Determinants and Expansion of Specificity in a Trichothecene UDP-Glucosyltransferase from *Oryza sativa*

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ABSTRACT: Family 1 UDP-glycosyltransferases (UGTs) in plants primarily form glucose conjugates of small molecules and, besides other functions, play a role in detoxification of xenobiotics. Indeed, overexpression of a barley UGT in wheat has been shown to control *Fusarium* head blight, which is a plant disease of global significance that leads to reduced crop yields and contamination with trichothecene mycotoxins such as deoxynivalenol (DON), T-2 toxin, and many other structural variants. The UGT Os79 from rice has emerged as a promising candidate for inactivation of mycotoxins because of its ability to glycosylate DON, nivalenol, and hydrolyzed T-2 toxin (HT-2). However, Os79 is unable to modify T-2 toxin (T-2), produced by pathogens such as *Fusarium sporotrichioides* and *Fusarium langsethii*. Activity toward T-2 is desirable because it would allow a single UGT to inactivate co-occurring mycotoxins. Here, the structure of Os79 in complex with the products UDP and deoxynivalenol 3-O-glucoside is reported together with a kinetic analysis of a broad range of trichothecene mycotoxins. Residues associated with the trichothecene binding pocket were examined by site-directed mutagenesis that revealed that trichothecenes substituted at the C4 position, which are not glycosylated by wild-type Os79, can be accommodated in the binding pocket by increasing its volume. The H122A/L123A/Q202L triple mutation, which increases the volume of the active site and attenuates polar contacts, led to strong and equivalent activity toward trichothecenes with C4 acetyl groups. This mutant enzyme provides the broad specificity required to control multiple toxins produced by different *Fusarium* species and chemotypes.

Family 1 UDP-glycosyltransferases (UGTs) of plants are a large class of enzymes that modify their low-molecular weight substrates through the addition of a sugar moiety from activated co-substrates such as UDP-glucose. Typically, plant genomes contain a vast number of UGTs that can be recognized by the characteristic PSPG motif, a 44-amino acid sequence associated with the UDP-sugar binding site. For example, 107 UGT genes have been identified in the genome of *Arabidopsis* and 180 (in rice) or more genes are present in diploid crop plants. The range of substrates to which the carbohydrates are transferred by plant UGTs is enormous and includes endogenous flavonoids, terpenoids, phenolics, saponins, sterols, alkaloids, and plant hormones, where the modifications serve a wide range of biological functions from defense mechanisms to regulation and changes in bioactivity.

Structural studies have revealed that family 1 plant UGTs adopt the GT-B protein fold. This consists of an N-terminal domain and a C-terminal domain that each exhibit a Rossmann fold. The C-terminal domain forms the binding site for the UDP-sugar (donor), whereas the N-terminal domain contributes mostly to the target substrate (acceptor) binding site. The active site lies in a groove between the N-terminal acceptor and the C-terminal donor binding domains. Because of the structural constraints demanded by binding similar nucleotide sugar ligands, there is considerably more sequence similarity between family members in the C-terminal domain than in the N-terminal domain.

Many plant UGTs demonstrate broad substrate specificity even as they maintain more stringent regioselectivity. This attribute is almost a prerequisite for plant glycosyltransferases, because there are many more glycosylated secondary...
metabolites observed than there are glycosyltransferases encoded in their genomes.\textsuperscript{11,12} Broad specificity is also a desirable feature in the application of plant UGTs to medicinal biochemistry when these are capable of generating glycosylated drug products or those that are able to glycosylate xenobiotics. Thus, understanding substrate specificity in plant UGTs in an effort to change or broaden specificity could prove to be very useful as a means of glycosylating a wider range of desired substrates. However, prediction of biological targets or specificity has proven to be difficult and in general can be determined only through experimentation.\textsuperscript{12,13} Engineering of the acceptor binding pocket of plant UGTs has been shown to increase activity\textsuperscript{14} and alter regioselectivity.\textsuperscript{15,16} An exciting potential application for plant UGT engineering is the broadening of specificity of UGTs capable of glycosylating xenobiotics such as trichothecene toxins, which are virulence factors for \textit{Fusarium} head blight (FHB) in cereal crops.\textsuperscript{17,18}

Toxicogenic \textit{Fusarium} species cause a significant number of crop diseases worldwide\textsuperscript{19,20} and lead to crop loss and contamination with a variety of mycotoxins, including the trichothecenes shown in Figure 1. Trichothecene mycotoxins are a highly diverse group of tricyclic sesquiterpenoid epoxides that serve as virulence factors of plant pathogenic \textit{Fusarium} species but are also toxic to both humans and livestock.\textsuperscript{17,18,21,22} A primary cause of toxicity is the inhibition of protein biosynthesis by targeting the eukaryotic ribosome.\textsuperscript{23,24} Glycosylation of deoxynivalenol (DON), one of the most common trichothecenes, reduces its bioavailability and toxicity.\textsuperscript{25,26} As a consequence, UDP-glycosyltransferases have emerged as a promising strategy for controlling FHB in cereal crops.\textsuperscript{27–29} UGTs with specificity toward DON have been identified in \textit{Arabidopsis thaliana}, barley, \textit{Brachypodium distachyon}, rice, and sorghum.\textsuperscript{25,30–33} Even so, there is a need for enzymes with broader or altered specificity because a wide range of trichothecenes are produced by the various \textit{Fusarium} species that cause diseases on small grain cereals, maize, or potato worldwide. To generate such enzymes, a more complete understanding of the structural and enzymatic features that control specificity and activity in UGTs is required. This study focuses on the family 1 plant UGT Os79 (Os04g0206600) from \textit{Oryza sativa} with the goal of understanding the structural determinants for specificity and expanding the number of substrates that it can accept.

The UGT Os79 already exhibits substantial transferase activity toward DON, nivalenol (NIV), isotrichodermol (IsoT), and HT-2 toxin (HT-2), but not to C4-acetylated compounds like T-2 toxin (T-2), DAS, 4-ANIV, or 4,15-diANIV (Figure 1). Recent structural studies of this enzyme with trichothecene bound in the active site provided insight into the general position of the substrate because it defines the approximate location of the acceptor binding site.\textsuperscript{34} However, it also left some uncertainty about how the other substrates bind in the active site because it represents a minimal inhibitor complex. The compound trichothecene is an inactive substrate because it lacks the 3-hydroxyl group that accepts the glucosyl moiety. It is also the most hydrophilic and least substituted member of the trichothecene family of mycotoxins. It is much smaller than other substrates such as HT-2 toxin. As such, it was unclear what structural features account for the wide specificity of Os79, and in particular why HT-2 is a substrate whereas T-2 is not, where the only difference is the absence of the C4 acetyl group in HT-2. To address these questions, we determined the structure of the product complex of Os79 with deoxynivalenol 3-O-glucoside (D3G) and UDP together with kinetic measurements on a series of closely related substrates that distinguish the effects of substitutions at positions 4, 8, and 15 of the trichothecene skeleton (Figure 1). The structural determinants for specificity that became apparent from these observations were tested by site-directed mutagenesis that yielded an enzyme with specificity considerably broader than that of the wild-type enzyme as this list now includes T-2, 4-ANIV, and DAS. This was accomplished without greatly compromising the activity toward the initial substrates and attests to the functional plasticity of this active site. In addition, the role of an absolutely conserved Thr/Ser in the GT-1 family of glycosyltransferases that coordinates the \(\beta\)-phosphate oxygen of UDP was examined to understand its role in substrate orientation in the active site.

**EXPERIMENTAL PROCEDURES**

**Trichothecenes.** Diacetoxyscirpenol (DAS), deoxynivalenol (DON), T-2 toxin (T-2), HT-2 toxin (HT-2), isotrichodermol (IsoT), trichothecene, 4-acetyl nivalenol (4-ANIV), 4,15-diacetyl nivalenol (4,15-dianIV), and nivalenol (NIV) used in this study (Figure 1) were obtained from the USDA-ARS, Mycotoxin Prevention and Applied Microbiology Research Unit, Peoria, IL. Deoxynivalenol 3-O-glucoside (D3G) was enzymatically produced and purified via high-performance liquid chromatography as previously described.\textsuperscript{13}

![Figure 1. Trichothecene mycotoxins examined in this study. The structures of T-2 toxin, DON, and DAS are shown explicitly. NIV, 4-ANIV, 4,15-diANIV, and HT-2 toxin differ from T-2 toxin or DON at the C15 and C4 positions as illustrated.](image-url)
Site-Directed Mutagenesis. The construction of the plasmid containing the Os79 wild-type gene used in this study was described previously. Briefly, the plasmid is a derivative of pET21 containing His6-tagged maltose binding protein (MBP) followed by a TEV protease cleavage site and the codon-optimized Os79 WT gene. Mutants of Os79 were generated using a single primer, polymerase chain reaction-based method based on “QuikChange” mutagenesis. The following mutagenesis primers were used: T291V, S′-GGTT-TTGGTGTCATACCGAGTGTCTCTTCTTTTCTGATTT-GCTTAAAC-3′; T291S, S′-GGTTTTGGTGTCATACCGGA-GGTTTTCTACTTTTTGATTTGCTAAAC-3′; Q202A, S′-TCTTCGCTTCTTCTGTGAGGAGAAGT-3′; Q202L, S′-TCTCCGCTTCTGTGAGGAGAAGT-3′; Q202E, S′-TCTCCGCTTCTGTGAGGAGAAGT-3′; F199Q, S′-CTT-CAACCTCTCCTGGCGCCATGTGCTGTGGAC-3′; H122A/L123A, S′-AGAQTGTTTTCATCTGACCTACCCCTATGCTAATGATGATGCTG-3′; S203A, S′-AGAGTTTTAGTCTACGATCCAGCGGCCATGTGCTGTGGAC-3′; H122A/L123G, S′-AGAQTGTTTTCATCTGACCCCTATGCTAATGATGATGCTG-3′; S203L, S′-GCTCTTCTTGAGGACATTTGAGAGCCAAGTGCTG-3′; S203A, S′-GCTCTTCTTGAGGACATTTGAGAGCCAAGTGCTG-3′. All mutations were verified by DNA sequencing using BigDye protocols (ABI PRISM). Reaction mixtures were resolved by the University of Wisconsin—Madison Biotechnology Center.

Expression of Os79 Mutants. Os79 mutants were overexpressed in Escherichia coli strain BL21 Codon Plus (DE3). Cultures from a single colony were used to inoculate 6 L of Lysogeny broth (LB) supplemented with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. Expression of Os79 was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside when cultures reached an OD600 of ∼0.8. Induction was performed at 16 °C for 20 h. Cells were harvested by centrifugation at 40000 rpm in a 59.4 Å, 32% MEPEG 5K, and 5 mM UDP-glucose and vitriplunged into liquid nitrogen. Os79 crystallized in space group P3121 with one chain in the asymmetric unit and the following unit cell dimensions: a = 104.5 Å, b = 104.5 Å, and c = 98.3 Å.

Crystallography of Os79 T291V-UDP. Os79 T291V-UDP was screened for initial crystallization conditions as described above. Single, diffraction quality crystals were grown at 4 °C by hanging drop vapor diffusion by mixing 2 μL of 12 mg/mL Os79 in 10 mM HEPES (pH 7.6), 50 mM NaCl, 30 mM D3G, and 5 mM UDP-glucose with 2 μL of a well solution containing 100 mM sodium acetate (pH 5.0), 40% pentaerythritol propoxylate 426, and 320 mM NaCl. Hanging droplets were nucleated after 24 h from an earlier spontaneous crystallization event using a cat’s whisker. Crystals grew to approximate dimensions of 100 μm × 100 μm × 300 μm within 15 days. The crystals were transferred to a cryoprotecting solution that contained 100 mM sodium acetate (pH 5.0), 40% pentaerythritol propoxylate 426, 320 mM NaCl, 30 mM D3G, and 5 mM UDP and vitriplunged into liquid nitrogen. Os79 crystallized in space group P3,21 with one chain in the asymmetric unit and the following unit cell dimensions: a = 104.5 Å, b = 104.5 Å, and c = 98.3 Å.

Crystallography of Os79 Q202A-UDP. Os79 Q202A was screened for initial crystallization conditions as described above. Single, diffraction quality crystals were grown at 4 °C by hanging drop vapor diffusion by mixing 2 μL of 11 mg/mL Os79 Q202A in 10 mM HEPES (pH 7.6) and 5 mM UDP-glucose with 2 μL of a well solution containing 100 mM sodium acetate (pH 5.0), 40% pentaerythritol propoxylate 426, 320 mM NaCl, and 30 mM D3G and vitriplunged into liquid nitrogen. Os79 crystallized in space group P3,21 with one chain in the asymmetric unit and the following unit cell dimensions: a = 104.5 Å, b = 104.5 Å, and c = 98.3 Å.

Crystallography of Os79 T291V-UDP. The Os79 T291V-UDP protein complex was screened for initial crystallization conditions as described above. Single, diffraction quality crystals were grown at 23 °C by hanging drop vapor diffusion by mixing 2 μL of 10 mg/mL Os79 T291V in 10 mM HEPES (pH 7.6), 50 mM NaCl, and 5 mM UDP-glucose with 2 μL of a well solution containing 50 mM HEPES (pH 7.5) and 32% MEPEG 5K. Hanging droplets were nucleated after 24 h from an earlier spontaneous crystallization event using a cat’s whisker. Crystals grew to approximate dimensions of 75 μm × 75 μm × 300 μm within 11 days. The crystals were transferred to a cryoprotecting solution that contained 50 mM HEPES (pH 7.5), 32% MEPEG 5K, and 5 mM UDP-glucose and vitriplunged by
being rapidly plunged into liquid nitrogen. Os79 crystallized in space group $P_2_1_2_1_2_1$ with one chain in the asymmetric unit and the following unit cell dimensions: $a = 59.4$ Å, $b = 83.2$ Å, and $c = 98.7$ Å.

### Crystallization of Os79 H122A/L123A

Os79 was screened for initial crystallization conditions as described above. Single, di-screens for initial crystallization conditions as described above.

UDP with 2$\mu$M Os79 in 10 mM HEPES (pH 7.6), 50 mM NaCl, and 5 mM UDP grew to approximate dimensions of 75 $\times$ 75 $\times$ 75 Å.

A spontaneous crystallization event using a cat's whisker. Crystals grew to approximate dimensions of $75 \mu m \times 75 \mu m \times 300 \mu m$ within 4 days. The crystals were transferred stepwise to a cryoprotecting solution that contained 50 mM HEPES (pH 7.0), 20% methyl ether polyethylene glycol 2000, 15% glycerol, and 5 mM UDP and vitri-$\mu$N70 by being rapidly plunged into liquid nitrogen. Os79 crystallized in space group $P_2_1_2_1_2_1$ with one chain in the asymmetric unit and the following unit cell dimensions: $a = 59.4$ Å, $b = 83.2$ Å, and $c = 98.7$ Å.

### Data Collection and Refinement

**X-ray data for the Os79 structures were collected at 100 K on Structural Biology Center beamline 19BM at the Advanced Photon Source in Argonne, IL. Diffraction data were integrated and scaled with HKL3000.**

Data collection statistics are listed in Table 1. The structures were determined by molecular replacement using coordinates from the Protein Data Bank (PDB entry STME) as the molecular replacement search model in Phaser. Final models were generated by alternating cycles of manual building and least-squares refinement using Coot, Phenix, and Refmac.

### Glucosyltransferase Enzymatic Assays

steady-state kinetic analyses of Os79 mutants with trichothecene substrates were performed in a coupled-continuous enzymatic assay at 23 °C in a 1 cm path length cuvette. Reactions were initiated by the addition of varying volumes of trichothecenes to a master mix containing Os79 (final concentration of 3.0 μM/mL), 3 units of rabbit muscle lactate dehydrogenase, 2 units of rabbit muscle pyruvate kinase, Lactic acid (Sigma-Aldrich, buffered aqueous glycerol solution, 1.5 mM phoshonoepipruvate, 100 μM β-NADH, 50 mM KCl, 10 mM MnCl$_2$ and 100 mM glycyglycine (pH 8.0) to yield a final volume of 100 μL. Lactic dehydrogenase, pyruvate kinase, phosphonoepipruvate, β-NADH, Os79, and UDP-glucose were added to a master mix containing the remaining reaction components prior to the initiation of each reaction. Reaction progress was followed by monitoring the decrease in A$_{340}$ caused by the oxidation of β-NADH. The rates of reaction were determined at various trichothecene concentrations and fit by nonlinear regression to the Michaelis–Menten equation using GraphPad Prism. In several cases, LC-HR-MS and LC-HR-MS/MS (Agilent 6550 iFunnel QTOF) were used to confirm the identity of the glycosylated product. In these cases, the assay that was used was identical to the coupled assay described above with the exception that none of the components for the coupling system were added. The reaction mixtures were filtered in centrifugal filters (Amicon) with a 30000 nominal molecular weight limit, lyophilized, and analyzed by LC-MS/MS.
Results and Discussion

The goal of this study was to identify the structural determinants that define acceptor specificity for the wild-type (WT) trichothecene UDP-glucosyltransferase from O. sativa, Os79, and test the resultant hypotheses through a combination of structural and kinetic studies of mutant proteins (Tables 1 and 2). As described, this study shows that the wild-type enzyme intrinsically exhibits broad specificity, without a great loss of catalytic efficiency. As part of this study, the role of a serine/threonine in the binding site, which serves both of these roles.

Table 2. Steady-State Kinetic Constants for WT Os79 and Selected Amino Acid Substitutions with a Variety of Trichothecene Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat (s⁻¹)</th>
<th>Kcat (µM)</th>
<th>kcat/Km (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>DON 1.07 ± 0.04</td>
<td>61 ± 6</td>
<td>1.75 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>T-2 toxin b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>HT-2 toxin 0.85 ± 0.02</td>
<td>22 ± 2</td>
<td>3.86 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>DAS b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>IsoT 0.79 ± 0.02</td>
<td>&lt;1.5</td>
<td>5.27 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>NIV 0.38 ± 0.02</td>
<td>35 ± 6</td>
<td>1.09 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>4-ANIV b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>4,15-diANIV b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>A384S DON 1.6 ± 0.07</td>
<td>78 ± 13</td>
<td>2.05 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>F199Q DON b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>Q143A DON b</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>Q202A DON 0.88 ± 0.04</td>
<td>77 ± 16</td>
<td>1.14 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>Q202E DON 2.4 ± 0.3</td>
<td>1072 ± 257</td>
<td>2.24 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>Q202L DON 0.8 ± 0.01</td>
<td>13 ± 1.7</td>
<td>6.15 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>S203L DON 3.8 ± 0.2</td>
<td>612 ± 67</td>
<td>6.21 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>S203A DON 0.77 ± 0.03</td>
<td>57 ± 10</td>
<td>1.33 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>H122A/L123G DON 0.50 ± 0.05</td>
<td>2512 ± 361</td>
<td>1.99 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>T-2 toxin 0.26 ± 0.02</td>
<td>512 ± 90</td>
<td>5.08 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>H122A/L123A T-2 toxin 1.58 ± 0.11</td>
<td>926 ± 116</td>
<td>1.71 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>H122A/L123A/ T-2 toxin DON 0.89 ± 0.08</td>
<td>1202 ± 220</td>
<td>7.40 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>Q202A 4-ANIV 1.06 ± 0.04</td>
<td>501 ± 48</td>
<td>2.11 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>T-2 toxin 2.53 ± 0.10</td>
<td>89 ± 11</td>
<td>2.84 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>DAS 1.78 ± 0.03</td>
<td>49 ± 3</td>
<td>3.63 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>H122A/L123A/ 0.85 ± 0.01</td>
<td>261 ± 9</td>
<td>3.26 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>Q202L 4-ANIV 0.96 ± 0.03</td>
<td>88 ± 11</td>
<td>1.09 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>T-2 toxin 0.91 ± 0.02</td>
<td>118 ± 10</td>
<td>7.63 × 10³</td>
</tr>
</tbody>
</table>

The His 27–Asp 120 catalytic dyad serves as a general base that accepts the proton from the 3-hydroxyl of the trichothecene acceptor as the nucleophilic oxygen attacks the anomeric C1′ atom of glucose. Thr 291 plays a critical role in positioning the phosphate and possibly protonating it before the release of UDP. It is possible that Thr 291 serves both of these roles.

As seen in Figure 2A, when the hydroxyl of Thr 291 is replaced with a methyl group in Os79 T291V, the phosphate of UDP adopts a substantially different position in the active site compared to that seen in WT Os79. In the WT Os79 structure, there is a hydrogen bond between the threonine hydroxyl and a phosphate oxygen atom that is ~2.5 Å long. This is clearly lost when threonine is replaced by a valine. The change in the position of UDP is likely a direct result of the substitution because crystals of Os79 T291V and WT protein structures were grown at the same pH under very similar crystallization conditions.

If this interaction is important, then it should be present in all plant GT-1 glycosyltransferases. Indeed, this appears to be true. Comparison of the structures of six plant UGT structures that have been determined with UDP bound in their active sites reveals that the presence of a hydroxyl moiety within hydrogen bonding distance of a β-phosphate oxygen is a conserved characteristic in all of these enzymes (Figure 2B). The proteins were aligned by superimposing an α-helix and two β-strands that surround the UDP binding site, because the overall architecture of these proteins varies substantially at the periphery of the protein, away from the UDP binding site. These three secondary structural elements that underlie the framework for the UDP binding site are very similar across the six proteins and align with an average rmsd of 0.30 Å for the α-carbons. In every case, there is a threonine or serine hydroxyl positioned within 3.0 Å of the phosphate oxygen. The hydroxyl-
containing residues in each of the six structures are as follows: S277 in 2VG8, S277 in 2VCH, S282 in 3HBF, S282 in 3HBJ, T280 in 2C1X, T280 in 2C9Z, and T291 in Os79.44−46 As shown in Figure 2B, in every structure the phosphate adopts almost exactly the same orientation. This provides further evidence that the change in the orientation of the phosphate of UDP in the T291V structure is caused by the lack of a hydroxyl group and the inability to form a hydrogen bond. This serine or threonine residue has not been identified as a catalytically critical residue in any publication about plant UGTs to date. Here, we demonstrate that it is critical for orienting the phosphate in the active site. While this does not preclude it from participating as a catalytic acid, it does confirm a structural role for this side chain. This is likely a broad finding that applies to many, if not all, plant UGTs. With the question regarding the role of Thr 291 in the catalytic mechanism of Os79 resolved, understanding the substrate specificity for Os79, and how it might be broadened, was the next goal of this study.

Steady-State Kinetic Parameters for WT Os79. Many plant UGTs demonstrate broad substrate specificity, a useful characteristic when considering enzymes that glycosylate xenobiotics. It was previously reported that Os79 exhibits broad specificity and can glycosylate DON, HT-2, IsoT, and NIV but not T-2.34 T-2 is commonly produced by Fusarium species in Europe, Asia, Africa, and Australia.47−51 Given the importance of detoxifying T-2, expanding the specificity of Os79 to include this trichothecene and others would be of great benefit. Given that the only difference between T-2 and HT-2 toxin is the C4 acetyl group that is present on T-2 in place of the hydroxyl on HT-2, it was hypothesized that this acetyl

Figure 2. Stereo comparison of UDP binding in six plant UGTs and the role of Thr 291 in positioning the UDP phosphate. (A) Electron density for UDP in the T291V structure (PDB entry 6BK1) and comparison with the WT Os79 structure (PDB entry STME). Three segments of Os79 T291V (blue) were aligned with WT Os79 (yellow) (rmsd of 0.06 Å). In the absence of the hydroxyl of Thr 291, the phosphate moiety adopts a different position in the active site. The density map was calculated with coefficients of the form F_o − F_c, where UDP was omitted from the final refinement. The map was contoured at 3σ. (B) The same three segments surrounding the UDP binding site (shown in ribbon representation) from six plant UGTs (PDB entries 2C9Z,46 2C1X,46 3HBJ,16,45 3HBF,45 2VCH,44 and 2VG844) were aligned with the corresponding sections of wild-type Os79 (yellow) (PDB entry STME) with an average rmsd of 0.30 Å for the structurally equivalent α-carbons. A loop near the terminal phosphate is shown with the conserved serine or threonine residue shown in stick representation (Thr 291 in Os79). The hydroxyl of this conserved residue is on average 2.7 Å from the oxygen of the β-phosphate. Figures 2−5 were prepared with Pymol.53
group prevented T-2 from binding in the active site of Os79. It can then be predicted that 4-acetyl nivalenol (4-ANIV), commonly known as FUS-X, will not be a substrate for Os79. 4-ANIV is acetylated at the C4 position but lacks the C15 acetyl and C8 isovaleryl groups of T-2 (Figure 1). Indeed, glycosylation could not be detected with our coupled assay with 4-ANIV, 4,15-diANIV, or DAS. This confirms that the 4-acetyl group is responsible for precluding T-2, 4-ANIV, 4,15-diANIV, and DAS from the acceptor binding pocket. To gain insight into the structural aspects of the acceptor binding pocket that contribute to its inability to accommodate an acetyl group at the C4 position, the structure of Os79 with the glycosylated trichothecene product D3G in the active site was determined.

**Structure of Os79 in Complex with UDP and D3G.** Os79 was crystallized in the presence of the product D3G in an effort to understand the structural components that are responsible for substrate specificity and to gain insights into the nature of trichothecene binding in the acceptor pocket. The structure of Os79 in complex with UDP and D3G was determined to 2.17 Å resolution (PDB entry 6BK3) (Table 1). The overall structure is very similar to the previously determined structure of Os79 with trichothecene (TRI) and UDP-2-fluoro-2-deoxy-D-glucose (U2F) bound in the active site with an rmsd of 0.7 Å for structurally equivalent α-carbons. Electron density corresponding to DON and UDP was observed; however, there was no clear electron density corresponding to the glucose moiety of D3G, and the moiety was not modeled (Figure 3A). This is likely the result of the flexibility of the glucose moiety.

There are two major conformational changes in Os79 with D3G bound compared to the structure with trichothecene bound in the active site (Figure 3B). A loop from Ser 288 to Val 297 is shifted 9.5 Å away from the active site, and the region that extends from Trp 316 to Lys 336, which is composed of a loop and two short α-helices, is shifted 5.9 Å away from the active site. These conformational changes highlight the flexibility of the acceptor binding region of the protein and may provide insights into the structural basis for UDP release after the donor sugar has been transferred. Aligning the structures with TRI or D3G bound in the active site reveals that the trichothecene skeleton of DON is rotated ∼45° compared to the backbone of TRI. This change is likely the result of the presence of glucose on C3 and would suggest that the trichothecene backbone rotates in the acceptor binding pocket after the reaction is completed and before the glycosylated product is released. It is likely that the orientation of trichothecene is more representative than D3G of the positioning of DON in the active site prior to glycosylation. Using the orientation of trichothecene as a reference, His 122, which is only 4.2 Å from C4, was identified as a residue that could clash with the C4 acetyl of the substrates that WT Os79 is unable to glycosylate. With the structural information as a guide, the role of His 122 and other residues identified as potential contributors to the specificity of Os79 was investigated by kinetic analysis.

**Steady-State Kinetic Assays of Os79 Mutants.** The remarkable substrate plasticity of Os79 prompted the question of whether there are critical residues in the trichothecene binding pocket that facilitate this ability. It also raised the question of whether it might be possible to expand the substrate specificity to accommodate trichothecenes with large substituents such as acetyl groups at the C4 position.
Examination of the structures with trichothecene and D3G suggested seven residues that might influence binding (Figure 4). Three of these seven side chains, Phe 199, Gln 202, and Ser 203, are located on an α-helix that forms the back of the acceptor binding pocket as presented in Figure 4. His 122 and Leu 123 are on a loop located at the bottom right of the binding pocket. Gln 143 and Ala 384 are on two separate loops on the back left side and the upper left side of the binding pocket, respectively. Steady-state kinetic constants were determined for eight mutant proteins, including single changes and combinations thereof (Table 2). The effect of each of these mutations is discussed below.

Role of Gln 202. Gln 202 is located on an α-helix at the back of the acceptor binding pocket, as presented in Figure 4. In our proposed structural model of DON binding in the acceptor pocket, the carboxamide oxygen of Gln 202 is within hydrogen bonding distance (2.3 Å) of the C7 hydroxyl of DON (Figure 4). To examine whether this residue contributes to specificity, it was changed to a glutamate, alanine, or leucine. The $K_M$ value of Os79 Q202E is 17.5-fold higher than the $K_M$ of WT with DON as a substrate, which is accompanied by a small increase in $k_{cat}$. The Q202E substitution maintains a similar residue size at the 202 position but adds a negative charge. It is difficult to predict the molecular origin of the kinetic effect of this electrostatic change without knowing the on rate or the off rate for the substrate as both of these enter into the definition of $K_M$. The Q202E substitution may still contribute a hydrogen bond to the enzyme–substrate complex. To test whether there is a hydrogen bond that plays a role in DON binding between this hydroxyl and Gln 202, the kinetic constants for Os79 Q202A were determined. There is no significant difference in the $K_M$ value of Os79 Q202A compared to that of the WT (Table 2), indicating that either Gln 202 does not play a role in DON binding or that a water molecule can substitute for the side chain. Interestingly, the Q202L substitution decreases the $K_M$ for DON by 4.7-fold without a major change in the value of $k_{cat}$ (Table 2), which is in contrast to the increase generated by the Q202E mutation. Together, these substitutions emphasize the impact that substitutions at position 202 can have on substrate and product binding. In summary, these mutations suggest that a polar interaction in this position is not important for activity and that an increased charge is detrimental.

Role of Phe 199. Phe 199 is located in the α-helix one helical turn from Gln 202, lies at the top of the active site as shown in Figure 4, and makes a substantial contribution to the primarily hydrophobic acceptor binding pocket. The side chain is ~5 Å above the hydrophobic trichothecene backbone. Changing this residue to glutamine maintains a residue with approximately the same volume but with much greater polarity. The introduction of the polar glutamine in the place of the hydrophobic phenylalanine eliminates enzymatic activity as measured in the coupled-continuous enzymatic assay with DON as a substrate. This, along with the fact that this residue is a conserved Phe in all UGTs that have activity toward DON, highlights the importance of this residue as a component of the acceptor binding pocket.

Role of Gln 143. Gln 143 is located on a loop at the lower left side of the acceptor binding pocket as shown in Figure 4. The Oε atom of Gln 143 is 3.5 Å from the C6 oxygen of the glucose moiety on U2F. Even though this is somewhat on the long side for a substantial hydrogen bond, this side chain appears to play an important role in substrate binding. The Q143A substitution does not demonstrate activity in the coupled-continuous enzymatic assay with DON as a substrate. This observation is consistent with the suggestion by Hiromoto et al. that the structurally equivalent residue in UGT78K6 (Asn 137) is important in donor recognition.52

Role of Ser 203. Ser 203 is located at the bottom of the α-helix in the back of the acceptor binding pocket adjacent to Gln 202. This side chain was changed to alanine to investigate the role of a polar residue in this position. The $K_M$ value of the S203A mutant is similar to that of WT Os79. To determine if a small residue is important at this position, the S203L substitution was created. There is a 10-fold increase in the $K_M$ of the S203L mutant but no change in $k_{cat}$ (Table 2), indicating that a bulkier residue at this position might hinder DON association or dissociation of DON from the active site. The hydroxyl of Ser 203 is 6.7 Å from the closest carbon of DON (C4), and given that the change to an alanine has little effect, it appears unlikely that it interacts directly with the substrate. It is more likely that a small residue is required at position 203 to allow Gln 202 to maintain a productive orientation. The S-fold
protein as measured by an end point assay and analyzed by LC-MS/MS as described in Experimental Procedures. These were to toxin as a substrate and 1.99 × 10² s⁻¹ M⁻¹ and 2512 μM, respectively, with DON as a substrate (Table 2). The activity of Os79 Q202 toward T-2 toxin as a substrate was not detected with the assay used in this study, possibly due to a very high Kₘ. Os79 H122A/L123G was largely insoluble. On the basis of these findings, Os79 H122A/L123A was expressed and purified. Changing Leu 123 to an alanine instead of glycine improved the solubility of the protein. The catalytic efficiency of the H122A/L123A mutant with with T-2 as a substrate is 1.17 × 10³, which is a 3.5-fold increase over that of the WT. The Kₘ value of 926 μM is similar to that of the H122A/L123G mutant. To improve the Kₘ, the H122A/L123A substitutions were combined with the Q202A substitution to make Os79 H122A/L123A/Q202A. The Kₘ of the triple mutant with T-2 as a substrate is 89 μM, and the catalytic efficiency is 2.84 × 10⁴; these values represent a 10-fold decrease in Kₘ and a 20-fold increase in catalytic efficiency compared to those of the H122A/L123A double mutant. Similar kinetic parameters are observed with DAS as a substrate. The Kₘ of the H122A/L123A/Q202A triple mutant with 4-ANIV as a substrate is 501 μM, which is a 5.5-fold increase compared with that of T-2 toxin and DAS. Interestingly, the Kₘ value of the H122A/L123A/Q202A triple mutant with DON as a substrate is 1202 μM, and the catalytic efficiency is 7.4 × 10⁵; these values represent a 20-fold increase and 23-fold decrease, respectively, compared to those of the WT. It is clear that although Os79 H122A/L123A/Q202A is capable of glycosylating a range of substrates that is broader than that of WT Os79, the triple substitution comes at the cost of decreasing the catalytic efficiency with DON as a substrate. Given the observation that the Q202L substitution decreases the Kₘ for WT Os79 with DON as a substrate, Os79 H122A/L123A/Q202L was made in an attempt to produce an enzyme with intermediate Kₘ values for both DON and T-2 toxin. The Kₘ values for Os79 H122A/L123A/Q202L are 118, 261, and 88 μM for T-2 toxin, DON, and 4-ANIV, respectively (Table 2).

Figure 5. Comparison of Os79 WT and H122A/L123A and Q202A active sites. The structures of Os79 H122A/L123A (PDB entry 6BK2) and Q202A (PDB entry 6BK0) were aligned to WT Os79 with rmsds of 0.17 and 0.16 Å, respectively (PDB entry 5TMD). The cross section of the acceptor binding pocket is shown in a stereoview with the surface of the two variant proteins colored white. The His 122, Leu 123, and Gln 202 residues from the WT protein are shown as sticks with wheat-colored carbon atoms and dots to illustrate their size. Carbons 4, 8, and 15 of the trichothecene backbone are labeled. The 2-deoxy-2-fluoroglucose moiety from the U2F-trichothecene complex is also shown in the active site.

The catalytic e decrease in Kₘ as a result of the Q202L mutation illustrates the importance of that side chain. This supports the possibility that the S203L mutation might prompt a change in the position of Gln 202 that could increase the Kₘ for DON.

Role of Ala 384. Ala 384 is positioned on a loop at the upper left side of the acceptor binding pocket ~5 Å from C3 of DON (Figure 4). To investigate whether a hydrophobic residue at this position is important for activity, this residue was changed to a serine. The Kₘ value of the A384S mutant is similar to that of WT Os79 (Table 2). Similar to Ser 203, this appears to be a second-sphere residue, where changes in polarity appear to have little in

Structure of Os79 Q202A and Os79 H122A/L123A Proteins. The kinetic measurements with a variety of trichothecene acceptors discussed previously in this study revealed that the active site of Os79 is unable to accommodate the C4 acetyl group of trichothecene substrates. The preceding mutations indicate the importance of the helix that carries Phe 199, Gln 202, and Ser 203 where this α-helix is opposite and slightly above C4, C15, and C8 of the trichothecene. Inspection of the acceptor binding pocket revealed that His 122 and Leu 123 are opposite but below Phe 199, Gln 202, and Ser 203. It is unlikely that His 122 is positioned only 4.2 Å from C4. Consequently, His 122 and Leu 123 were simultaneously targeted for mutagenesis. Initially, four amino acid substitutions provided Os79 the ability to glycosylate T-2 toxin as measured by an end point assay and analyzed by LC-MS/MS as described in Experimental Procedures. These were the Q202L, Q202A, and Q202V single substitutions and the H122A/L123G double substitution. No further work was performed on Q202V because it was intermediate between Q202L and Q202A. The catalytic efficiency and Kₘ values of Os79 H122A/L123G are 5.08 × 10² s⁻¹ M⁻¹ and 512 μM, respectively, with T-2 toxin as a substrate and 1.99 × 10² s⁻¹ M⁻¹ and 2512 μM, respectively, with DON as a substrate (Table 2). The activity of Os79 Q202 toward T-2 toxin as a substrate was not detected with the assay used in this study, possibly due to a very high Kₘ. Os79 H122A/L123G was largely insoluble. On the basis of these findings, Os79 H122A/L123A was expressed and purified. Changing Leu 123 to an alanine instead of glycine improved the solubility of the protein. The catalytic efficiency of the H122A/L123A mutant with with T-2 as a substrate is 1.17 × 10³, which is a 3.5-fold increase over that of the WT. The Kₘ value of 926 μM is similar to that of the H122A/L123G mutant. To improve the Kₘ, the H122A/L123A substitutions were combined with the Q202A substitution to make Os79 H122A/L123A/Q202A. The Kₘ of the triple mutant with T-2 as a substrate is 89 μM, and the catalytic efficiency is 2.84 × 10⁴; these values represent a 10-fold decrease in Kₘ and a 20-fold increase in catalytic efficiency compared to those of the H122A/L123A double mutant. Similar kinetic parameters are observed with DAS as a substrate. The Kₘ of the H122A/L123A/Q202A triple mutant with 4-ANIV as a substrate is 501 μM, which is a 5.5-fold increase compared with that of T-2 toxin and DAS. Interestingly, the Kₘ value of the H122A/L123A/Q202A triple mutant with DON as a substrate is 1202 μM, and the catalytic efficiency is 7.4 × 10⁵; these values represent a 20-fold increase and 23-fold decrease, respectively, compared to those of the WT. It is clear that although Os79 H122A/L123A/Q202A is capable of glycosylating a range of substrates that is broader than that of WT Os79, the triple substitution comes at the cost of decreasing the catalytic efficiency with DON as a substrate. Given the observation that the Q202L substitution decreases the Kₘ for WT Os79 with DON as a substrate, Os79 H122A/L123A/Q202L was made in an attempt to produce an enzyme with intermediate Kₘ values for both DON and T-2 toxin. The Kₘ values for Os79 H122A/L123A/Q202L are 118, 261, and 88 μM for T-2 toxin, DON, and 4-ANIV, respectively (Table 2).
The mutations all suggest that the volume of the active site of Os79 is an important determinant in broadening the specificity by allowing the acceptor binding pocket to accommodate the C4 acetyl group. However, this does not exclude the possibility that the mutations cause a structural change in the acceptor binding pocket. To address this question, the structures of Os79 H122A/L123A (PDB entry 6BK2) and Os79 Q202A (PDB entry 6BK0) were determined in the presence of UDP to resolutions of 1.47 and 1.29 Å, respectively (Table 1). These structures show that the mutations result in very little change in the overall structures of the protein. The 50 residues that line and surround the acceptor binding pocket of the Os79 Q202A and H122A/L123A structures aligned with the corresponding residues of the Os79 WT structure with rmsds of 0.12 and 0.16 Å, respectively, for structurally equivalent α-carbons. This indicates that the frameworks of the acceptor binding pockets of these proteins are almost identical to that of the WT enzyme. The main difference is the size of the trichothecene binding pocket. As shown in Figure 5, the H122A/L123A and Q202A mutations increase the volume of the active site. Specifically, these substitutions appear to open the side of the active site that would need to accommodate the C4 acetyl group. This is consistent with the hypothesis that the broad specificity is sanctioned by the general hydrophobicity and volume of the acceptor cavity that affords the C4 acetyl group enough space to allow toxins such as 4-ANIV, DAS, and T-2 to bind.

**CONCLUSIONS**

Broad specificity while maintaining a high catalytic efficiency is a puzzle in enzymology. This is particularly true for members of the plant UGT family that adopt the GT-B protein fold and have evolved to glycosylate a wide range of xenobiotics. As shown here, the wild-type trichothecene UDP-glucosyltransferase from rice, Os79, has a broad specificity that can modify substrates that differ in molecular weight by a factor of 1.8 (isotrichodermol and HT-2 toxin; 250.3 and 424.5, respectively) with catalytic efficiency of $k_{cat}/K_{M}$ in the range of $1 \times 10^{4} \text{ s}^{-1} \text{M}^{-1}$, but even here there are enigmatic observations. Foremost, the wild-type enzyme is unable to glycosylate T-2 toxin yet readily modifies HT-2, which compared to T-2 is deacetylated at the C4 position. Indeed, the wild-type enzyme is unable to accommodate substrates that are substituted at the C4 position (Figure 1).

The three-dimensional structure of the product complex (Os79-UDP-D3G) in combination with the previously determined structure of trichothecene bound to Os79 revealed that the acceptor pocket is mostly hydrophobic and includes only a few residues capable of forming hydrogen bonds. Mutagenesis of these polar residues that might interact with the trichothecene substrate had small effects on $k_{cat}$ and $K_{M}$, whereas mutagenesis of Phe 199 to a glutamine eliminated activity. These observations suggested that the hydrophobicity and volume of the active site are primary factors in substrate specificity. On the basis of this structural knowledge, the volume of the active site was increased by mutagenesis, the consequence being that Os79 H122A/L123A showed excellent activity toward T-2 toxin but reduced activity toward DON. Addition of the Q202L substitution created an enzyme that is a compromise that allows essentially equivalent activity toward both DON and T-2 toxin. This demonstrates that rational design of plant UGTs is a promising approach for the glycosylation of new small molecule substrates not only as a means of detoxifying a wider range of xenobiotics such as trichothecenes but also for the glycosylation of small molecules in medicinal biochemistry. The broad specificity of Os79 H122A/L123A/Q202L makes it an attractive gene for incorporation into transgenic plants that are susceptible to infection of both T-2 toxin and DON producing *Fusarium* species, like maize (*Fusarium sporotrichioides* and *Fusarium graminearum*), oat (*Fusarium langsethiae* and *Fusarium culmorum*), or potatoes (*Fusarium sambucinum* and *F. graminearum*).

**ASSOCIATED CONTENT**

**Accession Codes**

X-ray coordinates for the Os79-UDP deoxynivalenol 3-O-glucoside, Q202A-UDP, H122A/L123A-UDP, and T291V-UDP complexes have been deposited with the Research Collaboratory for Structural Bioinformatics as Protein Data Bank entries 6BK3, 6BK0, 6BK2, and 6BK1, respectively.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

4-ANIV, 4-acetyl nivalenol; FUS-X, or fusarenon-X; 4,15-diANIV, 4,15-diacetyl nivalenol; D3G, deoxynivalenol 3-O-glucoside; DAS, diacetoxyscirpenol; DON, deoxynivalenol; FHB, *Fusarium* head blight; GT-1, family 1 of the glucosyltransferase superfamily; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HT-2, hydrolyzed T-2 toxin; MBP, maltose binding protein; MEPEG 5K, methyl ether polyethylene glycol 5000; MPD, 2-methyl-2,4-pentanediol; NiNTA, nickel-nitrilotriacetic acid; NIV, nivalenol; Os79, product of the Os04g0206600 gene (original seq
NM_001058779); rmsd, root-mean-square deviation; T-2, T-2 toxin; TCEP, tris(2-carboxyethyl)phosphine; TEV, tobacco etch virus protease; TRI, trichothecene; U2F, UDP-2-fluoro-2-deoxy-β-D-glucose; UGTs, UDP-glycosyltransferases.

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natural product modification.


