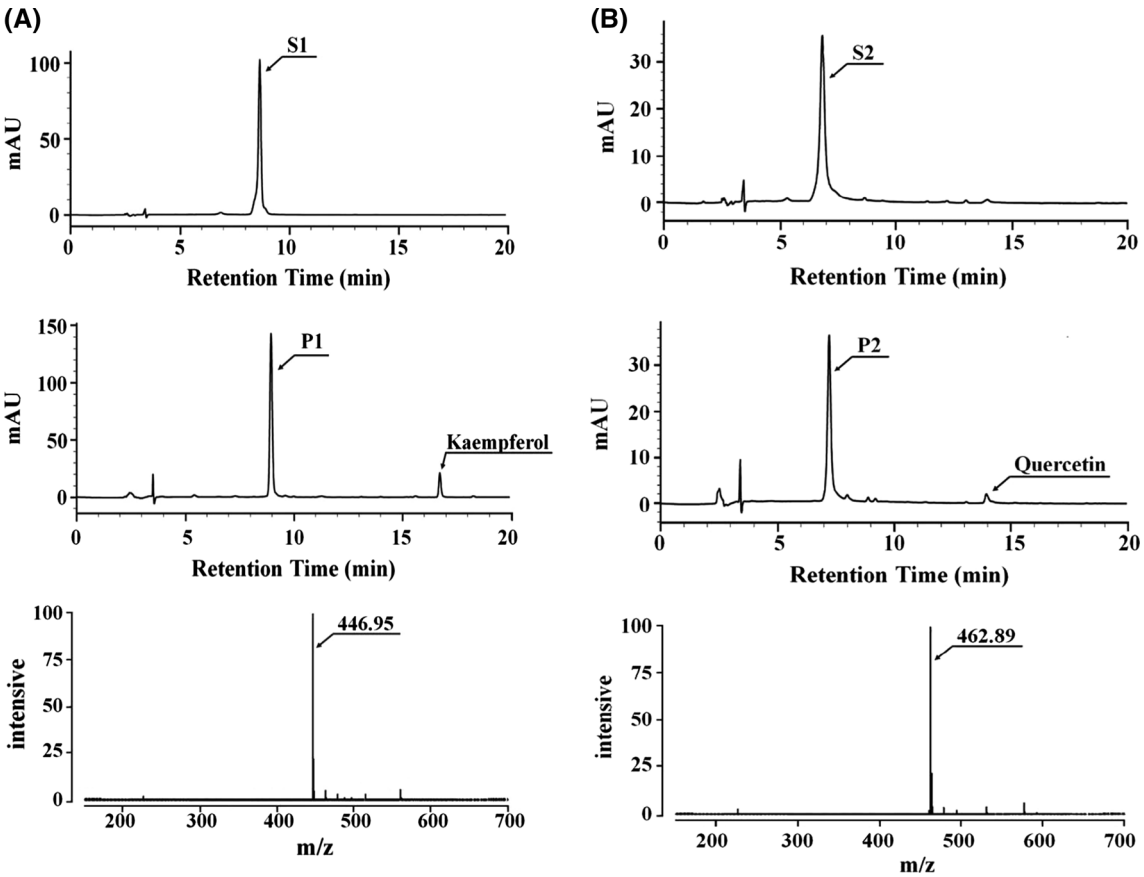

Fig. 4 HPLC and MS analysis of reaction products by *bGT173*. (**A**) Kaempferol 3-*O*-glucoside (S1) and reaction product of apigenin with *bGT173* (P1), (**B**) quercetin 3-*O*-glucoside (S2) and reaction product of eriodictyol with *bGT173* (P2)

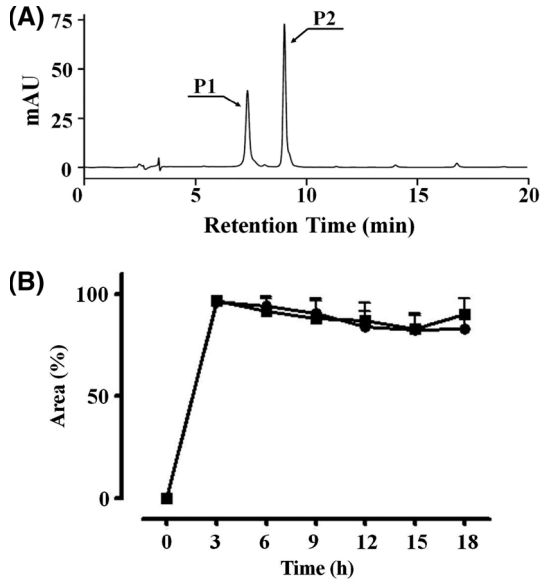

Fig. 5 Dual biotransformation of kaempferol and quercetin. (**A**) HPLC analysis of *bGT173*. Quercetin 3-*O*-glucoside (P1), Kaempferol 3-*O*-glucoside (P2) (**B**). *Closed circles* quercetin 3-*O*-glucoside; *closed squares* kaempferol 3-*O*-glucoside (area means relative quantity of glucoside per aglycon)

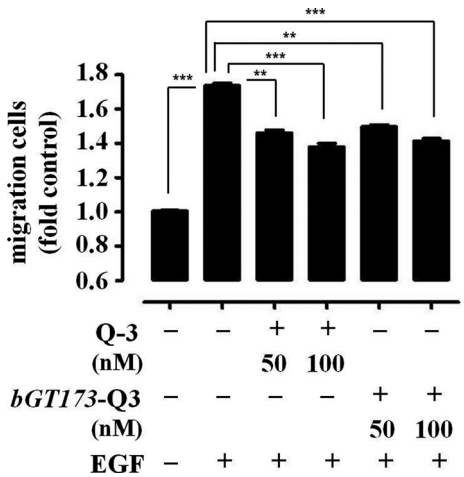

Fig. 6 Migration of pancreatic cancer cells following treatment with quercetin or quercetin-3-*O*-glucoside. SNU-213 human pancreatic cancer cells were treated with different doses of commercial chemical (Q-3) and biotransformation system-produced quercetin-3-*O*-glucoside (*bGT173-Q3*), and then incubated with 50 μg/L EGF. Migration was evaluated using the Transwell migration assay ($n = 3$; Tukey's post hoc test was applied to detect significant group effects as determined by analysis of variance, $p < 0.0001$; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 0% inhibition)

kaempferol and quercetin were the best acceptors of glucose, producing their corresponding 3-*O*-glucosides, suggesting *bGT173* prefers flavonols compared to flavones and flavanones. In a dual biotransformation assay, kaempferol and quercetin were effectively modified to their corresponding 3-*O*-glucosides in 3 h. These products could be clearly and easily separated by HPLC (Fig. 5(A)), suggesting *bGT173* biotransformation system is very useful for the production of flavonol-3-*O*-glucoside in a short time.

Pancreatic cancer is a highly lethal disease with a 5-year fatality rate of 97%. The reason of the poor survival rate is the difficulty in detecting the cancer because of lack of specific initial symptoms (Hingorani et al. 2003; Lee et al. 2015a, b). Flavonoids have many biological properties, such as inhibition of the cell cycle, cell proliferation, and apoptosis (Birt et al. 2001). In the present study, we demonstrate that the active flavonol-3-*O*-glucoside could be easily produced in *E. coli*, showing that biotransformation system-produced quercetin-3-*O*-glucoside (*bGT173-Q3G*) has the same activity compared to standard chemical.

In conclusion, we cloned a useful flavonol-3-*O*-GT gene from a Korean native citrus 'Byungkyool' for the first time and characterized its specific activity using various flavonoids. Our results provide insights into possible diverse functions of the flavonol glucosyltransferases in citrus and should aid further investigation of flavonoid glucosyltransferases in flavonoid metabolism and substrate regiospecificity.

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