Identification of a Bifunctional Maize C- and O-Glucosyltransferase

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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-hydroxylation (Zmf2H1, 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-dependent glucosyltransferase (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 Zmf2H1 and UGT708A6 show the formation of the flavones C-glycosides. UGT708A6 can also O-glycosylate flavones 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 are enzymes that catalyze the transfer of a sugar moiety to an acceptor molecule. The glycosyltransferases that use uridine diphosphate (UDP) sugar molecules as donors are referred to as UDP-dependent glycosyltransferases (UGTs), and they are members of glycosyltransferase family 1 (1, 2). This family contains most plant UGTs, which utilize different small molecules derived from specialized metabolisms 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, flavonoids, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins, and a few species also produce auronos, isoflavonoids, 3-deoxyanthocyanins, and phlobaphenes (9). In general, plants accumulate flavonoids in vacuoles as O-glycoside derivatives; 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), whereas C-glycosyl flavonoids identified in cucumber leaves 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 chemopreventive compounds against obesity (21). In addition, because of their potential antioxidant properties, they are commonly used as nutraceutical components in the human diet (22, 23).

Although 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 (Zmf2F1H), CYP93G5, converts flavanones into the corresponding 2-hydroxyflavanones (25), which are proposed to serve as substrates for C-glycosylation, followed by dehydration as 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 was 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 biocorversion 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 (Pergamino, Buenos Aires, Argentina). Maize plants were grown in greenhouse conditions with supplemental visible lighting to 1000 microeinstein 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 (7-day-old plants), and juvenile leaves (21-day-old plants). Flavonoid standards and UDP-glucose were purchased from Sigma-Aldrich and Indofine Chemical Co. (New Orleans, LA).

*Cloning and Expression of ZmUGTs and Purification of UGT708A6—*A full-length cDNA corresponding to GRMZM2G162783 (UGT708A6) was amplified by PCR using the primers UGT708A6-Ndel-forward and UGT708A6-Not-reverse harboring the Ndel and NotI restriction sites, respectively, for further cloning. PCRs were performed with GoTaq (Promega) and Pfu polymerases (Invitrogen) (10:1) using 1× buffer, 2 mM MgCl₂, 0.5 μM each primer, 0.5 mM each dNTP, 0.5 unit of enzyme, and cDNA from B73 leaves in a 25-μl final volume under the following cycling conditions: 2-min denaturation at 94 °C and 35 cycles at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 120 s followed by 7 min at 72 °C. Primers for cDNA were designed based on the sequence provided by the maize genome sequence (MaizeSequence, release 5b.60, GRMZM2G162783). 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 Ndel and NotI, and the insert was purified and cloned in pET28a vector generating the construct pET28-UGT708A6. Full-length cDNAs corresponding to GRMZM2G162755 (UGT708A5), GRMZM2G063550 (UGT707A8), and GRMZM2G180283 (UGT91L1) were obtained from Arizona Genomics Institute (Tucson, AZ). ZmUGTs were amplified from the bacterial artificial chromosome clones by PCR using the primers described in [supplemental Table 1](#) for further cloning in pET28 vector. PCRs were performed as described above for UGT708A6. The PCR products were purified from the gels, digested with the corresponding restriction enzymes, purified, and cloned into pET28 vector, and sequenced.

BL21(DE3) cells with the chaperone expression plasmid pGRO (28) were transformed with the construct pET28-ZmUGTs and the empty vector pET28. Cell cultures (200 ml of LB medium containing 30 mg liter⁻¹ kanamycin and 35 mg liter⁻¹ chloramphenicol) were grown at 37 °C until A₆₀₀ reached 0.4, and 1-arabinose (2 mg ml⁻¹) was added to induce chaperone proteins. The cultures were grown at 37 °C to mid-log phase (A₆₀₀ 0.5–0.6), and recombinant N-terminal His₉-ZmUGTs expression was achieved by induction with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside 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. The 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 mixture (Roche Applied Science). 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 nickel-nitrotriacetic acid 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 three washes with 7 volumes of washing buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 5% glycerol, 40 mM imidazole). Elution was carried out by five sequential additions of 1 ml of elution buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 5% glycerol, 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 four cycles of concentration and dilution using Amicon Ultra-15 30,000 (Millipore) and stored at −80 °C. The protein level was estimated both by comparison with dilution series of bovine serum albumin on a Coomassie Blue-stained SDS-polyacrylamide gel and by using the Bradford reagent (Bio-Rad; Ref. 29). The yield of 90% pure recombinant protein obtained in these conditions was 6 mg liter⁻¹ of culture.

To express each ZmUGT in yeast, the full-length cDNAs were amplified by PCR using primers harboring restriction sites (supplemental Table 1) and each pET28-ZmUGT construct as templates. The PCR product was purified, digested with the corresponding enzymes, and cloned in p5AX43 vector generating the plasmids p5AX43-ZmUGTs: 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 HindIII site. The p5AX43-ZmUGT plasmids and p5AX43 empty vector were transformed into competent WAT11 (31) yeast cells harboring pGZ25-Zmf2H1 or pGZ25 empty vector (25), respectively, following the Trafo protocol (32). Yeast colonies harboring the plasmids were selected by growth on synthetic complete medium (SC) agar plates lacking uracil, tryptophan, and leucine (SC Ura– Trp– Leu–).
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Bioconversion Experiments—For in vivo yeast activity assays, an individual recombinant yeast colony was grown for 40 h at 30 °C in 5 ml of liquid SC medium containing 2% (w/v) glucose. Then an aliquot of this culture corresponding to an A$_{600}$ of 1.0 was collected by centrifugation, washed in sterile water, and used to seed 5 ml of induction medium, SC medium containing 2% (w/v) galactose and 3% (w/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 liquid chromatography-mass spectrometry (LC-MS) analysis.

For in vivo E. coli activity assays, BL21(DE3) cells harboring pGRO (for expression of GroEL-GroES chaperone complex) and pET28-ZmUGTs or empty pET28a plasmids were grown at 37 °C in LB with appropriate antibiotics. Expression of chaperones and UGT proteins was induced by the addition of L-arabinose and 0.5 mm isopropyl 1-thio-β-D-galactopyranoside, respectively, as described above, and cultures were simultaneously supplemented with 40 µg ml$^{-1}$ flavonoids. Cultures were grown at 22 °C for 24–48 h and then centrifuged at 15,000 × g for 5 min. One-milliliter 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, whereas 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 Product Analyses by LC-MS—Reaction products were analyzed by LC-MS using a system consisting of an Agilent 1100 high-performance liquid chromatograph pump, and a Bruker microOTOF-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 Analyses by RT-(Quantitative (q)) PCR—Tissues from three independent biological replicates were frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted following the 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 qPCR in a iCycler iQ detection system with the Optical System Software version 3.0a (Bio-Rad) 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 PRIMER3 software (33). Three biological replicates were used for each sample plus a 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 and 40–45 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 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 sequenced. Primers used for UGT708A6 are listed in supplemental Table 1 (UGT708A6-RT-forward and UGT708A6-RT-reverse).

Extraction of Total Flavonoid from Maize Silks—Flavonoid extraction was performed as described previously (12). Fresh silks and 25-day-after pollination pericarps were rinsed with water and lyophilized for 1 day. Dry weight was measured, and the sample was ground to a powder with a mortar and pestle. The powder was extracted for 8 h with 12 volumes of acidic methanol (1% (v/v) HCl in methanol) followed by a second extraction with 12 volumes of chloroform and 6 volumes of distilled water. The extracts were vortexed and centrifuged for 2 min at 3000 × 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 glycosyltransferase domain (IPR002213) present on the maize genome (version 5b.60) using chloroz1 (GRMZM2G165390) as a model. These gene models were further used to generate a list to cross-reference to data publicly available from Morohashi et al. (25) (P1-rr and P1-ww pericarps and silks) and from publicly available data sets (root, shoot, and leaf from the B73 inbred line) RNA sequencing results (35). These data were further used to generate a heat map on the MeV Multiple Array Viewer (36).

Accession Numbers—Sequence data from ZmUGTs can be found in the maize genome sequence (version 3b.60 at Maize-Sequence) under the following accession numbers: UGT708A5 (GRMZM2G162755), UGT707A8 (GRMZM2G063550), UGT91L1 (GRMZM2G180283), and UGT708A6 (GRMZM2G162783).
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Expression and Phylogenetic Analysis of ZmUGTs—To determine a putative candidate for C-glycosylation reaction of flavonoids in maize, we followed two criteria. First, we evaluated how genes of candidates 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 bronze1 (GRMZM2G165390), one of the best studied maize UGTs and characterized UGTs capable of performing N- and O-glycosylation reactions (1, 2). Interestingly, ZmUGTs were highly up-regulated in ZmUGTs. After a 2-day fermentation in vivo by feeding 2-hydroxyxarinagenin as a flavonoid acceptor to E. coli cultures expressing each of the ZmUGTs. After a 2-day fermentation assay, flavonoids were extracted with ethyl acetate, and products were analyzed by LC-MS. Of all the glycosyltransferases tested (UGT708A5, UGT707A8, UGT707A1, and UGT707A6), only UGT707A6 was able to produce a compound (I) that was identified as apigenin 6-C-glucoside (isorivinexin) by comparison with an isorivinexin standard using LC-MS/MS (Fig. 3, A and C). The negative control, E. coli containing the empty vector, did not show production of this compound (Fig. 3A).

To verify the ability of UGT707A6 to convert 2-hydroxynaringenin to isorivinexin, we took advantage of a yeast strain that we had previously generated that expresses the A. thaliana cytochrome P450 reductase and ZmF2H1, accumulating small amounts of 2-hydroxynaringenin when fed with naringenin (25). Thus, yeast cultures expressing both ZmF2H1 and one of the ZmUGTs or harboring the corresponding combination of empty vectors were supplied with the flavonones naringenin or eriodictyol as substrates, and the glycoside products were analyzed by LC-MS. In these combinatorial assays, only when UGT707A6 was expressed along with ZmF2H1 were the 6-C-

![FIGURE 1. Alignment of plant secondary product glycosyltransferase motif from plant UGTs. The 10 highly conserved residues of the motif proposed in the interaction with the UDP-sugar are in bold.](image-url)
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FIGURE 2. Phylogenetic analyses of selected UGT proteins from higher plants. The numbers indicate bootstrap values (10,000 replicates). Bar, 0.1 amino acid substitution per site. Different glycosyltransferases are clustered in circles based on the regioselectivity upon the substrate acceptors. The following plant UGT sequences were analyzed: UGT78D2 (A. thaliana, NP_197207), UGT78D3 (A. thaliana, NP_197205), VvGT1 (Vitis vinifera, AAP81683), UGT78A2 (Aralia cordata, AB103471), Phf3GlcT (P. hybridum, AAD52985), VvGT5 (Vitis vinifera, BA12846), VvGT6 (V. vinifera, BA12847), UGT78G1 (Medicago truncatula, A6NX6C). Zm3GlcT (Zea mays, X13501), ThsGT (Torenia hybrida, AP076698), Pf5GlcT (Perilla frutescens, BAA36421), UGT75C1 (A. thaliana, Q0WW21), PhASGT (Petunia × hybridum, BAA89009.1), Vh5GlcT (Verbena × hybridum, BAA36423), OsCGT (O. sativa, ABC94602.1), UGT72B1 (A. thaliana, Q9M156), UGT706D1 (O. sativa, BAB68093), UGT707A3 (O. sativa, BAC83989), UGT71G1 (M. truncatula, AAW56092), UGT71F1 (Beta vulgaris, AY526081), FaGT6 (Frangaria × ananassa, DQ289587), UGT89C1 (A. thaliana, Q9LNE6), UGT73J1 (Alium cepa, AY62063), AcGT7 (A. cepa, AY62062), UGT73C6 (A. thaliana, AEC09298), UGT73C8 (M. truncatula, DQ875459), Sb7GlcT (Scutellaria baicalensis, AB031274), Nt7GlcT (Nicotiana tabacum, AF346431), FaGT7 (Frangaria × ananassa, DQ289588), Db7GlcT (Dorotheranthus bellidiformis, Y18871), UGT73A4 (B. vulgaris, AY526080), Pht1–6RhaT (P. hybridum, CA50376), UGT79G16 (Ipomoea purpurea, AB192315), Cm1–2RhaT (Citrus maxima, AY048882), UGT94B1 (Bellis perennis, AB190262), CrUGT3 (Catharanthus roseus, AB443870), and UGT94D1 (Sesamum indicum, BAF99027).

glucosyl derivatives of the respective flavones, isovitexin and isoorientin, identified as products (1 and 2) as compared with the respective standards by LC-MS/MS (Fig. 3, B–F). These compounds show the characteristic fragment ions of the C-glycoside moiety, [M + H – 90] and [M + H – 120] (Fig. 3, D and H). Furthermore, the formation of the C-glycoside products was verified due to the stability of these compounds under acid hydrolysis (10, 26) (not shown). In addition to isoorientin (luteolin 6-C-glycoside), another reaction product with an m/z of 449.1 and different retention time was observed (3). Further analysis of the relative intensity of the product ion found by positive electrospray ionization (LC-MS/MS) allowed validation of reaction product 3 as orientin (luteolin 8-C-glycoside) (Fig. 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 and eriodictyol as substrates, production
of new compounds was detected by LC-MS. Analysis of the extracts showed the presence of one naringenin derivative product (4) with an m/z of 435.1 [M + H⁺], whereas eriodictyol generated two new products (5 and 6), both with an m/z of 451.1 [M + H⁺] (Fig. 4, A and D). Interestingly, the fragmentation patterns of these new glycoside derivatives showed the typical neutral loss of 162 (transition 435.1 → 273.1 for naringenin and 451.1 → 289.1 for eriodictyol, respectively) corresponding to a hexose residue in a flavonoid O-glycoside (Fig. 4, C, F, and 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 with the respective standards by LC-MS/MS (Fig. 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-glycosylate 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 (Fig. 5A). When the recombinant UGT708A6 was assayed using the flavanones naringenin and 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-glucoside 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-glucosides.

On the other hand, when 2-hydroxynaringenin was assayed as a substrate, two reaction products with an m/z of 433.1 [M + H⁺] were observed, one corresponding to isovitexin (apigenin 8-C-glucoside, 1) in comparison with the available standard (Figs. 5B and 3C). Analysis of the relative intensity of the product ion found by positive electrospray ionization allowed the identification of selective ions for the C8 isomer ([\(^{13}\)C-H₂O-CO]⁺ and \(^{12}\)C-HCHO-CO]⁺ with m/z values of 297.3 and 256.4, respectively), indicating that the second reaction product (7) corresponds to vitexin (apigenin 8-C-glucoside) (45) (Fig. 5D). Together, both in vitro and in vivo bioconversion activity assays demonstrate that UGT708A6 is a bifunctional enzyme able to...
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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 is shown. 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 and E, ion chromatograms of standards naringenin 7-O-glucoside and eriodictyol 7-O-glucoside, respectively. C, F, and 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 with the standards (B and E).

catalyze both the C-glucosylation of 2-hydroxyflavanones and the O-glycosylation of flavanones.

Flavonoid Glycosides in Maize Pericarps and Silks—Supplemental Fig. 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, 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 (isoorientin and isovitexin) with the glycosylated substitutions at the C6 position were identified as was reported previously (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 that likely represent the different glycosylation positions of these compounds. 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 that contributes to flavonoid stability, solubility, storage, and biological activity changes (3). Although flavonoid glycosides have been described in maize, for example 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 have 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 ZmfF2H1 (CYP93G5) (25). Here, through bioconversion assays in yeast expressing ZmfF2H1 with UGT708A6, we have 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 pro-
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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* |
|----------------------|---------------------|----------------------|
|                      |                     |                      |
| 2.6, 3.6, 5.3        | 435                 | Naringenin O-hexoside|
| 7.5                  | 451                 | Erdiotyol O-hexoside |
| 6.2, 6.8             | 597                 | Naringenin di-O-hexoside |
| 8.5                  | 449                 | 6-C-Glucosyl luteolin (isoorientin) |
| 8                    | 433                 | 6-C-Glucosyl apigenin (isovitexin) |
| 8.8                  | 576                 | 6-C-Glucosyl luteolin O-rhamnoside (maysin) |

* Identification was based on MS/MS fragmentations using standards as references.

In rice, C-glycosyl flavone biosynthesis takes place through a pathway different from that of O-glycosyl flavone formation involving the generation of 2-hydroxyflavanones by CYP93G2 activity followed by the C-glycosylation catalyzed by OsCGT (10, 26). It has been proposed that an open form of 2-hydroxyflavanones is the actual substrate for OsCGT, resulting in the formation of 2-hydroxyflvanone C-glycoside products that are further dehydrated by a dehydratase (10, 26, 27). However, it is important to mention that we could not detect the 2-hydroxyflvanone C-glycoside products either by in vivo or in vitro experiments. The failure to detect these intermediates in C-glycosyl flavone biosynthesis could possibly be due to spontaneous dehydration of these unstable compounds during the reaction...
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**FIGURE 6. Proposed model for C-glycosyl flavone biosynthesis by UGT708A6.** F2H1, flavanone 2-hydroxylase 1; R, hydrogen 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.

process (10, 49, 55). In addition, the relative abundances 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), whereas both flavone 6-C-glucoside (isorientin) and flavone 8-C-glucoside (orientin) were detected for eriodictyol in a ratio of 1:8 (Fig. 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 also be detected in minor proportion (Fig. 5). These results could be explained by proposing that the actual substrate for the glycosyltransferase is the closed form of the 2-hydroxyflavanone (Fig. 6, compound B) as it has less structural flexibility than the open form (Fig. 6, compound C). Because 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 C6 and C8 positions; however, only flavone 6-C-glycosides have been described in silks of maize (47, 48). Taking into consideration the proposed biosynthesis pathway of the C-glycosyl flavone maysin (16), a possible explanation for this is that C6 isomer (isovitexin and isoorientin) consumption by the following rhamnosyltransferase enzyme involved in this pathway may favor the formation of this isomer. Nevertheless, we cannot rule out that flavone 8-C-glycosides are present in maize tissues not yet studied.

The R2R3-MYB PI transcription factor regulates maysin production in silks tissues of some maize varieties (37–39). Our results show that UGT708A6, the expression of which is regulated by PI in silks (25), generates isovitexin and isoorientin, intermediates involved in biosynthesis of apimaysin and maysin, respectively (16), suggesting that this enzyme could be involved in this biosynthetic pathway. Similarly to rice (27), when ZmF2H1 and UGT708A6 enzymes were co-expressed in yeast, the intermediate 2-hydroxyflavanones were not detected; it is 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, preventing 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, and 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.

**TABLE 2**

Analysis of UGT708A6 expression

| Maize tissues | Expression level relative to actin1 (×10³) |
|--------------|------------------------------------------|
| 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      | Not detected                             |

*UGT708A6 expression was 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 actin1 mRNA (J01238). Data are represented as the means obtained from biological triplicates ±S.D. of the samples.*

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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 flavonone 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.

REFERENCES
1. Osmani, S. A., Bak, S., and Muller, B. L. (2009) Substrate specificity of plant UDP-dependent glycosyltransferases predicted from crystal structures and homology modeling. Phytochemistry 70, 325–347.
2. Caputi, L., Malnoy, M., Goremykin, V., Nikiforova, S., and Martens, S. (2012) A genome-wide phylogenetic reconstruction of family 1 UDP-glycosyltransferases revealed the expansion of the family during the adaptation of plants to life on land. Plant J. 69, 1030–1042.
3. Bowles, D., Isayenkova, J., Lim, E. K., and Poppenberger, B. (2005) Glycosyltransferases: managers of small molecules. Curr. Opin. Plant Biol. 8, 254–263.
4. Bowles, D., and Lim, E. K. (2010) Glycosyltransferases of small molecules: their roles in plant biology. eLS 10.1002/9780470015902.a0021537.
5. Hassan, S., and Mathesius, U. (2012) The role of flavonoids in root-rhizosphere signaling: opportunities and challenges for improving plant-microbe interactions. J. Exp. Bot. 63, 3429–3444.
6. Payyavula, R. S., Babst, B. A., Nelsen, M. P., Harding, S. A., and Tsai, C. J. (2009) Glycosylation-mediated phenylpropanoid partitioning in Populus tremuloides cell cultures. BMC Plant Biol. 9, 151.
7. Vaistij, F. E., Lim, E. K., Edwards, R., and Bowles, D. J. (2009) Glycosylation-mediated phenylpropanoid partitioning in isolated young leaves of Arabidopsis thaliana. Plant Cell 21, 1840–1854.
8. Hassan, S., and Mathesius, U. (2012) The role of flavonoids in root-rhizosphere signaling: opportunities and challenges for improving plant-microbe interactions. J. Exp. Bot. 63, 3429–3444.
9. Payyavula, R. S., Babst, B. A., Nelsen, M. P., Harding, S. A., and Tsai, C. J. (2009) Glycosylation-mediated phenylpropanoid partitioning in Populus tremuloides cell cultures. BMC Plant Biol. 9, 151.
10. Brazier-Hicks, M., Edwards, R. (2013) Metabolic engineering of the flavone pathway in maize. Curr. Opin. Plant Biol. 27, 248–254.
11. Gietz, R. D., and Sugino, A. (1988) A rapid and sensitive method for the quantitation of yeast protein using the bicinchoninic acid method. Anal. Biochem. 166, 162–168.
12. Casati, P., and Walbot, V. (2005) Differential accumulation of maysin and homology modeling. Phytochemistry 70, 325–347.
13. Caputi, L., Malnoy, M., Goremykin, V., Nikiforova, S., and Martens, S. (2012) A genome-wide phylogenetic reconstruction of family 1 UDP-glycosyltransferases revealed the expansion of the family during the adaptation of plants to life on land. Plant J. 69, 1030–1042.
14. McNally, D. J., Wrums, K. V., Labbé, C., Quideau, S., and Belanger, R. R. (2003) Complex C-glycosyl flavonoid phytoalexins from Curcuma sativus. J. Nat. Prod. 66, 1280–1283.
15. El-Alfy, T. S., El-Gohary, H. M., Sokkar, N. M., Hosny, M., and Al-Mahdy, D. A. (2011) A new flavonoid C-glycoside from Celtis australis L. and Celtis occidentalis L. leaves and potential antioxidant and cytotoxic activities. Sci. Pharm. 79, 963–975.
16. Taps, K., Sakarkar, D. M., and Kakde, R. B. (2008) Flavonoids as nutraceuticals: a review. Trop. J. Pharm. Res. 7, 1089–1109.
17. Markham, K. R., Tanner, G. J., Cassi-Lit, M., Whitecross, M. I., Nayudu, M., and Mitchell, K. A. (1998) Possible protective role for 3-hydroxyflavonones in maize against Helicoverpa armigera. Photochem. Photobiol. 63, 1167–1173.
18. Markham, K. R., Tannner, G. J., Cassi-Lit, M., Whitecross, M. I., Nayudu, M., and Mitchell, K. A. (1998) Possible protective role for 3’A'-dihydroxyflavones induced by enhanced UV-B in a UV-tolerant rice cultivar. Photochem. Photobiol. 70, 1913–1919.
19. McNally, D. J., Wrums, K. V., Labbé, C., and Belanger, R. R. (2003) Synthesis of C-glycosyl flavonoid phytoalexins as a site-specific response to fungal penetration in cucumber. Mol. Plant Pathol. 63, 293–303.
20. McNally, D. J., Wrums, K. V., Labbé, C., Quideau, S., and Belanger, R. R. (2003) Complex C-glycosyl flavonoid phytoalexins from Curcuma sativus. J. Nat. Prod. 66, 1280–1283.
21. Lee, E. M., Lee, S. S., Chung, B. Y., Cho, J. Y., Lee, I. C., Ahn, S. R., Jang, S. I., and Kim, T. H. (2010) Pancreatic lipase inhibition by C-glycosidic flavones isolated from Eremodochloa ophiurioides. Molecules 15, 8251–8259.
22. Patil, B. S., Jayaparakasha, G. K., Chidambahara Murthy, K. N., and Vikram, A. (2009) Bioactive compounds: historical perspectives, opportunities, and challenges. J. Agric. Food Chem. 57, 8141–8160.
23. Brazier-Hicks, M., and Kakde, R. B. (2008) Flavonoids as nutraceuticals: a review. Trop. J. Pharm. Res. 7, 1089–1109.
24. Morohashi, K., Casas, M. I., Falcone Ferreyra, M. L., Mejia-Guerra, M. K., Pourcel, L., Yilmaz, A., Feller, A., Carvalho, B., Emiliani, J., Rodrigues, E., Pellegrenit, S., McMullan, M., Casati, P., and Groetewold, E. (2012) A genome-wide regulatory framework identifies maize pericarp color 1 controlled genes[C][W]. Plant Cell 24, 2745–2764.
25. Du, Y., Chu, H., Chu, I. K., and Lo, C. (2010) CYP93G2 is a flavanone 2-hydroxylase required for C-glycosylflavone biosynthesis in rice[1][W]. Plant Physiol. 154, 324–333.
26. Casati, P., and Walbot, V. (2005) Differential accumulation of maysin and flavonoids in cereals. Plant Physiol. 138, 1167–1173.
A Bifunctional Glycosyltransferase from Maize

melsbach, D. S., and Costello, C. E. (1989) in 197th and 198th National Meetings of the American Chemical Society and the 1989 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, December 17–22, 1989 (Hedin, P. A., ed) pp. 251–263, American Chemical Society, Washington D. C.

39. Snook, M. W., Widstrom, N., and Gueldner, R. C. (1989) Reversed-phase high-performance liquid chromatographic procedure fro the determination of maysin in corn silks. *J. Chromatogr.* 477, 439–447

40. Futtek, D. B., Schiefelbein, J. W., Johnston, F., and Nelson, J. O. (1988) Sequence comparisons of three wild-type *Bronze-1* alleles from *Zea mays*. *Plant Mol. Biol.* 11, 473–481

41. Ralston, E. J., English, J. J., and Dooner, H. K. (1988) Sequence of three bronze alleles of maize and correlation with the genetic fine structure. *Genetics* 119, 185–197

42. Sekhon, R. S., Lin, H., Childs, K. L., Hansey, C. N., Buell, C. R., de Leon, N., and Kaeppler, S. M. (2011) Genome-wide atlas of transcription during maize development. *Plant J.* 66, 553–563

43. Kroon, J., Souer, E., de Graaff, A., Xue, Y., Mol, J., and Koes, R. (1994) Cloning and structural analysis of the anthocyanin pigmentaion locus Rt of *Petunia hybrida*: characterization of insertion sequences in two mutant alleles. *Plant J.* 5, 69–80

44. Brazier-Hicks, M., Offen, W. A., Gershater, M. C., Revett, T. J., Lim, E. K., Bowles, D. J., Davies, G. J., and Edwards, R. (2007) Characterization and engineering of the bifunctional N- and O-glycosyltransferase involved in xenobiotic metabolism in plants. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20238–20243

45. Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B., and Vincente, F. (2008) New features on the fragmentation and differentiation of C-glycosidic flavone isomers by positive electrospray ionization and triple quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* 22, 1834–1842

46. Zhang, J., Subramanian, S., Zhang, Y., and Yu, O. (2007) Flavone synthases from *Medicago truncatula* are flavanone-2-hydroxylases and are important for nodulation. *Plant Physiol.* 144, 741–751

47. Grotewold, E., Chamberlin, M., Snook, M., Siame, B., Butler, L., Swenson, J., Maddock, S., St Clair, G., and Bowen, B. (1998) Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. *Plant Cell* 10, 721–740

48. Snook, M. E., Widstrom, N. W., Wiseman, B. R., Byrne, P. F., Harwood, J. S., and Costello, C. E. (1995) New C-4′-hydroxy derivatives of maysin and 3′-methoxymaysin isolated from corn silks (*Zea mays*). *J. Agric. Food Chem.* 43, 2730–2745

49. Kerscher, F., and Franz, G. (1988) Isolation and some properties of an UDP-glucose:2-hydroxyflavanone-6(or 8)-C-glycosyltransferase from *Fagopyrum esculentum* M. cotyledons. *J. Plant Physiol.* 132, 110–115

50. Dürr, C., Hoffmeister, D., Wohlert, S. E., Ichinose, K., Weber, M., Von Mulert, U., Thorson, J. S., and Bechthold, A. (2004) The glycosyltransferase UrdGT2 catalyzes both C- and O-glycosidic sugar transfers. *Angew. Chem. Int. Ed. Engl.* 43, 2962–2965

51. Kumano, T., Richard, S. B., Noel, J. P., Nishiyama, M., and Kuzuyama, T. (2008) Chemoenzymatic syntheses of prenylated aromatic molecules using *Streptomyces* prenyltransferases with relaxed substrate specificities. *Bioorg. Med. Chem.* 16, 8117–8126

52. Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008) Glycosyltransferases: structures, functions and mechanisms. *Ann. Rev. Biochem.* 77, 521–555

53. Shao, H., He, X., Achnine, L., Blount, J. W., Dixon, R. A., and Wang, X. (2005) Crystal structures of a multifunctional triterpene/flavonoid glycosyltransferase from *Medicago truncatula*. *Plant Cell* 17, 3141–3154

54. Schreiber, S. L. (2007) Rethinking relationships between natural products. *Nat. Chem. Biol.* 3, 352

55. Akashi, T., Aoki, T., and Ayabe, S. (1998) Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.; Fabaceae) which represents licodione synthase and flavone synthase II. *FEBS Lett.* 431, 287–290