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Identification of a bifunctional maize *C*- and *O*-glucosyltransferase*

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*Running title: A bifunctional glycosyltransferase from maize

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Background: Plant UDP-glycosyltransferases add sugars to acceptors like flavonoids, either via hydroxyls (*O*-linkage) or carbons (*C*-linkage)
Results: A maize glycosyltransferase produces both flavonoid *C*-glycosides and *O*-glycosides
Conclusion: This is the first description of a bifunctional *C*-/*O*-glycosyltransferase with a dual role in nature
Significance: This enzyme might be involved both in the biosynthesis of the natural insecticide maysin, and also in the formation of *O*-glycosides

ABSTRACT

Flavonoids accumulate in plant vacuoles usually as *O*-glycosylated derivatives, but several species can also synthesize flavonoid *C*-glycosides. Recently, we demonstrated that a flavanone 2-hydroxylase (*Zm*F2H1, CYP93G5) converts flavanones to the corresponding 2-hydroxy derivatives, which are expected to serve as substrates for *C*-glycosylation. Here, we isolated a cDNA encoding a UDP-glucose-dependent glycosyltransferase (UGT708A6), and its activity was characterized by *in vitro* and *in vivo* bioconversion assays. *In vitro* assays using 2-hydroxyflavanones as substrates and *in vivo* activity assays in yeast co-expressing *Zm*F2H1 and UGT708A6 show the formation of the flavones *C*-glycosides. UGT708A6 can also *O*-glycosylate flavanones in bioconversion assays in *Escherichia coli* as well as by *in vitro* assays with the purified recombinant protein. Thus, UGT708A6 is a bifunctional glycosyltransferase that can produce both *C*- and *O*-glycosidated flavonoids, a property not previously described for any other glycosyltransferase.

Glycosyltransferases (GTs) are enzymes that catalyze the transfer of a sugar moiety to an acceptor molecule. The GTs that use uridine diphospho (UDP) sugar molecules as donors are referred as UDP-dependent glycosyltransferases

(UGTs) and they are members of GTs family 1 (1,2). This family contains most plant UGTs, which utilize different small molecules derived from specialized metabolism as acceptors, such as terpenoids, flavonoids, saponins, plant hormones and xenobiotics (2). Thus, plant UGTs are involved in different cellular processes that include specialized metabolism, modification of plant hormones, detoxification of xenobiotics and plant-pathogen interactions. The glycosylation of specialized metabolites, such as flavonoids, affect their properties, enhancing their stability and solubility, and are believed to be important for the compartmentalization, storage and biological activity of many specialized metabolites (3-8). Flavonoids are classified in six major subgroups: chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins, and few species also produce aurones, isoflavonoids, 3-deoxyanthocyanins and phlobaphenes (9). In general, plants accumulate flavonoids in vacuoles as *O*-glycoside derivates; however, bryophytes, ferns, gymnosperms and several angiosperms also produce flavonoid *C*-glycosides (10,11). In particular, cereals like wheat, rice and maize mainly accumulate *C*-glycosyl flavones that are involved in protection against UV-B radiation and defense against pathogens (12-14). For example, maysin, the *C*-glycosyl flavone predominant in silk tissues of some maize varieties, is a natural insecticide against the corn earworm *Helicoverpa zea* (15,16), while *C*-glycosyl flavonoids identified in cucumber leaves would act as phytoalexins in defense against powdery mildew fungi (17,18). From another perspective, there is an increasing interest for *C*-glycosyl flavones because of their benefits for human health and their possible applications in the prevention of diverse diseases (19,20). For example, *C*-glycosyl flavones inhibit pancreatic lipases, allowing their applications as chemo preventive compounds against obesity (21). In addition, due to their potential antioxidant properties, they are commonly used as nutraceutical components in the human diet (22,23).

While the early metabolic steps resulting in flavanone formation and the branching point for the formation of different classes of flavonoids are well characterized in plants (24), the genes involved in the biosynthesis of glycosyl flavones in maize have not yet been fully identified (16). We have previously demonstrated that a flavanone 2-hydroxylase (*Zm*F2H1), CYP93G5, converts flavanones into the corresponding 2-hydroxyflavanones (25), which are proposed to serve as substrates for *C*-glycosylation, followed by dehydration as it has been described in other grasses (9,26,27). However, the specific enzyme responsible for *C*-glycosylating 2-hydroxyflavanones in maize remains unknown. Thus, the aim of this study is to identify a *C*-glycosyltransferase involved in the formation of *C*-glycosyl flavones in maize. Here, we show that UGT708A6 is a *C*-glycosyltransferase that can catalyze the addition of a glucose molecule to 2-hydroxyflavanones, generating *C*-glycosyl flavones. Surprisingly, UGT708A6 can also accept flavanones as substrates to form *O*-glycosidated products. These dual activities were confirmed by both *in vivo* bioconversion assays and *in vitro* assays with the recombinant protein, revealing that UGT708A6 is a bifunctional enzyme with the ability to form both *C*-glycoside and *O*-glycoside derivatives using as acceptors 2-hydroxyflavanones and flavanones, respectively.

EXPERIMENTAL PROCEDURES

Plant Material, Growth Conditions and Chemicals- B73 seeds were obtained from the Instituto Nacional de Tecnología Agropecuaria (INTA, Pergamino, Buenos Aires, Argentina). Maize plants were grown in greenhouse conditions with supplemental visible lighting to 1000 $\mu E\ m^{-2}\ s^{-1}$ with 15 h of light and 9 h of dark. Samples were collected from hypocotyls, radicles (3 day-old plants), anthers, roots (21 day-old plants), seedlings (7day-old plants) and juvenile leaves (21 day-old plants).

Flavonoid standards and UDP-glucose were purchased from Sigma-Aldrich (St. Louis, MO) and Indofine Chemical Company (New Orleans, LA).

Cloning and expression of ZmUGTs. Purification of UGT708A6s- A full-length cDNA corresponding to GRM*ZM*2G162783 (UGT708A6) was amplified by PCR using the primers UGT708A6-NdeI-forward and UGT708A6-Not-reverse harboring the *NdeI* and *NotI* restriction sites, respectively, for further cloning. PCR reactions were performed with GoTaq (Promega) and *Pfu* Polymerases

(Invitrogen) (10:1) under the following conditions: 1X buffer, 2 mM $MgCl_2$, 0.5 µM of each primer, 0.5 mM of each dNTP and 0.5 U of enzyme, and cDNA from B73 leaves in 25 µl of final volume under the following cycling condition: 2 min denaturation at 94°C; 35 cycles at 94°C for 20 sec, 60°C for 30 sec, and 72°C for 120 sec, followed by 7 min at 72°C. Primers for cDNA were designed based on the sequence provided by the maize genome sequence (www.maizesequence.org, release 5b.60, GRM*ZM*2G162783). The PCR product was purified from the gel, cloned in pGEMT-easy vector (Promega) and sequenced. The pGEMT-UGT708A6 construct was digested with the corresponding restriction enzymes, *NdeI* and *NotI,* the insert was purified and cloned in pET28a vector generating the construct pET28-UGT708A6. Full-length cDNA corresponding to GRM*ZM*2G162755 (UGT708A5), GRM*ZM*2G063550 (UGT707A8) and GRM*ZM*2G180283 (UGT91L1) were obtained from Arizona Genomics Institute (AGI, Tucson, USA). *Zm*UGTs were amplified from the BAC clones by PCR using the primers described in Supplemental Table 1 for further cloning in pET28 vector. PCR reactions were performed as described above for UGT708A6. The PCR products were purified from the gels, digested with the corresponding restriction enzymes, purified, cloned into pET28 vector and sequenced.

BL21(DE3) cells with the chaperone expression plasmid pGRO (28) were transformed with the construct pET28-*Zm*UGTs and the empty vector pET28. Cell cultures (200 mL LB medium containing 30 mg/l kanamycin and 35 mg/l chloramphenicol) were grown at 37°C until OD_{600} reached 0.4 and L-arabinose (2 mg/ml) was added to induce chaperone proteins. The cultures were grown at 37°C to mid log phase (OD_{600} 0.5–0.6) and the recombinant N-terminal His_6-*Zm*UGTs expression was achieved by induction with 0.5 mM IPTG for 20 h at 22 °C.

For the purification of UGT708A6, cells were harvested by centrifugation at 3000*g* for 20 min at 4°C. Pellet was resuspended in binding buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% glycerol) containing 0.1% Tween-20, 1 mM phenylmethylsulfonyl fluoride and complete EDTA-free protease inhibitor cocktail (Thermo). Cells were disrupted by sonication and then centrifuged at 12,000*g* for 20 min at 4°C to obtain soluble cell extracts. The protein was bound to a Ni-NTA resin (Invitrogen) by rocking at 4 °C for 1 h, and then the resin was loaded onto a column, washed three times with 15 volumes of binding buffer, followed by 3 washes with 7 volumes of washing buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 5% glycerol and 40 mM imidazole). Elution was carried out by 5 sequential additions of 1 ml of elution buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 5% glycerol and 200 mM imidazole). Finally, the recombinant protein was desalted in desalting buffer (25 mM Hepes-NaOH pH 7.5, 10 mM 2-mercaptoethanol, 5% glycerol) by 4 cycles of concentration and dilution using Amicon Ultra-15 30K (Millipore) and stored at -80 ºC. Protein level was estimated both by comparison with dilution series of bovine serum albumin on a Coomassie Blue-stained SDS-PAGE and also using the Bradford reagent (Bio-Rad, 29). The yield of 90% pure recombinant protein obtained in these conditions was 6 mg/L of culture.

To express each *Zm*UGT in yeast, the full-length cDNA were amplified by PCR using primers harboring restriction sites (Supplemental Table 1) and each pET28-*Zm*UGT construct as templates. The PCR product was purified, digested with the corresponding enzymes and cloned in p5AX43 vector generating the plasmids p5AX43-*Zm*UGTs: p5AX43-UGT708A5, p5AX43-UGT707A8, p5AX43-UGT91L1 and p5AX43-UGT708A6. The p5AX43 vector corresponds to a modified version of plasmid YEplac181 (30) in which the glyceraldehyde 3-phosphate dehydrogenase promoter was inserted at the *Hind*III site. The p5AX43-*Zm*UGT plasmids and p5AX43 empty vector were transformed into competent WAT11 (31) yeast cells harboring pGZ25-*Zm*F2H1 or pGZ25 empty (25), respectively following the Trafo Protocol (32). Yeast colonies harboring the plasmids were selected by growth on synthetic complete media (SCD) agar plates lacking uracil, tryptophan and leucine (SCD Ura-Trp-Leu).

Bioconversion experiments- For *in vivo* yeast activity assays, an individual recombinant yeast colony was grown for 40 h at 30°C in 5 ml liquid SC Ura- Trp- Leu- medium containing 2% (w/v) glucose. Then an aliquot of this culture corresponding to an OD_{600} = 1.0 was collected by centrifugation, washed in sterile water and used to

seed the 5 ml induction medium, SC Ura- Trp- Leu- containing 2% (w/v) galactose and 3% (v/v) glycerol. The flavonoid substrates were then added to a final concentration of 40 µg ml^{-1}. After incubation for 48 h at 30°C, flavonoids were extracted with ethyl acetate from 1 ml culture aliquots, by adding 500 µL of ethyl acetate and vortexing for 1 min. Solvent layers were separated by centrifugation at 13,000 rpm for 1 min, and flavonoids (both the aglycones and the glycosides) were recuperated in the organic layer. The organic layer was then twice re-extracted with 500 µL of ethyl acetate, and the organic layers were combined. The organic phase was dried in a SpeedVac, and resuspended in methanol for subsequent LC-MS analysis.

For *in vivo E. coli* activity assays, BL21(DE3) cells harboring pGRO (for expression of GroEL/GroES chaperone complex) and pET28-*Zm*UGTs or empty pET28a plasmids were grown at 37°C in LB with appropriate antibiotics. Chaperones and UGT proteins expression were induced by the addition of L-arabinose and 0.5 mM IPTG, respectively, as it was described above and cultures were simultaneously supplemented with 40 µg ml^{-1} of flavonoids. Cultures were grown at 22°C for 24–48 h and then centrifuged at 15 000 *g* for 5 min. One ml medium aliquots were extracted with ethyl acetate as described above, vacuum dried, and resuspended in methanol for subsequent LC-MS analysis.

Acid hydrolysis was performed to differentiate between *O*- and *C*-glycosylated products, as an acidic treatment hydrolyzes *O*-glycosidic linkages, while *C*-linked conjugates are stable to this treatment. After extraction with ethyl acetate, an equal volume of 2 N HCl was added to the samples followed by incubation at 90 °C for 1 h. One volume of 100% methanol was added to prevent the precipitation of aglycones.

In vitro UGT708A6 activity assays- The reaction mixture contained 50 mM Hepes-NaOH pH 7.5 10 mM 2-mercaptoethanol, 100 µg ml^{-1} flavonoid substrates, 2 mM UDP-glucose and 5 µg of recombinant purified protein in a final volume of 100 µl. Reactions were initiated by the addition of the enzyme and terminated by extraction with ethyl acetate. Activity assays were performed at 30°C for up to 60 min.

Glycoside products analyses by Liquid Chromatography-Mass Spectrometry (LC-MS)- Reaction products were analyzed by LC-MS using a system consisting of an Agilent 1100 high-performance liquid chromatography pump, and a Bruker micrOTOF-Q II mass spectrometer in a positive-ion mode configured with a Turbo-ion spray source setting collision energy 25 eV. Samples (10 µl) were chromatographed on a Phenomenex Hypersil GOLD C18 (3 µm; 2.0 by 150 mm) at 200 µl/min with a linear gradient from 20 % MeCN to 100 % in 0.1 % formic acid over 10 min. The eluate was delivered unsplit into the mass spectrometer source. Compounds were identified by comparison of mass spectra to those of authentic commercial standards (Sigma-Aldrich and Indofine Chemical Company). Absorbance units were detected at 295 and 360 nm.

Gene expression analyzes by RT-(q)PCR- Tissues from three independent biological replicates were frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted following Trizol Protocol (Invitrogen) followed by DNase treatment (Promega). cDNAs were synthesized from 4 µg of total RNA using Superscript Reverse Transcription Enzyme II (Invitrogen) with oligo-dT as a primer. The resulting cDNAs were used as templates for quantitative PCR (qPCR) in a iCycler iQ detection system with the Optical System Software version 3.0a (BioRad), using the intercalation dye SYBR Green I (Invitrogen) as a fluorescent reporter and Platinum Taq Polymerase (Invitrogen). Primers were designed to generate unique 150-250 bp-fragments using the PRIMER3 software (33). Three biological replicates were used for each sample plus negative control (reaction without reverse transcriptase). To normalize the data primers for *Actin1* (J01238) were used (Supplemental Table 1). Amplification conditions were as follows: 2 min denaturation at 94°C; 40 to 45 cycles at 94°C for 10 s, 57°C for 15 s, and 72°C for 20 s, followed by 5 min at 72°C. Melting curves for each PCR product were determined by measuring the decrease of fluorescence with increasing temperature (from 65°C to 95°C). To confirm the size of the PCR products and to check that they corresponded to a unique and expected PCR product, the final PCR products were separated on a 2% (w/v) agarose gel, stained with SYBR green (Invitrogen) and also sequenced. Primers used for *UGT708A6* are listed in Supplemental Table 1 (*UGT708A6*-RT-forward, *UGT708A6*-RT-reverse).

Extraction of total flavonoid from maize silks- Flavonoid extraction was performed as previously described (12). Fresh silks and 25-DAP pericarps were rinsed with water, and lyophilized for 1d. Dry weight was measured and ground to a powder with a mortar and pestle. The powder was extracted for 8 h with 12 volume of acidic methanol (1% [v/v] HCl in methanol), followed by a second extraction with 12 volume of chloroform and 6 volume of distilled water. The extracts were vortexed, centrifuged for 2 min at 3,000*g* and organic phases were collected. Flavonoid extracts were analyzed by LC-MS/MS.

Phylogenetic analysis- The tree was constructed using MEGA 4.0 Software with the Neighbor-Joining method based on ClustalW multiple alignments (34).

Computational Analyses from High Throughput Available Data- The heat map was generated with all the gene models with the glycosyl transferase domain (IPR002213) present on the maize genome (version 5b.60) using *bronze 1* (GRMZ*M*2G165390) as a model. These gene models were further used to generate a list to cross reference to data publicly available from Morohashi et al., 2012 (P1-*rr* and P1-*ww* pericarps and silks) and from publicly available data sets (root, shoot, and leaf from B73 inbred) RNA-Seq results (35). These data were further used to generate a heat map on MeV Multiple Array Viewer (36).

Accession Numbers- Sequence data from *Zm*UGTs can be found in the maize genome sequence (version 3b.60 at maizesequence.org) under the following accession numbers: UGT708A5 (GRMZ*M*2G162755), UGT707A8 (GRMZ*M*2G063550), UGT91L1 (GRMZ*M*2G180283) and UGT708A6 (GRMZ*M*2G162783).

RESULTS

Expression and phylogenetic analysis of ZmUGTs- In order to determine a putative candidate for *C*-glycosylation reaction of flavonoids in maize, we followed two criteria. First, we evaluated how candidates' genes were expressed in different maize tissues, and whether they are regulated by the P1 transcription factor, extensively known to be involved in the regulation of *C*-glycosyl flavone biosynthesis (16,37-39). Therefore, we built a list of 157 putative UGTs in maize using *bronze 1* (GRMZ*M*2G165390), one of the best studied maize UGTs and involved in anthocyanin biosynthesis (40,41), as a starting point. We next intersected this list with RNA-Seq data publicly available from maize leaves, shoots and roots from the B73 inbred; and RNA-Seq data from silks and pericarps with contrasting *P1* alleles in the common A619 genetic background and referred here as *P1-rr* and *P1-ww* (Supplemental Figure 1), (25,42). From these results, we selected four genes that were highly up regulated in *P1-rr*, compared to *P1-ww* pericarps: UGT708A5, UGT91L1, UGT707A8 and UGT708A6. These candidate *Zm*UGTs contain the characteristic PSPG (Plant Secondary Product Glycosyltransferase) motif characteristic of plant UGTs, with ten conserved amino acids proposed to be involved in the interaction with the UDP-sugar molecule (Figure 2).

Our second criterion was that any gene model taken into consideration would have sequence similarity with previously characterized UGTs capable of performing *C*-glycosyl bond formation, such as the rice *C*-glycosyl transferase (10) (Figure 1). With this, we generated a phylogenetic tree with selected UGTs that use mainly flavonoids as substrate acceptors. The tree shows five well defined clusters characterized by the regioselectivity of some of these enzymes (Figure 1). Enzymes in cluster 1 transfer UDP-sugars onto the 7-hydroxyl group of their substrates; cluster 2 includes UGTs that utilize flavonoid glycosides as acceptors, and catalyze the formation of sugar-*O*-sugar links. Clusters 3 and 4 are constituted by UGTs that transfer sugars onto the 3- and 5-hydroxyl groups of the acceptors, respectively. Finally, cluster 5 included members characterized by having a broad plasticity in the position of glycosylation (3', 3 and 7-hydroxyl groups), and by the formation of more than one glycoside product. From this analysis, we placed UGT91L1 in cluster 2, which includes UGTs that utilize flavonoid glycosides as acceptors, and catalyze the formation of sugar-*O*-sugar links, like Ph1-6RhaT from *Petunia hybrida*, which add rhamnose to the 6-*O*-glucose of anthocyanidin (43). UGT708A5, UGT707A8 and UGT708A6 were included in cluster 5 as well. It is important to take into consideration that phylogenetically distant UGTs can have similar substrate specificity, whereas evolutionary close UGTs may accept different

substrates; and that the selectivity for acceptors cannot be inferred only by the similarity in their primary sequences (1,2). Interestingly, UGT708A6, included in cluster 5 together with the *C*-glycosyltransferase from *Oryza sativa* (*Os*CGT) and the bifunctional *N*- and *O*-glycosyltransferase from *Arabidopsis thaliana*, UGT72B1 (10,44), shows the highest identity (67%) to the *Os*CGT a rice UDP-glucosyltransferase that uses 2-hydroxyflavanones as flavonoid acceptors (10).

Thus, we predict that UGT708A6 is among the best candidates to catalyze the *C*-glycosylation reaction in the C-glycosyl flavone biosynthetic pathway, since it is up regulated in *P1-rr* tissues and has the highest identity to a previously described CGT.

Bioconversion assays in E. coli and yeast- In order to evaluate if UGT708A6 or more of the selected *Zm*UGTs are involved in the *C*-glycosyl flavone pathway catalyzing the reaction that follows that of *Zm*F2H1 as it was described in rice, the full open reading frames of each UGT were cloned in the pET28a vector, and the proteins were expressed in *E. coli* as N-terminal fusion proteins with a His_6 tag as described in Experimental Procedures section.

Glycosyltransferase activity was assayed *in vivo* by feeding 2-hydroxynaringenin as a flavonoid acceptor to *E. coli* cultures expressing each of the *Zm*UGT. After a two-day fermentation assay, flavonoids were extracted with ethyl acetate, and products were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS). Of all the glycosyltransferases tested (UGT708A5, UGT707A8, UGT91L1 and UGT708A6), only UGT708A6 was able to produce a compound (**1**) that was identified as apigenin 6-*C*-glucoside (isovitexin) by comparison with an isovitexin standard using LC-MS-MS (Figure 3A and C). The negative control, *E. coli* containing the empty vector, did not show the production of this compound (Figure 3A).

In order to verify the ability of UGT708A6 to convert 2-hydroxynaringenin to isovitexin, we took advantage of a yeast strain that we had previously generated that expresses the *A. thaliana* cytochrome P450 reductase and *Zm*F2H1, accumulating small amounts of 2-hydroxy naringenin when fed with naringenin (25). Thus, yeast cultures expressing both *Zm*F2H1 and each *Zm*UGT or harboring the corresponding combination of empty vectors were supplied with the flavanones naringenin or eriodictyol as substrates, and the glycoside products were analyzed by LC-MS. In these combinatorial assays, only when UGT708A6 was expressed along with *Zm*F2H1, the 6-*C*-glucosyl derivatives of the respective flavones, isovitexin and isoorientin, were identified as products (**1** and **2**) as compared to the respective standards by LC-MS-MS (Figure 3B-F). These compounds show the characteristic fragment ions of the *C*-glycoside moiety, [M+H-90] and [M+H-120] (Figure 3D and H). Furthermore, the formation of the *C*-glucoside products was verified due to the stability of these compounds under acid hydrolysis (10,26) (not shown). In addition to isoorientin (luteolin 6-*C*-glucoside), another reaction product with m/z of 449.1 and different retention time was observed (**3**). Further analysis of the product ions relative intensity found by positive electrospray ionization (LC-MS-MS) allowed validating the reaction product **3** as orientin (luteolin 8-*C*-glucoside) (Figure 3H) (45).

Previous experiments showed that a yeast dehydratase activity was responsible for converting 2-hydroxyflavanones into the corresponding flavones (46). To verify that the flavones generated by dehydration from the 2-hydroxyflavanones are not the actual substrate acceptors for the UGT708A6 *C*-glycosyltransferase activity, flavones (apigenin and luteolin) were fed to yeast cultures expressing only UGT708A6; however, no glycosylation products were detected. In addition, to verify the specificity of UGT708A6, different flavonoids were fed to *E. coli* cultures expressing this enzyme. No glycoside product was detected when flavonols (quercetin and kaempferol), flavones (apigenin, luteolin and chrysin) and anthocyanidins (cyanidin) were used as substrates. However, when *E. coli* cultures were fed with the flavanones naringenin or eriodictyol as substrates, production of new compounds were detected by LC-MS. Analysis of the extracts showed the presence of one naringenin derivative product (**4**) with a m/z of 435.1 [$M+H^+$], while eriodictyol generated two new products (**5** and **6**), both with a m/z of 451.1 [$M+H^+$] (Figure 4A and D). Interestingly, the fragmentation patterns of these new glycoside derivatives showed the typical neutral loss of 162 (transition 435.1 → 273.1 for narigenin, and 451.1

→ 289.1 for eriodictyol, respectively) corresponding to a hexose residue in a flavonoid *O*-glycoside (Figure 4C, and F-G). These results were confirmed by acid hydrolysis (not shown). Finally, the *O*-glycoside flavonoid products were identified as naringenin 7-*O*-glucoside (**4**) and eriodictyol 7-*O*-glucoside (**5**) as compared to the respective standards by LC-MS-MS (Figure 4).

Hence, the results described for the bioconversion assays in *E. coli* and yeast show that UGT708A6 is a novel enzyme able not only to *C*-glucosylate 2-hydroxyflavanones, but also to *O*-glucosylate flavanones.

In vitro activities of the recombinant purified UGT708A6 protein- To verify that UGT708A6 is a glucosyltransferase able to produce both *O*- and *C*-glucosyl products as shown in the bioconversion experiments in *E. coli* and yeast; we purified the recombinant protein expressed in *E. coli* to perform *in vitro* activity assays (Figure 5A). When the recombinant UGT708A6 was assayed using the flavanones naringenin or eriodictyol as acceptors and UDP-glucose as a donor, products corresponding to the flavanone *O*-glucosides were detected (not shown). Similarly as observed by *in vivo* assays in *E. coli*, when naringenin was assayed as a substrate, the formation of one naringenin *O*-glycoside compound was detected, whereas eriodictyol generated two *O*-glycosides derivatives, which could correspond to the glucose molecule bound to different -OH groups. Furthermore, the sensitivity of these compounds to acid hydrolysis confirmed that they correspond to *O*-glycosides.

On the other hand, when 2-hydroxynaringenin was assayed as a substrate, two reaction products with m/z 433.1 [M+H$^+$] were observed; one corresponding to isovitexin (apigenin 6-*C*-glucoside, **1**), in comparison with the available standard (Figure 5B, Figure 3C). Analysis of the product ions relative intensity found by positive electrospray ionization allowed the identification of selective ions for the C-8 isomer ([^{0,3}X-H_2O-CO]$^+$ and [^{0,2}X-CHO-CO]$^+$ with m/z of 297.3 and 256.4, respectively), indicating that the second reaction product (**7**) corresponds to vitexin (apigenin 8-*C*-glucoside) (45) (Figure 5D).

Together, both *in vitro* and *in vivo* bioconversion activity assays demonstrate that UGT708A6 is a bifunctional enzyme able to catalyze both the *C*-glucosylation of 2-hydroxyflavanones and the *O*-glucosylation of flavanones.

Flavonoid glycosides in maize pericarps and silks- Supplemental Figure 1 shows that *UGT708A6* is expressed in pericarps and silks, and its expression is positively regulated by *P1*, showing significantly higher mRNA levels in *P1-rr* than in *P1-ww* pericarps and silks (25). Thus, in order to correlate UGT708A6 activities with the flavonoid glycosides present in these organs, methanolic extracts of maize *P1-rr* pericarps and silks were analyzed by LC-MS/MS. As shown in Table 1, both *C*-glycosyl flavones derived from apigenin and luteolin (isorientin, isovitexin) with the glycosylated substitutions at the 6*C* position were identified, as it was previously reported (47,48) (Table 1). Interestingly, we could identify flavanone *O*-glycosides (both for naringenin and eriodictyol), in accordance with the detected expression of *UGT708A6* in these tissues (25). In addition, successive losses of hexoyl units were observed for naringenin *O*-glycosides, indicating the presence of di-*O,O* hexosides. Isomers with different retention times were detected for naringenin *O*-glycosides, likely representing these compounds' different glycosylation positions. Overall, metabolic profiling analysis demonstrates that this enzyme could catalyze the biosynthesis of both *C*- and *O*-glycoside products *in planta.*

DISCUSSION

Glycosylation is an important step in flavonoid biosynthesis, which contributes to flavonoid stability, solubility, storage and biological activity changes (3). Although flavonoid glycosides have been described in maize since the characterization of a glycosyltransferase involved in anthocyanin biosynthesis (*bronze1*), information about other glycosyltransferases implicated in flavonoid metabolism have not been reported (40,41). Here, we characterized a maize glycosyltransferase, UGT708A6, involved in the biosynthesis of *C*-glycosyl flavones by *in vitro* and *in vivo* bioconversion activity assays. Previously, we have demonstrated that the first step in the formation of the *C*-glycosyl flavone involves the conversion of flavanones into 2-hydroxyflavanones by *Zm*F2H1 (CYP93G5) (25). Here, through bioconversion assays in yeast expressing *Zm*F2H1 with UGT708A6, we demonstrated the formation of isovitexin and

isoorientin, the 6-*C*-glucosyl derivatives of the flavones apigenin and luteolin, respectively. Furthermore, both *in vitro* activity assays with the recombinant purified UGT708A6 protein and bioconversion assays in yeast showed the formation of both isomers of apigenin *C*-glucosides (vitexin and isovitexin) and luteolin *C*-glucosides (orientin and isorientin), respectively. These results indicate that UGT708A6 is a *C*-glycosyl transferase that uses 2-hydroxyflavanones as substrates to generate *C*-glycosyl flavones, similarly to a flavonoid *C*-glycosyltransferase from *Fagopyrum esculentum*, and the rice glycosyltransferase CGT (10,26,27, 49).

In addition, both bioconversion assays in *E. coli* expressing UGT708A6 and *in vitro* experiments showed that UGT708A6 can also *O*-glucosylate the flavanones naringenin and eriodictyol, generating one and two different glucoside products, respectively. Consequently, these results show that UGT708A6 is a bifunctional enzyme which has the ability to form both *C*-glycoside and *O*-glycoside links with the flavonoid acceptors 2-hydroxyflavanones and flavanones, respectively; a property which has been only described for a modified glycosyltransferase from *Streptomyces fradiae* using an unnatural substrate (UrdGT2, (50)). Interestingly, carbon–carbon-based and carbon–oxygen-based prenylation of a diverse collection of hydroxyl-containing aromatic acceptors like naringenin was described for bacterial prenyltransferases (51). These enzymes have a similar bimolecular nucleophilic substitution (S_N2)-like reaction mechanism to that of plant UGTs (52). The reaction involves a carbon-mediated nucleophilic attack on C1 of geranyl diphosphate (GPP), with the diphosphate moiety stabilized by Mg^{2+} coordination, and the basic character of the diphosphate binding site serving as a leaving group. On the other hand, based on crystal structures and genetic evidences, plant *O*-glycosyltransferases contain a highly conserved histidine residue in its active site, which acts as a general base to abstract a proton from the acceptor substrate. A nearby aspartate residue interacts with the histidine forming a triad substrate-His-Asp that helps to stabilize the histidine charge after deprotonating the flavonoid substrate (53). It was proposed that the deprotonated acceptor displaces the UDP by attacking the C1 carbon center of the UDP-sugar to form the b-glucoside product (53). Protein sequence alignments showed that UGT708A6 has the conserved His-Asp residues corresponding to the active site of *O*-glycosyltransferases (Figure 2). On the substrate site the distribution of charges in the deprotonated phenolic structure of ring A of flavonoids can permutate between the C and the adjacent O substituent. Thus, in a similar way as it was suggested for prenyltransferases (52) and also described for isopentenyl pyrophosphate transferases involved in terpene biosynthesis (54), the dual function of UGT708A6 may be explained by the phenolic character of the substrate, that alternatively can mediate either the *C*- or *O*-nucleophilic attack on C1 of UDP.

In rice, *C*-glycosyl flavone biosynthesis takes place through a pathway different from the *O*-glycosyl flavone formation, involving the generation of 2-hydroxyflavanones by CYP93G2 activity, followed by the *C*-glycosylation catalyzed by *Os*CGT (10,26). It has been proposed that an open form of 2-hydroxyflavanones is the actual substrate for the *Os*CGT glycosyltransferase, resulting in the formation of 2-hydroxy flavanone *C*-glycoside products which are further dehydrated by a dehydratase (10,26,27). However, it is important to mention that we could not detect the 2-hydroxy flavanone *C*-glycoside products, neither by *in vivo* or by *in vitro* experiments. No detection of these intermediates in *C*-glycosyl flavone biosynthesis could be probably due to spontaneous dehydration of these unstable compounds during the reaction process (10,49,55). In addition, the relative abundance of *C*-glycosyl flavone isomers derived from naringenin and eriodictyol were different. The main product detected for naringenin was the flavone 6-*C*-glucoside (isovitexin), while both flavone 6-*C*-glucoside (isoorientin) and flavone 8-*C*-glucoside (orientin) were detected for eriodictyol, in a ratio 1:8 (Figure 3E). A similar result of the *in vivo* assays in yeast was obtained using 2-hydroxy naringenin *in vitro,* but formation of flavone 8*C*-glucoside (vitexin) could be also detected in minor proportion (Figure 5). These results could be explained by proposing that the actual substrate for the glycosyltransferase is the closed form of the 2-hydroxyflavanone (Figure 6, compound B), as it has less structural flexibility than the open form (Figure 6, compound C). Since the only structural differences between

the two substrates are the substitutions on the B ring of the flavanone, these hydroxyl groups should be important for substrate accommodation in the active site of the enzyme, something difficult to obtain with an open-chain flavanone.

Overall, the results described in this study indicate that UGT708A6 can generate *C*-glycosides, with the glucose molecule at the 6*C*- and 8*C*-position, however; only flavone 6-*C*-glycosides have been described in silks of maize (47,48). Taking in consideration the proposed biosynthesis pathway of the *C*-glycosyl flavone maysin (16), a possible explanation for this can be that the *C*6- isomer (isovitexin and isorientin) consumption by the following rhamnosyl transferase enzyme involved in this pathway may favor the formation of this isomer, thus being a spontaneous non-enzymatic step. Nevertheless, we can not rule out that flavone 8-*C*-glycosides are present in maize tissues not yet studied.

The R2R3-MYB *P1* transcription factor regulates maysin production in silk tissues of some maize varieties (37-39). Our results show that UGT708A6, which expression is regulated by P1 in silks (25), generates isovitexin and isoorientin, intermediates involved in apimaysin and maysin biosynthesis, respectively (16); suggesting that this enzyme could be involved in this biosynthetic pathway. Similarly to rice (27), when *Zm*F2H1 and UGT708A6 enzymes were co-expressed in yeast, the intermediate 2-hydroxyflavanones were not detected; being also likely that UGT708A6 is not the limiting activity in the *C*-glycosyl flavone biosynthesis in maize. However, *UGT708A6* shows a relatively constitutive expression pattern in different maize tissues (Table 2), consistent with the microarray database from a genome-wide atlas of transcription (42); consequently, this pattern of expression could allow the generation of flavanone *O*-glycosides in different maize tissues, as well as their storage in vacuoles avoiding their toxicity and increasing their stability. Nevertheless, we cannot rule out that other non-characterized glycosyltransferase enzymes are also responsible for the formation of these compounds, as well as it cannot be excluded that additional transcription factors could be involved in the regulation of *UGT708A6* expression in maize tissues. Thus, additional studies are required to reveal the involvement of UGT708A6 in other branches of flavonoid biosynthesis, besides the *C*-glycosyl flavone pathway.

In summary, we have identified and characterized the first occurring *C*-/*O*-glycosyltransferase, a dual role that has not yet been described for any glycosyltransferase in nature. This enzyme could be involved in the formation of the insecticidal *C*-glycosyl flavone maysin, but can also catalyze the formation of flavanone *O*-glycosides. Further studies concerning the catalytic mechanism of UGT708A6 will provide useful information to be applied in genetic engineering of other glycosyltransferases to develop therapeutic compounds more stable than *O*-glycosides to enzymatic degradation by glycosidases.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Phylogenetic analyses of selected UGT proteins from higher plants. The numbers indicate bootstrap values (10,000 replicates). Bar = 0.1 amino acid substitutions per site. Different glycosyltransferases are clustered in circles based on the regioselectivity upon the substrate acceptors. The following plant UGT sequences were analyzed: UGT78D2 (*Arabidopsis thaliana*, NP_197207), UGT78D3 (*Arabidopsis thaliana*, NP_197205), UGT78D1 (*Arabidopsis thaliana*, NP_197205), VvGT1 (*Vitis vinifera*, AAB81683), UGT78A2 (*Ariata cordata*, AB103471), PhF3GlcT (*Petunia hybrida*, AAD55985), VvGT5 (*Vitis vinifera*, BAI22846), VvGT6 (*V. vinifera*, BAI22847), UGT78G1 (*Medicago truncatula*, A6XNC6) *Zm*3GlcT (*Zea mays*, X13501) Th5GT (*Torenia hybrida*, AB076698), Pf5GlcT (*Perilla frutescens*, BAA36421), UGT75C1 (*Arabidopsis thaliana*, Q0WW21), PhA5GT (*Petunia x hybrida* BAA89009.1), Vh5GlcT (*Verbena x hybrida*, BAA36423), OsCGT (*Oryza sativa*, ABC94602.1), UGT72B1 (*A. thaliana*, Q9M156), UGT706D1 (*O. sativa*, BAB68093), UGT707A3 (*Oryza sativa*, BAC83989), UGT71G1 (*Medicago truncatula*, AAW56092), UGT71F1 (*Beta vulgaris*, AY526081), FaGT6 (*Fragaria x ananassa*, DQ289587), UGT89C1 (*A. thaliana*, Q9LNE6), UGT73J1 (*Allium cepa*, AY62063), AcUGT73G1 (*A. cepa*, AY62062), UGT73C6 (*A. thaliana*, AEC09298), UGT73C8 (*Medicago truncatula*, DQ875459), SbUF7GT, (*Scutellaria baicalensis*, AB031274), Nt7GlcT (*Nicotiana tabacum*, AF346431), FaGT7 (*Fragaria x ananassa* DQ289588), Db7GlcT (*Dorotheanthus bellidiformis,* Y18871), UGT73A4 (*Beta vulgaris*, AY526080), Ph1-6RhaT (*P. hybrida*, CAA50376), UGT79G16 (*Ipomoea purpura*, AB192315), Cm1-2RhaT (*Citrus maxima*, AY048882), UGT94B1 (*Bellis perennis*, AB190262), CrUGT3 (*Catharanthus roseus*, AB443870), UGT94D1 (*Sesamum indicum*, BAF99027).

FIGURE 2. Alignment of PSPG motif from plant UGTs. The ten highly conserved residues of the motif proposed in the interaction with the UDP-sugar are in bold.

FIGURE 3. *C*-glycosylation of 2-hydroxyflavanones in *E. coli* and yeast expressing UGT708A6. LC-MS analysis of 2-hydroxynaringenin bioconversion in *E. coli* expressing UGT708A6 (A), and bioconversion in yeast co-expressing *Zm*F2H1 and UGT708A6 fed with naringenin (B) or eriodictyol (E). The reaction

products generated molecular ions of *m/z* 433 (A and B), and 449 (E), respectively; neither *E. coli* nor yeast cells transformed with empty vectors showed the production of any of the product peaks. (C, F) Ion chromatograms of standards isovitexin and isorientin, respectively. (D, G and H) MS/MS fragmentation profile of product **1** detected in both *E. coli* and yeast and products **2** and **3** that corresponds to isovitexin, isoorientin and orientin, respectively, as compared to the corresponding standards. (C, F)

FIGURE 4. *O*-glycosylation of flavanones in *E. coli* expressing UGT708A6. LC-MS analysis of naringenin (A) or eriodictyol (D) bioconversion in *E. coli* harboring the pET28-UGT708A6 construct or the empty vector. The reaction products generated molecular ions of *m/z* 435.1 (A), and 451.1 (D), respectively; *E. coli* cells transformed with the empty vector did not show the production of any of the product peaks. (B, E) Ion chromatograms of standards naringenin 7-*O*-glucoside and eriodictyol 7-*O*-glucoside, respectively. (C, F, G) MS/MS fragmentation profiles of the detected products **4**, **5** and **6** that correspond to naringenin 7-*O*-glucoside (product **4**) and eriodictyol 7-*O*-glucoside (product **5**) as compared to the standards (B, E).

FIGURE 5. *In vitro* activity of UGT708A6 assayed with 2-hydroxynaringenin as a substrate acceptor. (A) SDS-PAGE analysis (12%) of the recombinant purified UGT708A6 protein. The numbers on the left side of the gel indicate the molecular mass of standard proteins in kD, that of UGT708A6 on the right side of the gel. (B) LC-MS analysis of purified UGT708A6 activity. The reaction products generated molecular ions of *m/z* 433.1. (C, D) MS/MS fragmentation profiles of detected products **1** and **7** that correspond to isovitexin and vitexin, respectively; the fragmentation patterns of the standards isovitexin (C) and vitexin (D) are shown inside the graphs.

FIGURE 6. Proposed model for *C*-glycosyl flavone biosynthesis by UGT708A6. F2H1: Flavanone 2-hydroxylase 1, R: H or OH. A: Flavanone. B: 2-hydroxyflavanone, closed form. C: 2-hydroxyflavanone, open form. D: 2-hydroxyflavanone 6-*O*-glycoside, closed form. E: 2-hydroxyflavanone 6-*O*-glycoside, open form F: 2-hydroxyflavanone 8-*O*-glycoside. G: flavone 6-*C*-glycoside. H: flavone 8-*C*-glycoside.

TABLE 1. Accumulation of *C*- and *O*- glycosides in maize *P1-rr* pericarps and silks determined by LC-MS/MS.

Retention time (min)	Precursor ion (m/z)	Compound assignment[a]
2.6, 3.6, 5.3	435	Naringenin *O*-hexosides
7.5	451	Eriodictyol *O*-hexoside
6.2, 6.8	597	Naringenin di *O*,*O*-hexosides
8.5	449	*6C*- glucosyl luteolin (isorientin)
8	433	*6C*- glucosyl apigenin (isovitexin)
8.8	576	*6C*- glucosyl luteolin *O*-rhamnoside (maysin)

[a] Identification was based on MS/MS fragmentations using standards as references.

TABLE 2. Analysis of *UGT708A6* expression. *UGT708A6* expression evaluated by RT-qPCR in different tissues of the maize B73 inbred line: hypocotyls, radicles, roots (21 day-old plants), seedling (7 day-old plants), and juvenile leaves (21 day-old plants). Each reaction was normalized using the C_t values corresponding to the *actin1* mRNA (J01238). Data are represented as the means obtained from biological triplicates +/- the S.D. of the samples.

Maize tissues	**Relative expression level to *actin 1* (x 10^3)**
Hypocotyls	0.734 +/- 0.120
Radicles	1.083 +/- 0.200
Roots	0.163 +/- 0.030
Seedlings	0.037 +/- 0.005
Juvenile leaves	0.393 +/- 0.044
Anthers	no detected

Figure 1

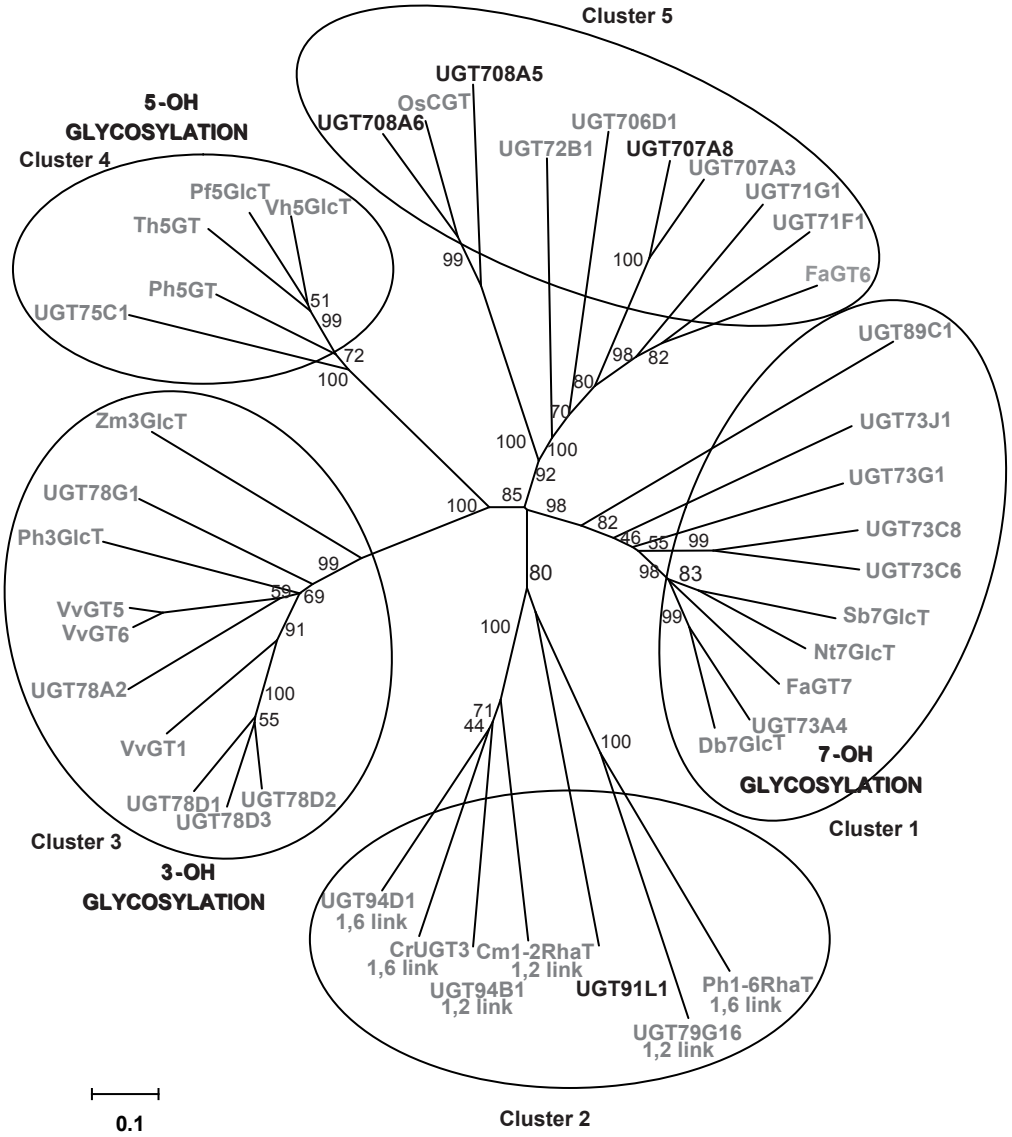


Figure 2

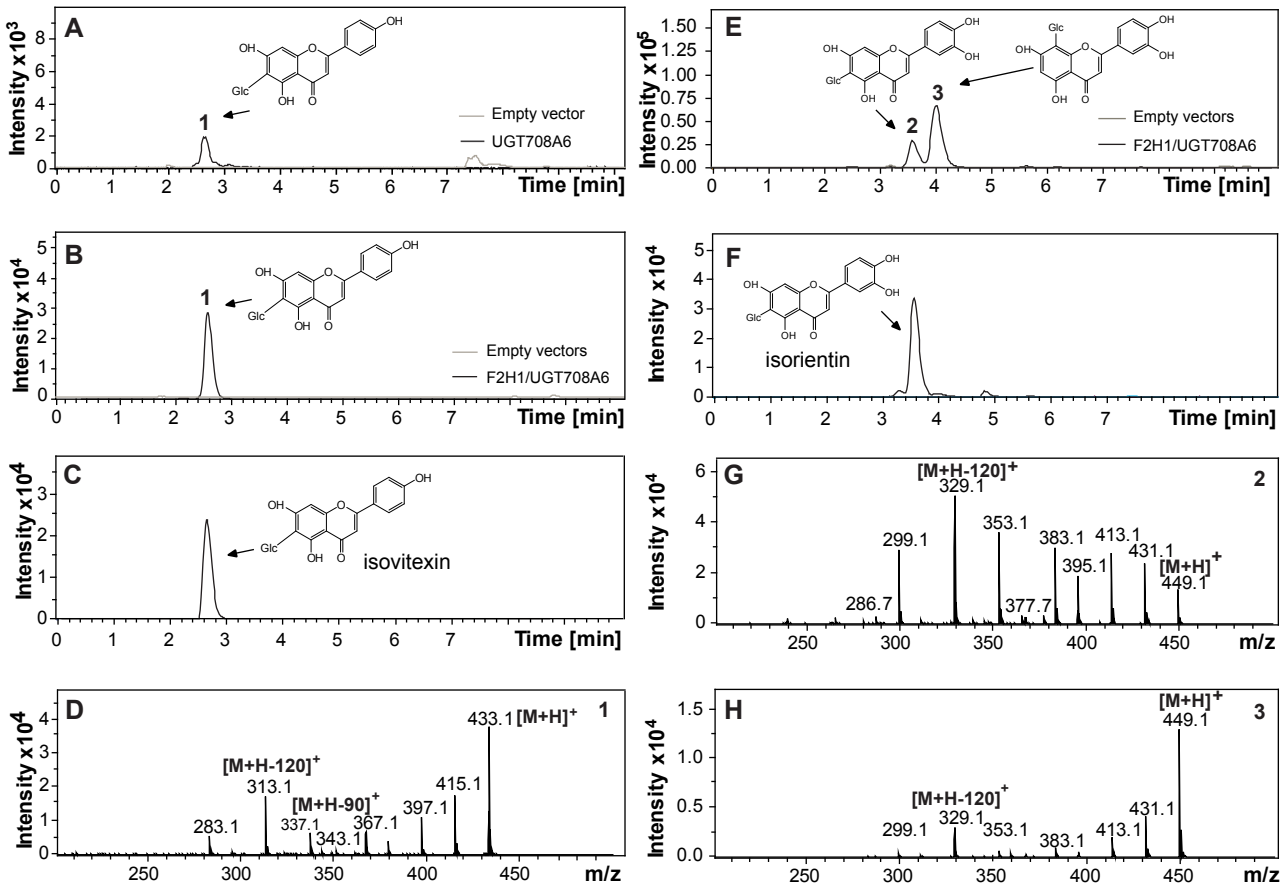


Figure 4

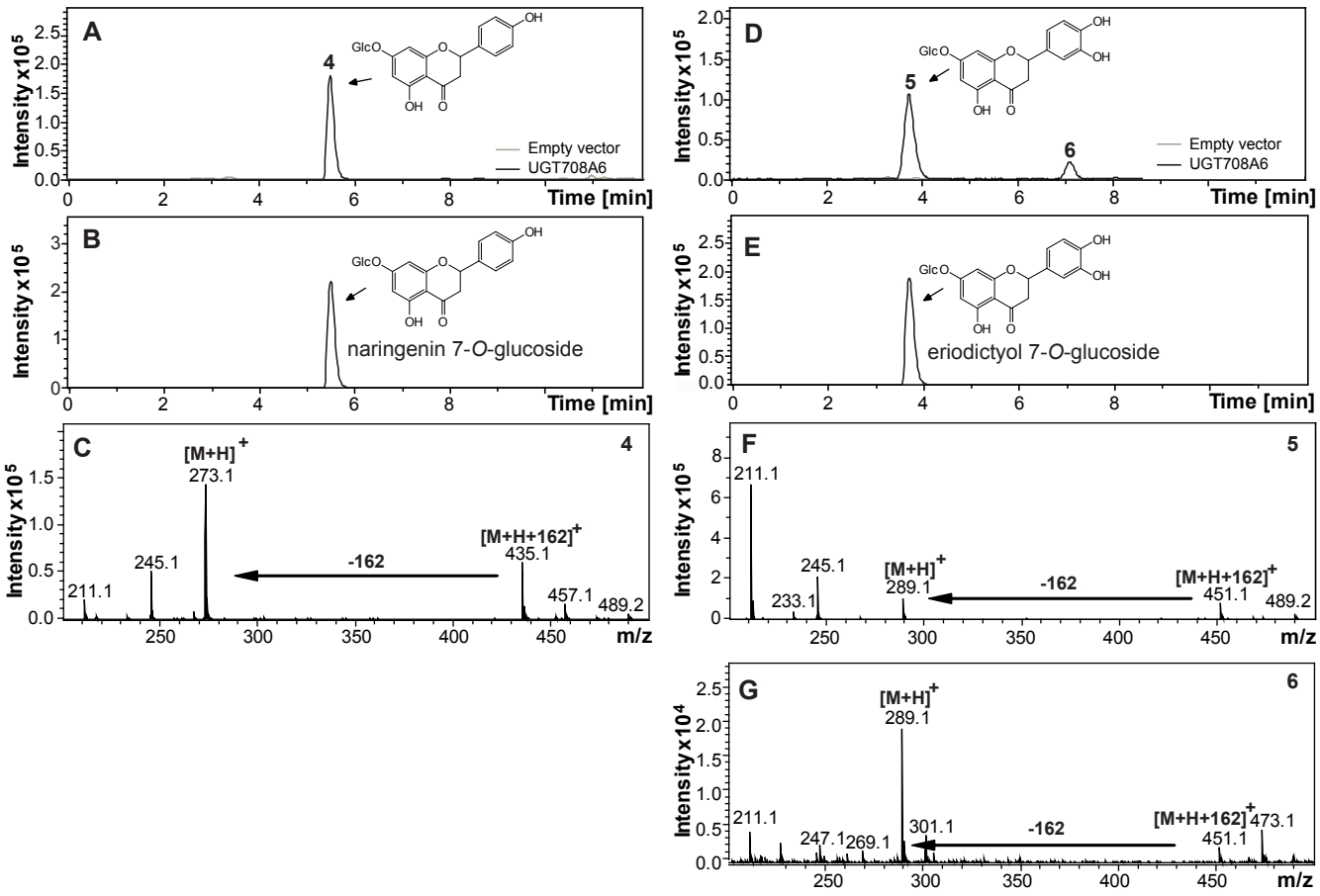
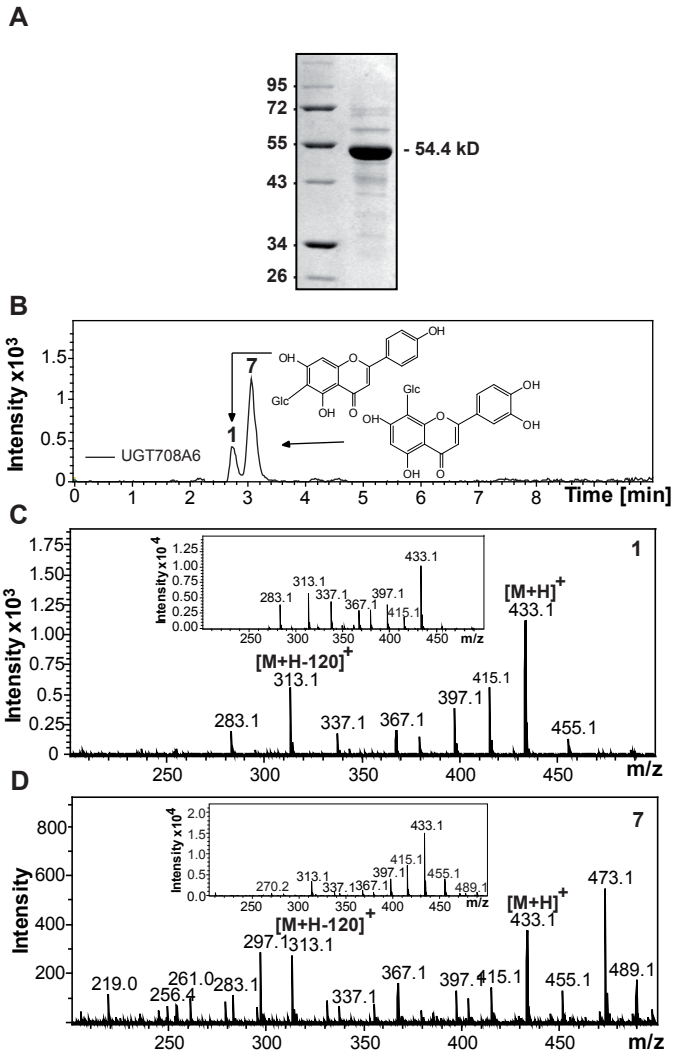
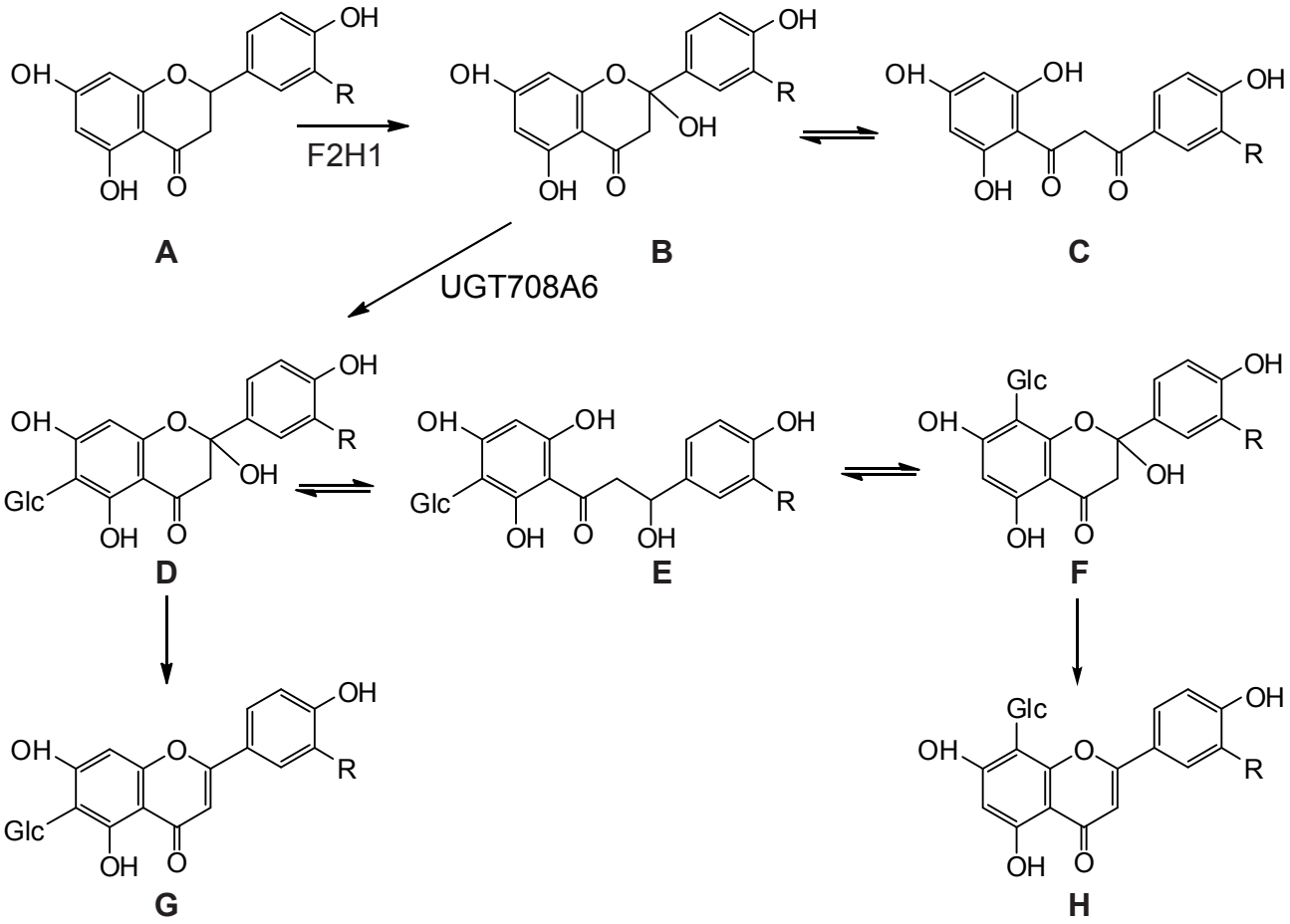


Figure 5





Identification of a bifunctional maize *C*- and *O*-glucosyltransferase*

María Lorena Falcone Ferreyra, Eduardo Rodriguez, María Isabel Casas, Guillermo Labadie, Erich Grotewold, Paula Casati

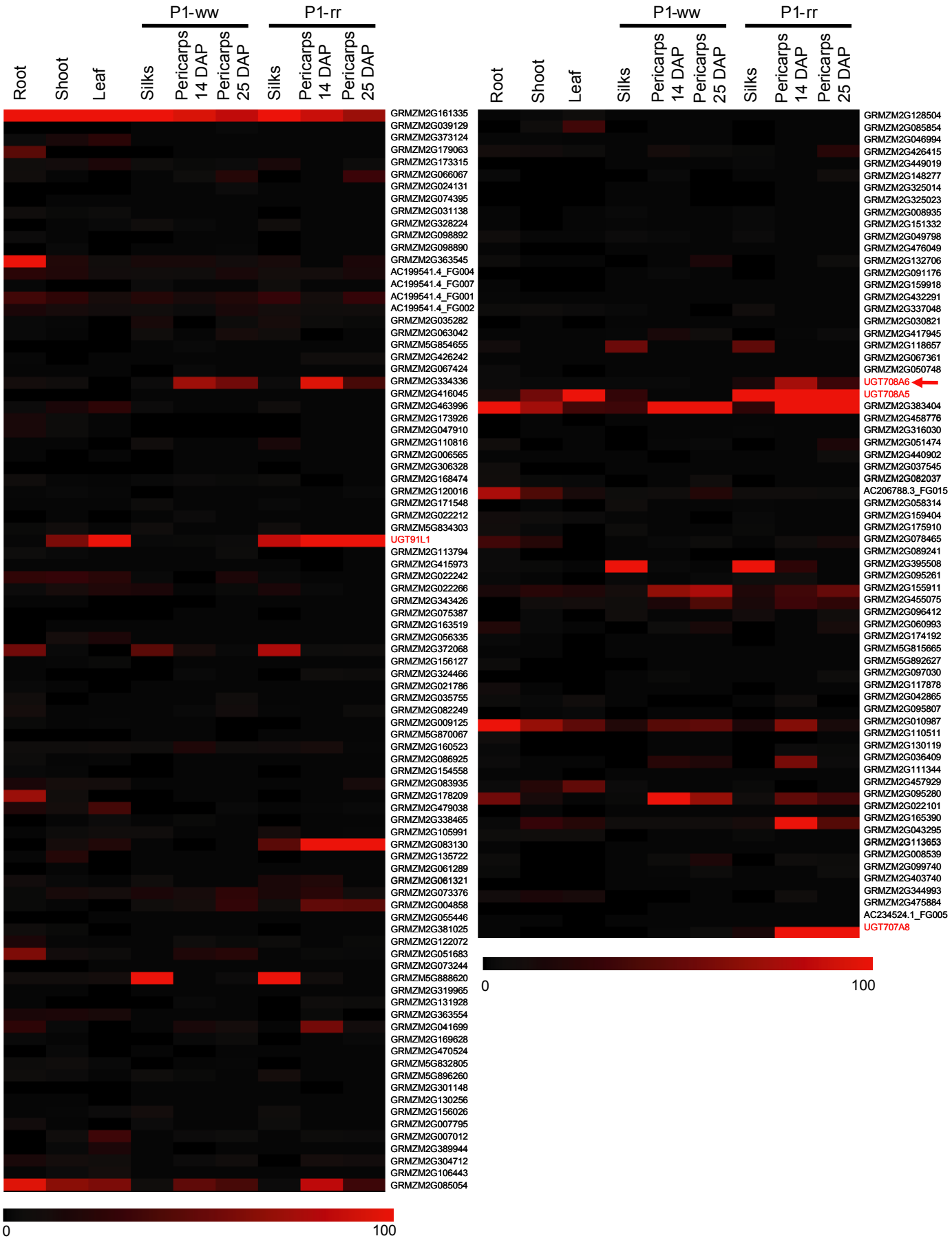
LEGENDS OF SUPPLEMENTAL FIGURES

Supplemental Figure 1. Expression Patterns of maize *glycosyltransferase* Genes. Heat map representation of the expression patterns of *glycosyltransferase* genes. Expression data were derived from previous studies by (24) (*P1-rr* and *P1-ww* pericarps and silks) and from publicly available data sets (root, shoot, and leaf from B73) RNA-Seq results (32).

Supplemental Table 1. Primers used for cloning and RT-qPCR.

Name and purpose	Sequence
UGT708A6-NdeI-forward Cloning in pET28	5'ACAGCATATGGCGGCCAACGGCGGTGACCACACG3'
UGT708A6-Not-reverse Cloning in pET28	5'TGTCAGCGGCCGCACTAGTCTACTTACGCTCCGCGTCC3'
UGT708A6-XbaI-forward Cloning in p5AX43	5'GACTATCTAGAATGGCGGCCAACGGCGGT3'
UGT708A6-KpnI-reverse Cloning in p5AX43	5'CGACGGGTACCCTACTTACGCTCCGCGTC3'
UGT708A6-RT-forward qPCR	5'CGCGGAGCGTAAGTAGGC'3
UGT708A6-RT-reverse qPCR	5'GGAGGCATCGCGCGGTGA'3
UGT708A5-NdeI-forward Cloning in pET28	5'ACAGCATATGGCTCCGCCGCCGGCAATGCAGAG3'
UGT708A5-BamH-reverse Cloning in pET28	5'TGTCAGGATCCACTAGTTCAAGCTCCTCCCTTAAGCTT3'
UGT707A8-NdeI-forward Cloning in pET28	5'ACAGCATATGGCGGCAACGGCATACCCCACTGT3'
UGT707A8-BamH-reverse Cloning in pET28	5'TGTCAGGATCCACTAGTTCATATTATTCTGGCCGGAGA3'
UGT91L1-NdeI-forward Cloning in pET28	5'ACAGCATATGGCCGCCGCCGACTCCTCCCCGCT3'
UGT91L1-BamH-reverse Cloning in pET28	5'TGTCAGGATCCACTAGTTCAGTTCTTGTAGGTTCTCAG3'
UGT708A5-XbaI-forward Cloning in p5AX43	5'GACTATCTAGAATGGCTCCGCCGCCGGCA3'
UGT708A5-BamHI-reverse Cloning in p5AX43	5'CGACGGGATCCTCAAGCTCCTCCCTTAAG3'
UGT707A8-XbaI-forward Cloning in p5AX43	5'GACTATCTAGAATGATGGCGGCAACGGCATAC3'
UGT707A8-KpnI-revers Cloning in p5AX43e	5'CGACGGGATCCTCATCATATTATTCTGGCCGG3'
UGT91L1-XbaI-forward Cloning in p5AX43	5'GACTATCTAGAATGGCCGCCGCCGACTCC3'
UGT91L1-KpnI-reverse Cloning in p5AX43	5'CGACGGGTACCTCAGTTCTTGTAGGTTCTCAG3'
ZmActine1-forward qPCR	5'CTTCGAATGCCCAGCAAT3'
ZmActine1-reverse qPCR	5'CGGAGAATAGCATGAGGAAG3'

Supplemental Figure 1



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