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Crystal Structure of *Medicago truncatula* UGT85H2 – Insights into the Structural Basis of a Multifunctional (Iso)flavonoid Glycosyltransferase

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(Iso)flavonoids are a diverse group of plant secondary metabolites with important effects on plant, animal and human health. They exist in various glycosidic forms. Glycosylation, which may determine their bioactivities and functions, is controlled by specific plant uridine diphosphate glycosyltransferases (UGTs). We describe a new multifunctional (iso)flavonoid glycosyltransferase, UGT85H2, from the model legume *Medicago truncatula* with activity towards a number of phenylpropanoid-derived natural products including the flavonol kaempferol, the isoflavone biochanin A, and the chalcone isoliquiritigenin. The crystal structure of UGT85H2 has been determined at 2.1 Å resolution, and reveals distinct structural features that are different from those of other UGTs and related to the enzyme's functions and substrate specificities. Structural and comparative analyses revealed the putative binding sites for the donor and acceptor substrates that are located in a large cleft formed between the two domains of the enzyme, and indicated that Trp360 may undergo a conformational change after sugar donor binding to the enzyme. UGT85H2 has higher specificity for flavonol than for isoflavone. Further substrate docking combined with enzyme activity assay and kinetic analysis provided structural insights into this substrate specificity and preference.

Keywords: glycosylation; uridine diphosphate glycosyltransferase; isoflavonoid; crystal structure; substrate specificity

Introduction

Glycosylation of small molecules has key roles in many biological processes, including biosynthesis of various bioactive compounds, regulation of hormone activity, and metabolism of toxins.[1,2] Small-molecule glycosylation is catalyzed by uridine diphosphate glycosyltransferases (UGTs), members of family 1 of the glycosyltransferase superfamily, which contains over 80 families of enzymes‡.[3,4] UGTs utilize UDP-activated sugars as donors and transfer their sugar moiety to various acceptors. Glycosylation is often the final step in the biosynthesis of many plant natural products,[5] enhancing their solubility and stability, and facilitating their storage and accumulation in plant cells. Consistent with the variety and complexity of plant natural products, a large number of *UGT* gene sub-families have evolved for the glycosylation of these molecules.[5,6] Plant UGTs are characterized by a highly conserved signature putative secondary plant glycosyltransferase (PSPG) motif, but share relatively low levels of sequence identity, especially within the regions related to acceptor binding, and this feature may be essential to account for the recognition of the huge

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Abbreviations used: UGT, uridine diphosphate glycosyltransferase; rmsd, root-mean-square deviation; PSPG, putative secondary plant glycosyltransferase; TCA, trichloroacetic acid.

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‡ http://afmb.cnrs-mrs.fr/CAZY/index.html

variety of acceptors and the synthesis of the large number of products. The relationship between primary amino acid sequence, substrate specificity and product regioselectivity of plant UGTs is complex and remains to be determined.

Flavonoids and isoflavonoids, a diverse group of plant natural products, are synthesized *via* the phenylpropanoid pathway. Most (iso)flavonoids exist in plants in the glycosylated forms,[7] and glycosylation, controlled by a large group of specific UGTs, is one of the major factors determining their different functions, bioactivities, and bioavailabilities. (Iso)flavonoids play important roles in growth and development, and in defense against microorganisms and pests.[8,9] These compounds also possess antioxidant activity with potential health benefits for humans and animals.[9–11] The isoflavonoids, identified primarily in the Leguminosae, have been ascribed various estrogenic, antiangiogenic, antioxidant, and anticancer activities, leading to their popularity as dietary supplements.[12–15] They may have additional health-promoting activities, including chemoprevention of osteoporosis and other postmenopausal disorders,[16–18] and have received considerable attention as "nutraceuticals". UGTs play central roles in determining the diversity of (iso)flavonoid structures and regulating their functions and activities, but the glycosylation processes are complex and not well understood.

We previously reported the X-ray crystal structure of *Medicago truncatula* UGT71G1.[19] This structure, along with the more recently reported structure of grape *Vv*GT1,[20] provides an initial structural basis for understanding enzyme catalytic mechanism and specificity for plant UGTs. These enzymes comprise two Rossmann-like domains, belonging to the GT-B fold, and in this respect are similar to the bacterial Gtfs of the GT1 family,[21–23] and other GT-B fold enzymes of the GT superfamily, although they share very low levels of sequence identity. The two plant UGT structures reveal that nucleotide sugar donors are bound to the C-terminal domains of the enzymes with the acceptors binding to the N-terminal domains. Their three-dimensional structures share high levels of similarity, although the amino acid sequence identity is only ~20%. Both enzymes recognize multiple acceptors; *M. truncatula* UGT71G1 recognizes the triterpenes medicagenic acid and hederagenin and may be involved in triterpene saponin biosynthesis *in vivo*,[24] whereas grape *Vv*GT1 is a cyanidin 3-*O*-glycosyltransferase. Both enzymes recognize flavonols such as quercetin. UGT71G1 produces five different quercetin monoglucosides, with the 3′-*O*-glucoside predominating,[19,25] whereas *Vv*GT1 produces only the 3-*O*-glucoside.[20] These structures provided significant insights into (iso) flavonoid glycosylation. However, identification and structural study of specific (iso)flavonoid UGTs is necessary for understanding the glycosylation of this important group of bioactive compounds.

Here, we report the functional characterization and structural analysis of a new UGT, UGT85H2, from *M. truncatula*. Substrate screening showed that UGT85H2 has activity towards several flavonoid-related secondary metabolites, including isoflavones, flavonols and chalcone. Further kinetic study indicated its higher catalytic efficiency and preference for flavonol than for isoflavone or chalcone. The three-dimensional structure of UGT85H2 was solved at 2.1 Å resolution, and revealed distinct features including several insertions and deletions around the substrate binding site, which may determine the enzyme's function and substrate specificity. The structure, combined with the results of substrate docking, kinetic and mutational analyses, sheds light on the enzyme's complex structure–function relationship as regards regio-specificity and preference for different (iso)flavonoid substrates and products.

Results

Substrate specificity and kinetics of UGT85H2

Substrate screening with purified UGT85H2 enzyme was performed against a range of polyphenolic compounds (Figure 1) using UDP-glucose, UDP-galactose or UDP-glucuronic acid as sugar donors. The enzyme showed activity towards the flavonols kaempferol and quercetin, the isoflavones genistein and biochanin A, and the chalcone isoliquiritigenin (Figure 1(a)) with UDP-glucose as donor. No activity was detected with the compounds shown in Figure 1(b).

UGT85H2 catalyzed the regioselective glycosylation of biochanin A and genistein at their 7-*O*-positions. Regioselectivity was also observed for UGT85H2 toward kaempferol, producing the 3-*O*-glucoside. When quercetin was used as substrate, two products were formed, with quercetin 3-*O*-glucoside as the major product, but the substitution position of the minor product was not determined. UGT85H2 produced a single product with the chalcone isoliquiritigenin, and the position of glycosylation is yet to be determined. No product was observed when UDP-galactose or UDP-glucuronic acid were used as sugar donors, except in the assays with biochanin A, when single products were detected but in trace amounts. These results indicate that UDP-glucose is the preferred sugar donor for UGT85H2.

An enzyme kinetic study was performed to further explore the substrate specificity (Table 1). The K_m for the preferred donor UDP-glucose was 485 μM, first determined with biochanin A as acceptor substrate. UDP-glucose was used as donor for determining kinetic parameters of acceptor substrates. K_m values for the flavonols kaempferol and quercetin were the lowest (~2.9 μM), indicating strong binding affinity for the enzyme, followed by biochanin A and isoliquiritigenin. The K_m for genistein was ~2.0 mM, indicating very weak affinity with the enzyme. UGT85H2 exhibited the highest catalytic efficiency and specificity (k_{cat}/K_m)