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Isolation of Legume Glycosyltransferases and Active Site Mapping of the *Phaseolus lunatus* Zeatin O-glucosyltransferase ZOG1

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Abstract O-Glycosides of the cytokinin zeatin are found in many plant tissues. They provide protection against degradative enzymes and may serve as cytokinin reserves. Two zeatin glycosyltransferase (GT) genes, an O-glucosyltransferase (*ZOG1*) from *Phaseolus lunatus* and an O-xylosyltransferase (*ZOX1*) from *P. vulgaris*, were previously isolated. Five novel bean and soybean GT genes with high sequence identity to *ZOG1* were isolated, sequenced, and expressed, along with two such genes from rice and one from tomato. None of the recombinant proteins showed GT activity with zeatin. By comparing the ZOG1 sequence to the 3D model of *Medicago truncatula* UGT71G1, four regions possibly important to zeatin binding were identified, and mutation studies identified one amino acid within each region (R59, D87, L127, and F149) whose mutation strongly impaired enzyme activity. The new bean and soybean GTs differ from ZOG1 in one (*Pl*GT2 and *Gm*GT2) to three (*Gm*GT1) of these residues. Mutation of one such GT (*Pl*GT2) to render it identical to ZOG1 at the four implicated residues conferred low enzyme activity, providing further support for the importance of these amino acids in recognizing zeatin as substrate.

Keywords Cytokinin · Zeatin · Glucosyltransferase · Xylosyltransferase · Site-directed mutagenesis · *Phaseolus*

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Introduction

Cytokinins are plant hormones essential for plant growth and development (Mok and Mok 1994). The natural cytokinins are adenine derivatives, most frequently with an isoprenoid side chain at the N^6 position, although aromatic cytokinins are also known to occur (Strnad 1997). Isoprenoid cytokinins are synthesized in plants by isopentenyltransferases from ADP or ATP and isopentenyl pyrophosphate (Kakimoto 2001; Takei and others 2001), after which the side chain can be hydroxylated to form zeatin-type compounds (Chen and Leisner 1984; Takei and others 2004). The hydroxylated side chain allows for O-glucosylation, whereas the adenine ring can be glucosylated at the N3, N7, or N9 position.

Glucosylation is an important metabolic conversion. Glucosylation at the N positions generally results in an

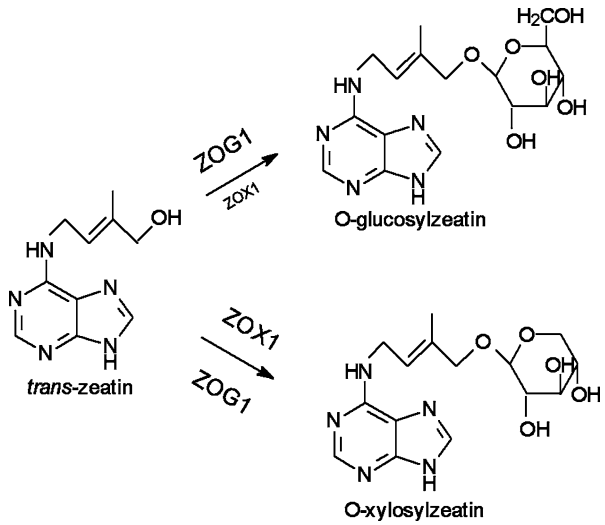

Fig. 1 Chemical structures of zeatin and the major products of ZOG1 and ZOX1

irreversible loss of activity (Letham and others 1983). Zeatin O-glucoside, however, is highly active in bioassays, sometimes even more active than zeatin itself (Mok and others 1992). Because the glucosylated side chain is too large to confer activity, as confirmed by cytokinin receptor assays (Spíchal and others 2004), the high activity is due to slow conversion to the aglycone. O-Glucosylation provides protection from cytokinin oxidases/dehydrogenases, which can rapidly degrade zeatin but not its O-glucoside (McGaw and Horgan 1983; Armstrong 1994). O-Glucosides accumulate in the vacuoles (Fusseder and Ziegler 1988) and may be cytokinin storage forms.

Enzymes and genes mediating glycosylation of zeatin (Figure 1) have been identified in several plant species. The first zeatin O-glucosyltransferase (EC 2.4.1.203) was isolated from immature *Phaseolus lunatus* seeds (Dixon and others 1989), and a variant of this enzyme, zeatin O-xylosyltransferase (EC 2.4.2.40), was obtained from *P. vulgaris* (Turner and others 1987). Generation of monoclonal antibodies to these enzymes enabled the cloning of the corresponding genes, *ZOG1* and *ZOX1* (Martin and others 1999a,b). Zeatin O-glucosyltransferase genes have recently been identified in *Arabidopsis* (Hou and others 2004), and two genes encoding O-glucosyltransferases with preference for *cis*-zeatin were obtained from maize (Martin and others 2001b; Veach and others 2003). An N-glucosyltransferase (EC2.4.1.118) converting zeatin to the N7- and N9-glucosides was found in radish (Entsch and Letham 1979; Entsch and others 1979) and N-glucosyltransferase genes have been identified in the *Arabidopsis* genome (Hou and others 2004).

To determine the effects of overexpressing cytokinin glucosyltransferases on plant development, transgenic tobacco and maize plants harboring the *ZOG1* gene, under the control of an inducible (Tet) or a constitutive (35S) promoter, were generated (Martin and others 2001a; Pineda Rodo 2007). Leaf discs of the Tet:*ZOG1* transformants required tenfold higher zeatin concentrations for formation of callus and shoots; this shows that *ZOG1* expression leads to sequestering of zeatin. Also, plants constitutively expressing the transgene exhibited characteristics indicative of cytokinin deficiency such as shorter stature, thinner stems, narrower leaves, increased root mass, and root formation on the lower stems of tobacco. Exceptions were the occasional branching of transformed tobacco plants (Martin and others 2001a) and the increased chlorophyll of transformed maize plants (Pineda Rodo 2007), traits usually associated with increased cytokinin levels or sensitivity. This suggests that secondary changes can take place in particular tissues or stages of development.

Glycosyltransferases (GTs) are currently classified into 90 families (http://www.cazy.org/fam/acc_GT.html). The cytokinin GTs belong to Family 1, a large family of enzymes present in both prokaryotes and eukaryotes. About one-half of these enzymes, including the cytokinin GTs, are characterized by a highly conserved UDP-binding signature sequence (PSPG motif) in the carboxy terminal region (Hundle and others 1992; Hughes and Hughes 1994; Lim and Bowles 2004). The *Arabidopsis* genome contains 117 sequences with this consensus motif (Li and others 2001; Paquette and others 2003; Lim and Bowles 2004). The substrates of these GTs are generally small molecules, including secondary metabolites, plant hormones, and xenobiotics such as pesticides and herbicides (Jones and Vogt 2001).

Recently, crystal structures were published for two plant GTs belonging to Family 1, a triterpene/flavonoid GT of *Medicago truncatula* (Shao and others 2005) and a flavonoid GT of *Vitis vinifera* (Offen and others 2006). The active sites of these enzymes are located in a deep cleft present at the interface between two domains, with the C-terminal domain providing specificity for UDP, as expected (Hefner and Stöckigt 2003; Hans and others 2004; Thorsøe and others 2005). The binding determinants for the acceptor substrates, in contrast, are primarily in the N-terminal domain. Our work with ZOG1/ZOX1 chimeras has shown that the preference for glucose or xylose is located in the N-terminal domain (Martin and others 2000).

We are interested in identifying amino acid residues of ZOG1 and ZOX1 important for zeatin binding. Here we use the *Medicago* GT structure to identify residues of ZOG1 potentially interacting with zeatin. Site-directed mutagenesis of *ZOG1* and some close relatives is then used to probe the importance of these residues in recognizing zeatin as a substrate. Sequence comparisons raise the possibility that zeatin GT activity has arisen independently in different plant families.

Materials and Methods

Isolation of *ZOG1* Homologs

ZOX1 from *Phaseolus vulgaris* was isolated by PCR and inverse PCR using primers based on *ZOG1* as previously described (Martin and others 1999a). To isolate related GTs, genomic DNA was extracted from *Glycine max*, *P. vulgaris*, and *P. lunatus* using the CTAB method (Doyle and Doyle 1990). The genomic DNA was digested with Sau 3A1, fractionated on a sucrose gradient to obtain fragments between 15–23 kb, and cloned with the Lambda FIX® II/Xho I Partial Fill-In Vector Kit following the manufacturer's instructions (Stratagene, La Jolla, CA). The genomic libraries were screened with ^{32}P-labeled *ZOG1*. DNA was isolated from positive plaques, digested, and *ZOG1* probe positive bands were identified and subcloned into Puc18 for sequencing. Primers with *Nco*I (forward) and *Xba*I (reverse) restriction sites were designed to clone the open reading frames (ORFs) from positive clones into pHT, a modified Ptrc99 vector (Amersham Biosciences, Piscataway, NJ) containing seven His residues at the N-terminus of the protein for expression analysis. Two genes from *G. max, GmGT1* (AF489873) and *GmGT2* (AF489874), two additional genes from *P. lunatus*, *PlGT2* (AF489876) and *PlGT3* (AF489877), and one additional gene from *P. vulgaris, PvGT2* (EF680377), were obtained. Two *Oryza sativa* ssp. *japonica* genes, *OsGT1* (CAD41646) and *OsGT2* (CAD41647), were identified by BLAST searches with ZOG1. The corresponding cDNAs were obtained from a cDNA library of rice leaves (kindly provided by Pioneer Hi-Bred International, Inc., Johnston, IA). Clones were sequenced and primers were designed to amplify the ORFs for insertion into the pHT vector. Two maize (*Zea mays*) *cisZOG* genes (*ZmcisZOG1* and *ZmcisZOG2*) were previously isolated and characterized (Martin and others 2001b; Veach and others 2003). The tomato (*Lycopersicon esculentum*) *LeGT1* gene (AY082661) was obtained by screening a λEMBL3 genomic library of tomato cultivar Rutgers (kindly provided by Dr. A. Theologis, UC Berkeley) with ^{32}P-labeled *ZOG1*. DNA was extracted from positive clones, digested with *Eco*RI and/or *Bam*HI, electrophoresed on a 1.1% Sea Plaque agarose gel, transferred to a membrane, and probed with ^{32}P-labeled *ZOG1*. DNA from bands corresponding to labeled fragments was purified and cloned into pUC18. The putative ORF was PCR amplified from genomic DNA and cloned into the pHT vector for expression analysis.

Western Blot Analysis

Selected proteins (0.1 μg) were separated by 12% SDS-PAGE and then transferred onto PVDF membrane by electroblotting, followed by probing with antibodies raised to ZOX1 according to the method described earlier (Martin and others 1999b). The immunoblots were labeled with horseradish-labeled anti-mouse IgG and bands were detected with enhanced chemiluminescence reagent (ECL). The chemiluminescence signal was recorded with GeneSnap image acquisition software (Syngene, Frederick, MD) and converted to a TIF file using CorelDRAW (Corel Corporation, Eden Prairie, MN).

Enzyme Models

Models of the zeatin-binding pocket were based on the 3D structure of *Medicago truncatula* UGT71G1 with UDP-Glc, PDB code 2ACW (Shao and others 2005), and were made using the PyMOL graphics program (DeLano 2002).

Site-Directed Mutagenesis

Plasmids of PHT *ZOG1*, *PlGT2*, and *ZOX1* were purified with a plasmid midi kit (Qiagen, Valencia, CA). The QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was utilized to synthesize the R54A and R59A single-site mutations by following the manufacturer's instructions. For synthesis of the following single-site-directed mutants of ZOG1 (Q55A, L58A, R59K, R59Q, E85A, D86A, D87A, D87E, D87N, L127A, L127F, and L127M), of *Pl*GT2 (T152F, CTC151SFS, and ΔPNN [84–86]), and of ZOX1 (QV142HS), plasmids were sent to Molecular Cloning Laboratories (South San Francisco, CA). Primer sequences are available in Supplementary Table 1. Plasmids were purified and sequenced to ensure that the correct mutations were present.

Overexpression and Purification of Proteins

One Shot® TOP10 *E. coli* cells were transformed with the plasmids according to kit instructions. Cells were grown at 37°C in modified 2×YT medium containing 16 g bacto-tryptone, 10 g bacto-yeast (both from BD Biosciences, Palo Alto, CA), and 5 g NaCl with the addition of 2 mM $MgSO_4$, pH 7.0, and 60 μg/ml carbenicillin (US Biological, Swampscott, MA) until cells reached an absorbance of $A_{600} = 0.4–0.6$. Cultures were induced with 0.4 mM isopropyl 1-thio-β-galactopyranoside (IPTG, US Biological) and grown overnight at 24°C. Cells were pelleted at 6,000 rpm at room temperature for 15 min and resuspended in one-half the original amount of modified 2×YT, 60 μg/ml carbenicillin, and 200 μg/ml chloramphenicol (Sigma, St. Louis, MO) to inhibit protein synthesis and allow protein refolding (Studier 2005). Cultures were grown an additional 2 h. Cells were pelleted at 5,000 rpm at 4°C for 15 min and either used immediately or frozen at −80°C.

Extraction and protein purification by immobilized metal affinity chromatography (IMAC) were performed according to Talon Metal Affinity Resin instructions (BD Biosciences), with the addition to the extraction buffer of 5 mM Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP, Pierce Biotechnology, Rockford, IL) as a reductant, 0.5 mM phenylmethanesulfonyl fluoride (PMSF, Sigma) and 1 µl/ml protease inhibitor cocktail (Sigma) as protease inhibitors, 1 µl/ml benzonase (Novagen, Madison, WI) as a nuclease, and 0.5 mg/ml lysozyme (Sigma) to lyse cells. After loading and washing the column, proteins were eluted with 500 mM imidazole (Sigma). Cross contamination of proteins during purification was avoided by using separate Talon columns for each mutated protein. After addition of 2.5 mM ethylenediaminetetraacetic acid (EDTA, Sigma), the eluate was concentrated with a 30 MWCO Centriprep filter (Millipore; Bedford, MA). Proteins were washed with 50 mM Tris (Sigma), pH 7.5, and reconcentrated with the Centriprep and then with a 50 MWCO Ultrafree filter (Millipore). Total protein concentration was assessed by measuring absorbance at 280 nm with a spectrophotometer (Beckman Coulter DU 800, Fullerton, CA). Typical yield was 0.5 mg/L culture. Although the purification yielded primarily the desired protein, minor contaminants still occurred and amounts varied from one mutant to another. For this reason, the specific protein concentration was determined by comparing ZOG1 or mutant bands with 0.1 µg BSA on a 12% SDS polyacrylamide gel.

Enzyme Assays and Analysis of Reaction Products

To determine enzyme activity, 0.1 µg of recombinant protein was incubated with 5 mM DTT, 5 mM UDP-Glc, 50 µM zeatin (all from Sigma), and 76 nCi [^{3}H]zeatin (OlChemIm, Olomouc, Czech Republic) in 150 µl at pH 7.5 (buffered with 55 mM Tris) at 30°C for 30 min. Following incubation, 18 µl of trichloroacetic acid (Sigma) was added and the mixture was centrifuged at 12,000 rpm for 2 min to precipitate proteins. Reaction products in the supernatant were separated by a Beckman model 110A dual-pump HPLC system with a reversed-phase C_{18} column (Ultrasphere ODS C_{18}, 5-µm particle size, 4.6 × 250 mm; Altrex, Alameda, CA). The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 4.8 with triethylamine (TEA, Sigma). Samples were eluted with an isocratic solution of 15% methanol (J.T. Baker) in TEA buffer with a flow rate of 1 ml/min and 1-ml fractions were collected. Radioactivity in these fractions was determined in Ecolite scintillation fluid (MP Biomedicals, Solon, OH) with a Beckman LS 7000 scintillation counter. Radioactivity in peaks was calculated after subtraction of background radioactivity. Data were obtained from two assays from each of two separate purifications. To determine if mutants with low or no activity in these assays had activity, an assay using ten times the amount of enzyme (1 µg protein) was performed. Two assays with proteins from different purifications were performed.

Sequence Identity Determinations and Phylogeny

Sequence identities and phylogeny were determined with the Genetics Computer Group package GCG9 (Madison, WI).

Results

Isolation and Characterization of Additional GTs

Thus far only two zeatin glycosyltransferases have been identified in legumes, the zeatin O-glucosyltransferase of *P. lunatus* and the zeatin O-xylosyltransferase of *P. vulgaris* (Martin and others 1999a,b). To discover more zeatin-specific legume GTs, we screened genomic libraries with *ZOG1* or *ZOX1* and PCR based on degenerative primers. This yielded two additional *P. lunatus* GTs, one from *P. vulgaris*, and two from *Glycine max*. The protein sequences are 70–85% identical (Figure 2A), but assays of the proteins showed that none had enzyme activity with zeatin and either UDP-Glc or UDP-Xyl as substrates. We presume that they have other, thus far unknown substrates. Interestingly, the monoclonal antibody to ZOX1 (Martin and others 1990) showed low recognition of these enzymes (Figure 2B).

A phylogenic tree was created with the bean and soybean GTs and some other related GTs (Figure 3A). Two zeatin N-glucosyltransferase and three O-glucosyltransferase genes were identified in the *Arabidopsis* genome (Hou and others 2004). We identified one additional *Arabidopsis* GT, At2g36790, with trace zeatin O-glucosyltransferase activity (0.01 nmol mg^{-1} s^{-1}). Some of the *Arabidopsis* GTs with low activity toward zeatin have other known substrates such as flavonoids (At2g36790) or brassinosteroids (At2g36800) (Jones and others 2003; Poppenberger and others 2006). Also shown in Figure 3A are two maize *cisZOG* genes (Martin and others 2001b; Veach and others 2003) and two related rice genes. The two maize enzymes prefer *cis*-zeatin over *trans*-zeatin, but the rice GTs and a tomato GT did not have any activity with *trans*- or *cis*-zeatin. The tomato GT has higher identity to ZOG1 (56%) than the most active *Arabidopsis* ZOG, At1g22400 (27%). Although the bean and soybean GTs are closely related and all cluster together on the phylogenic tree, the *Arabidopsis* zeatin O- and N-glucosyltransferases are only distantly related to this group of genes (Figure 3A). Interestingly, GTs of different

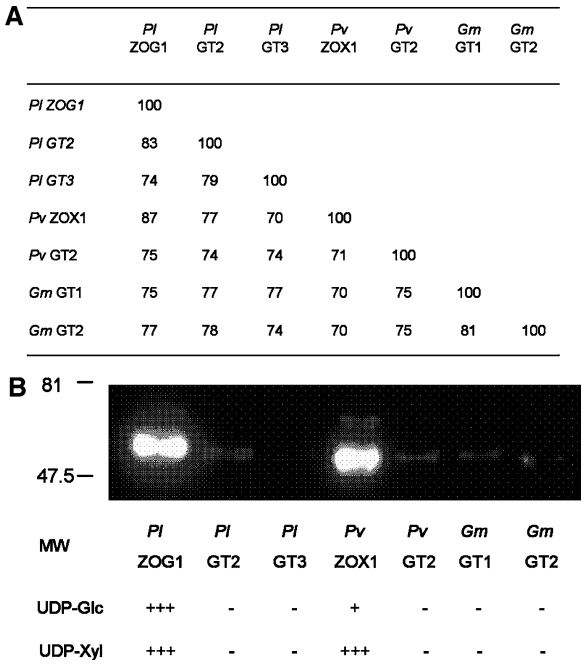

Fig. 2 Sequence relationships and antigenicity of legume GTs. (**A**) Amino acid identity of bean and soybean glucosyltransferases. (**B**) Western blot of bean and soybean GTs developed with a monoclonal antibody against *Pv*ZOX1. The strong antigenicity of *Pl*ZOG1 and *Pv*ZOX1 coincided with recognition of zeatin as substrate. *Gm*, *Glycine max*; *Pl*, *Phaseolus lunatus; Pv, P. vulgaris*

genera with the same or similar cytokinin substrates often show no close phylogenic relationship. Moreover, in each species or family there are several other GTs that are closely related to cytokinin GTs but have different substrates. Thus, overall sequence similarity is not a direct predictor of zeatin glycosylation activity.

Sequence Comparisons and Enzyme Modeling

Of the two published crystal structures for plant GTs belonging to Family 1, the triterpene/flavonoid glucosyltransferase of *M. truncatula* UGT71G1 (Shao and others 2005) showed 27% identity with ZOG1 over the entire sequence and that of *V. vinifera* VvGT1 (Offen and others 2006) aligned well only with the C-terminal portion of ZOG1. For this reason, we selected the *M. truncatula* enzyme as the better template for use in designing ZOG1 mutants.

At this low level of sequence similarity alignment details are unreliable and so, rather than create a specific homology model based on a single alignment, we viewed the *M. truncatula* GT structure to map out which regions of the N-terminal domain were involved in lining the acceptor binding pocket (Figure 4A). The UDP-Glc is totally enclosed in the back of the pocket, just above the catalytic His (H22) of the N-terminal domain. Removing the

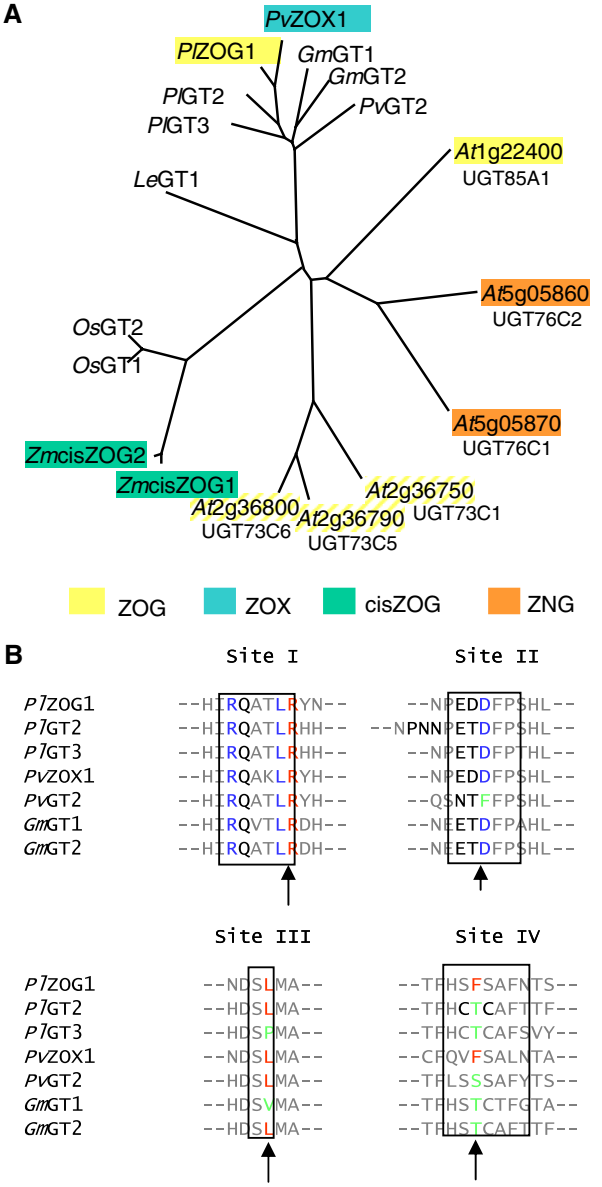

Fig. 3 (**A**) Phylogenic relationships between zeatin glycosyltransferases and closely related enzymes of bean, soybean, tomato, maize, rice, and *Arabidopsis*. Enzymes showing activity with cytokinins are highlighted in color. Zeatin O-glucosyltransferases (ZOGs) are highlighted in yellow and those with very low activity in hatched yellow; the zeatin O-xylosyltransferase (ZOX) in blue; *cis*-zeatin O-glucosyltransferases (cisZOGs) in green; and zeatin N-glucosyltransferases (ZNGs) in orange. (**B**) Sequence comparisons between the legume GTs at Sites I-IV. Amino acids essential for zeatin glycosylation by ZOG1 (as determined by complete loss of activity with zeatin as substrate by mutation to Ala) are highlighted in red; those of importance (as determined by partial loss in activity by mutation to Ala) in blue; those of little or no importance (no or slight loss of activity by mutation to Ala) in black. Amino acids differing from essential or important amino acids are indicated in green. Amino acids not included in site-directed mutagenesis are indicated in gray. *At*, *Arabidopsis thaliana*; *Le*, *Lycopersicon esculentum*; *Gm*, *Glycine max*; *Pl*, *Phaseolus lunatus; Pv, P. vulgaris*; *Os*, *Oryza sativa*; *Zm*, *Zea mays*

C-terminal domain reveals that the acceptor binding pocket is lined by four segments (I-IV) from the N-terminal domain (Figure 4B), and these sites were mapped onto the ZOG1 sequence (Figure 4C). The four ZOG1 segments that line the pocket are site I at the entry (RQATLR, ZOG1 54–59), site II just inside the opening and on the opposite wall (EDDFPS, 85–90), site III on the back pocket floor (SL 126–127)), and site IV on the back pocket wall (HSFSAFN, 147–153).

Characterization of Activities of ZOG1 Mutants

Within the four sites, we selected for mutagenesis nine residues that were conserved between ZOG1 and ZOX1, since both use zeatin as an acceptor: site I (R54, Q55, L58, R59), site II (E85, D86, D87), site III (L127), and site IV (F149). For the first round of mutations, all residues were changed to Ala, which is a substitution that shortens the side chain to a minimal CB atom without significantly changing the backbone conformational properties (Cunningham and Wells 1989). For follow-up mutations of those residues whose Ala substitution showed less than one-third of the wild-type activity, a series of more conservative substitutions were used to assess what aspect of the side chain was important for activity (for example, size or shape or charge). Arg was mutated to Lys (conserving its positive charge) or Gln (conserving its rough shape and length but not charge); Asp was mutated to Glu (conserving its negative charge) and Asn (conserving its shape but not its charge); Leu was mutated to Phe or Met (both conserving its nonpolar nature and rough size but with two somewhat different shapes); and Phe was mutated to Tyr (conserving its shape but adding a hydroxyl) and Leu (conserving its nonpolar nature and rough size but slightly varying its shape).

Each mutant behaved like wild-type ZOG1 during expression and purification, and protein yields were good. Conversion of zeatin to its glucoside was determined for wild-type recombinant ZOG1 and for each mutant (Table 1). The spiking with radiolabeled zeatin facilitated detection of very small amounts of reaction product. Five of the nine Ala mutants had greater than 45% of wild-type activity and these were not pursued further. At the other four positions three mutants (R59A, L127A, and F149A) had no activity and the fourth (D87A) had 27% of wild-type activity. Mutant enzymes showing no activity in these assays also had no activity when the amount of enzyme was increased tenfold and the specific activity of D87A was the same for assays with 0.1 or 1 μg enzyme.

The residues whose mutation strongly impaired the recognition of zeatin as a substrate, R59, D87, L127, and F149, were then mutated to amino acids that are more

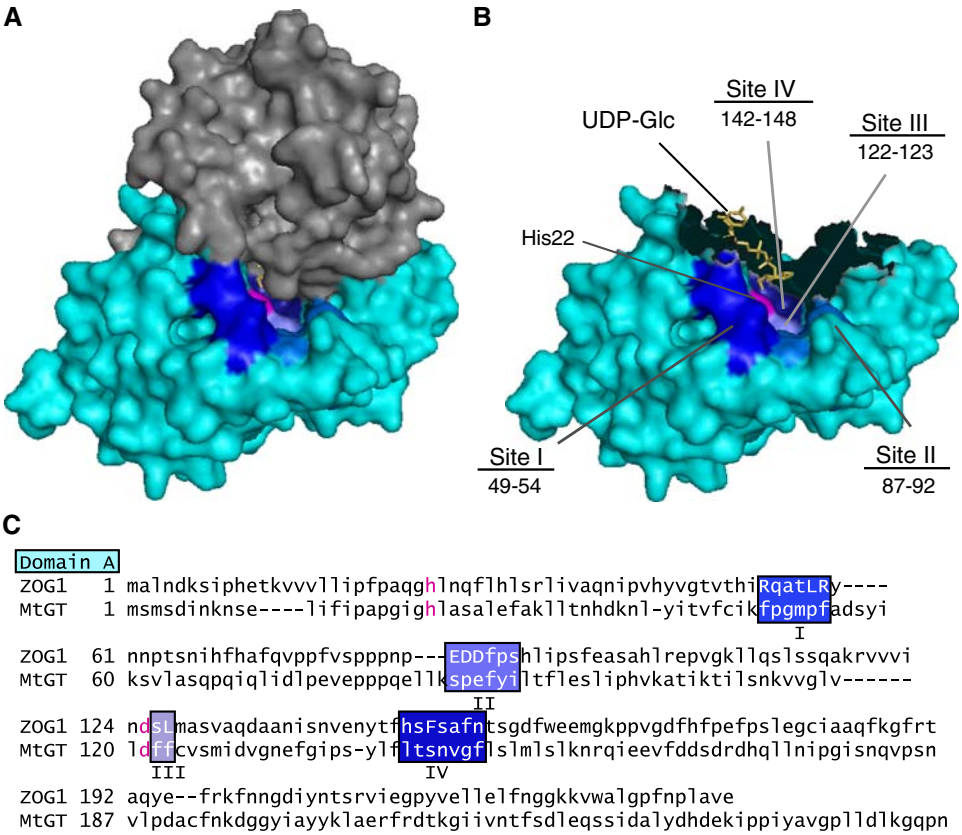

Fig. 4 Mapping acceptor-interacting regions of *M. truncatula* UGT71G1 (*Mt*GT) onto *P. lunatus* ZOG1. (**A**) Surface representation of the *Mt*GT structure shows how the N-terminal Domain A (residues 3–255; cyan) and C-terminal Domain B (residues 256–465; gray) surround a deep pocket at the bottom of which resides the glucose of UDP-Glc. (**B**) Removing Domain B allows identification of the four major segments from Domain A (labeled sites I–IV and shown in shades of blue) that contribute to the acceptor-binding pocket. The catalytic histidine (His22, magenta) and UDP-Glc (yellow stick figure) are also shown. (**C**) Alignment of the ZOG1 and UGT71G1 Domain A sequences highlighting recognition sites I–IV (blue) and the catalytic His22 and Asp121 (magenta). Panels A and B were created with PyMOL (DeLano 2002)

Table 1 Conversion of zeatin to O-glucosylzeatin

Enzyme	Mutation	Substrate conversion[a] (nmol mg^{-1} s^{-1})	% of ZOG1
*Pl*ZOG1		12.0 ± 1.6	100
	R54A	9.2 ± 0.2	76
	Q55A	12.3 ± 1.0	102
	L58A	6.3 ± 0.7	52
	R59A	0.0 ± 0.0	0
	R59K	1.3 ± 0.5	10
	R59Q	0.0 ± 0.0	0
	E85A	10.9 ± 1.0	91
	D86A	10.5 ± 1.0	88
	D87A	3.2 ± 0.4	27
	D87E	5.5 ± 0.7	46
	D87N	1.9 ± 0.4	16
	L127A	0.0 ± 0.0	0
	L127F	0.0 ± 0.0	0
	L127M	1.4 ± 0.4	11
	F149A	0.0 ± 0.0	0
	F149L	0.7 ± 0.1	6
	F149Y	1.0 ± 0.0	0
*Pl*GT2		0.0 ± 0.0	0
	Δ86–88	0.0 ± 0.0	0
	T152F	0.4 ± 0.0	3
	CTC151SFS	0.6 ± 0.2	5
*Pv*ZOX1		0.4 ± 0.1	3
	QV142HS	0.0 ± 0.0	0

Pl = *Phaseolus lunatus*; *Pv* = *P. vulgaris*

[a] Conversion was determined with 50 μM zeatin, 0.07 μCi [^{3}H]zeatin, 5 mM UDP-Glc, and 0.1 μg enzyme at 30°C for 30 min. Detection level ≈ 0.001 nmol zeatin. Assays of *Pl*GT3, *Pv*GT2, *Gm*GT1, and *Gm*GT2 had no conversion

similar in structure to the original amino acid (R59Q, R59K; D87N, D87E; L127F, L127M; and F149Y, F149L). For R59, activity was not restored by a mutation to Gln but was slightly restored by the more conservative Lys (10%). For D87, an Asn mutant had even lower activity, but the Glu mutant had activity restored to 46%. The mutation of L127 to Phe did not give activity, whereas mutation to Met gave slight activity (11%). Similarly, F149Y had no activity and F149L had slight activity (6%).

Site-Directed Mutagenesis of *Pl*GT2

Sequence comparisons among all bean/soybean GTs and ZOG1/ZOX1 with regard to the four residues identified as potential zeatin recognition elements (Figure 5B; Supplementary Figure 1) showed that they differ in one (*Pl*GT2 and *Gm*GT2) to three (*Gm*GT1) residues. To test whether

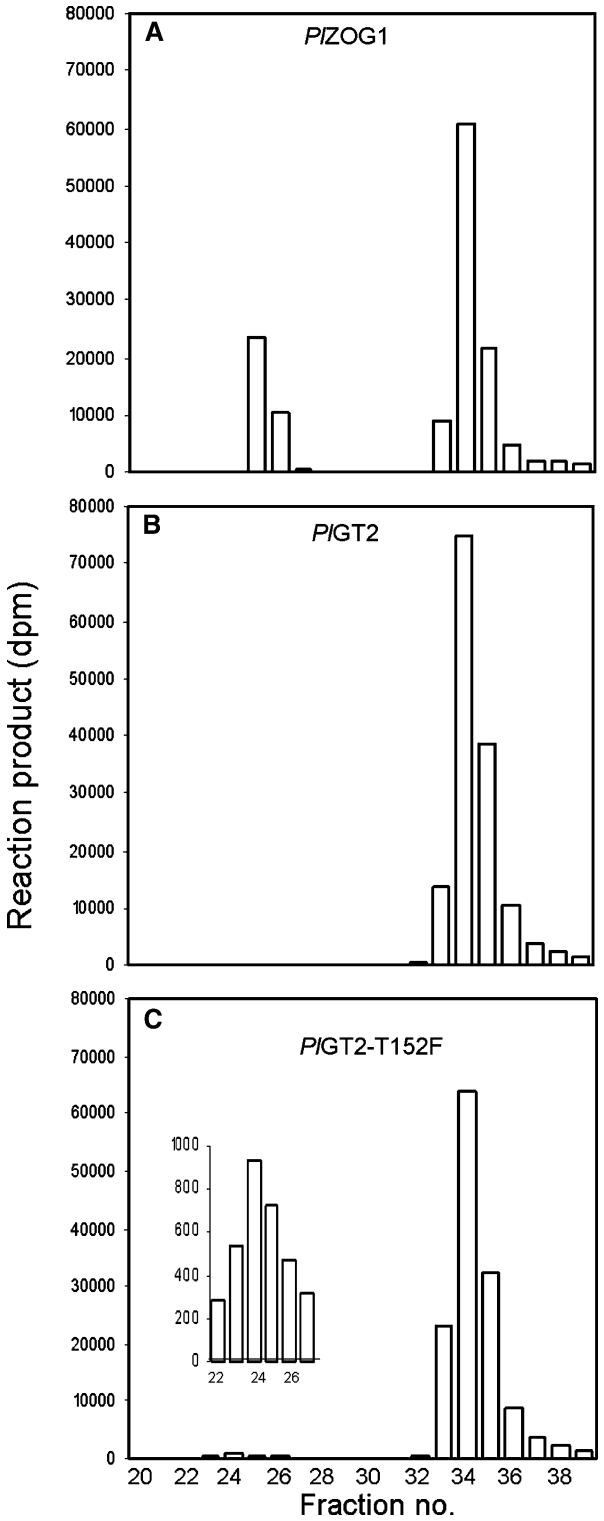

Fig. 5 Radioactivity in relevant fractions after fractionation of reaction mixtures by HPLC. (**A**) *Pl*ZOG1. (**B**) *Pl*GT2. (**C**) Mutant T152F of *Pl*GT2. The reaction mixture contained 50 μM zeatin, 0.07 μCi [^{3}H]zeatin, 5 mM UDP-Glc, and 0.1 μg enzyme. Zeatin eluted in fractions 33–37 and its O-glucoside in fractions 23–26

Table 2 Conversion of zeatin to O-xylosylzeatin

Enzyme	Mutation	Substrate conversion[a] (nmol mg^{-1} s^{-1})	% of ZOX1
ZOG1		10.1 ± 1.3	68
ZOX1		14.8 ± 0.1	100
	QV142HS	1.4 ± 0.2	9

[a] Conversion was determined with 50 μM zeatin, 0.07 μCi [^{3}H]zeatin, 5 mM UDP-Xyl, and 0.1 μg enzyme at 30°C for 30 min. Detection level ≈ 0.001 nmol zeatin. Assays of ZOG1 mutants R59A, D87A, L127A, and F149A, *Pl*GT2, *Pl*GT2 mutants T152F, CTC151SFS, and Δ86–88, *Pl*GT3, *Pv*GT2, *Gm*GT1, and *Gm*GT2 had no conversion

we could create a gain-of-function mutant of *Pl*GT2, T152 (corresponding to F149 of ZOG1) was changed to Phe to make it identical to ZOG1 in all four critical amino acids. Indeed, the mutated enzyme had low but measurable activity in assays with zeatin and UDP-Glc (Figure 5; Table 1). Changing the residues around T152 (CTC to SFS) had no further effect (Table 1). One other difference between ZOG1 and *Pl*GT2 is a three-residue (PNN) insertion in front of site II (Figure 3B; Supplementary Figure 1). Deletion of these three amino acids did not result in any activity. Specific activities were similar in assays with higher enzyme levels (1 μg).

Site-Directed Mutagenesis of ZOX1

ZOX1, which also has zeatin as the main substrate, is identical to ZOG1 in the four potential zeatin-interacting residues. In fact, sites I, II, and III are entirely identical. Site IV, however, differs between the two enzymes in a few amino acid residues (Figure 3B). The main distinction between the two enzymes resides in acceptance of sugar donor substrates, with ZOX1 having only very low glucosyltransferase activity (Tables 1 and 2). To determine whether the differences in site IV have any bearing on this sugar donor preference, the amino acids adjacent to F144 of ZOX1 (equivalent to F149 of ZOG1) were changed to resemble ZOG1 (QV142HS). This mutant expressed well but showed significant decreases in both glucosyltransferase (Table 1) and xylosyltransferase (Table 2) activities.

Discussion

Using the *M. truncatula* GT structure (Shao and others 2005) as a model for ZOG1 and ZOX1 (Figure 4), one can see that UDP-Glc is buried deeply between the N- and C- terminal domains and that the acceptor substrate zeatin sits in a tunnel created by the N-terminal domain. Unlike UGT71G1, an apparently promiscuous enzyme with broad acceptor specificity (Shao and others 2005; He and others 2006), ZOG1 glycosylates only the closely related *trans*-zeatin and *m*-topolin (Mok and others 2005) and is highly specific for the O-position of *trans*-zeatin. The high level of acceptor selectivity for ZOG1 implies a highly selective binding pocket for the acceptor.

To identify residues involved in zeatin recognition, we mutated residues in all four segments suggested by our modeling to contribute to the binding site. The good yields and the behavior of all expressed mutants provides evidence that all of the mutants studied folded well and did not have stability problems. The four residues we identified as important for activity (R59, D87, L127, and F149) are good candidates for zeatin-binding residues; alternatively, the mutations may have locally altered the protein structure so that a different catalytic or ligand-binding residue is not properly positioned. Although a loss of activity is generally easier to engineer, it is a gain in activity that is much more challenging and informative. That is why the gain of zeatin GT activity for two mutants of *Pl*GT2 (Table 1) that introduced a Phe in the T152 position provides stronger evidence that this group could be involved in the zeatin-binding site.

We isolated several other bean and soybean genes, each with very high identity to ZOG1, but none encoding enzymes with zeatin GT activity. These enzymes are clearly much more closely related to each other than to true zeatin GTs in *Arabidopsis*, rice, and corn, raising the interesting possibility that glucosylation of zeatin may have been acquired independently in different plant families. ZOG1 and ZOX1 belong to a GT group that diverges greatly from any of the 12 evolutionary groups of *Arabidopsis* GTs (Li and others 2001). Interestingly, the amino acid identity between ZOG1 and the major *Arabidopsis* zeatin O-glucosyltransferase, UGT85A1 (At1g22400) (Hou and others 2004), is quite low (27%), yet ZOG1 and UGT85A1 are identical in two of the putative zeatin-interacting residues, R59 and D87.

A double mutation in site IV of ZOX1 (QV142HS) to make it resemble ZOG1 not only failed to give an increase in glucosyltransferase activity, but it resulted in a significant loss in both glucosyltransferase and xylosyltransferase activity. This is consistent with these residues being in the zeatin-recognition region rather than the sugar-recognition region. Studies of ZOX1/ZOG1 chimeras mapped the difference in sugar donor acceptance between the two enzymes to amino acids 25–195 (Martin and others 2000), and additional work showed that the exchange of residues 134–195 of ZOG1 with those of ZOX1 was detrimental to enzyme activity (R. Martin, M.C. Mok, and D.W.S. Mok, unpublished results). Our present results indicate that the HS/QV difference is one factor contributing to this problem and suggest that further investigation of the sugar donor specificity should focus on residues 25–134.

In conclusion, this study identified four amino acids critical to activity of ZOG1. The importance of Phe149 was further supported by the gain-of-function mutation of *Pl*GT2. These results build a foundation for further studies in the understanding of zeatin GTs that we hope will eventually result in the ability to engineer guided alterations in the specificity of enzymes in this family. However, we realize the difficulty of identifying functions of selected residues and therefore we are pursuing crystallization trials for ZOG1 and ZOX1.

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