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Comparative Sequence Analysis of TRI1 of Fusarium

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Abstract: Trichothecene mycotoxins are a class of secondary metabolites produced by multiple genera of fungi, including certain plant pathogenic Fusarium species. Functional variation in the TRI1 gene produces a novel Type A trichothecene called NX-2 in strains of F. graminearum. Using a bioinformatics approach, a systematic analysis of 52 translated TRI1 sequences of Fusarium species, including five F. graminearum NX-2 producers and four F. graminearum non-NX-2 producers, was conducted to explain the functional difference of TRI1p of FGNX-2. An assessment of several signature motifs of fungal P450s revealed amino acid substitutions in addition to the post-translational N-X-S/T sequons motif, which is indicative of N-linked glycosylation of this TRI1-encoded protein characteristic of NX-2 producers. There was evidence of selection bias, where TRI1 gene sequences were found to be under positive selection and, therefore, under functional constraints. The cumulative amino acid changes in the TRI1p sequences were reflected in the phylogenetic analyses which revealed species-specific clustering with a distinct separation of FGNX-2 from FG-non-NX-2 producers with high bootstrap support. Together, our findings provide insight into the amino acid sequence features responsible for the functional diversification of this TRI1p.

Keywords: cytochrome P450; Fusarium; TRI1; NX-2 trichothecene

Key Contribution: Specific amino acid sequence features in the primary TRI1p structure may be responsible for the functional diversification of TRI1p in NX-2-producers.

1. Introduction

Trichothecenes mycotoxins are a large class of sesquiterpene metabolites, which are produced by several genera of plant pathogenic fungi including several species of Fusarium. Trichothecenes are considered to be virulence factors and their production improves the pathogenic potential of Fusarium species in a number of economically important plant host species [1–4]. Trichothecenes can be classified into four types based on (i) the carbonyl group at the 8-position, (ii) the position of macrolide rings, and (iii) the number of epoxy rings [5]. Type A (e.g., T-2 toxin and HT-2 toxin) and Type B trichothecenes (e.g., deoxynivalenol (DON) and nivalenol (NIV)) are harmful after acute and chronic exposure, highly cytotoxic, pro-inflammatory with emetogenic properties and are efficient inhibitors of eukaryotic protein synthesis [6–8]. The Food and Drug Administration (FDA) in the USA and the European Commission, Europa EU, enforce maximum allowable limits of trichothecene contamination of food products used in national and international trade (International Trade and Food Safety/AER-828, Economic Research Service/USDA; https://www.ers.usda.gov/webdocs/publications/41603/15640_aer828h_1_.pdf?v=42055).

Biosynthesis of trichothecenes is carried out by expression of a core TRI gene cluster [4]. Depending on the Fusarium species, oxygenase (TRI1) and acyltransferase (TRI16) genes in addition to an acetyltransferase gene (TRI101) can be located external to the core [9]. The TRI1 gene of different Fusarium
species encode calonectrin oxygenase (TRI1p) [9] and its expression is regulated by transcription factors TRI6p and TRI10p [5,10]. Specifically, for *F. sporotrichioides*, the TRI1 gene (*FsTRI1*) encodes a cytochrome P450 monoxygenase that hydroxylates C-8 of calonectrin in the biosynthesis of Type A trichothecenes. For *F. graminearum*, a homologue of this gene (*FgTRI1*) encodes a cytochrome P450 dioxygenase, trichothecene 7,8-dihydroxylase, that catalyzes the hydroxylation of both C-7 and C-8 of calonectrin to generate 7,8-dihydroxyacalonectrin and/or generate the 7- and 8-monohydroxylated calonectrin in the biosynthesis of DON and NIV [10–13] (Figure 1).

![Chemical structures](image)

Reaction: EC: 1.14.14; ID: RXN 17048 with reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen into the other donor

Standard Gibbs Free Energy (ΔG°): -170.78952 kcal/mol

**Figure 1.** Hydroxylation reactions catalysed by trichothecene 7,8-dihydroxylase (MetaCyc Accession No. G-44257 [13] and UniProt Accession No. Q7Z886 (https://www.uniprot.org/uniprot/Q7Z886).

Functional analyses of TRI genes of the TRI biosynthetic gene cluster explained, in part, the basis for the structural diversity of trichothecene analogs produced by *Fusarium* species [4,9,12,14]. Proctor et al. [4] reported that TRI gene gain, loss, and subsequent alterations in function of these genes are the primary effectors of this structural diversity. As a result, chemotype shifts can occur as a result of the introduction of new genotypes into new areas [15]; for example, detection of 3-ADON genotypes of *F. graminearum* sensu stricto in Canada [16], 3-ADON genotypes of *F. asiaticum* in Southern China [17], and the NIV genotype of *F. asiaticum* in the southern parts of the USA [18] and Brazil [19].

The value of studying TRI genes has been demonstrated by the detection of a novel type A trichothecene toxin (NX-2) produced by specific strains of *F. graminearum* (FGNX-2) [20,21]. NX-2 and 3-ADON producing strains primarily generate the 3-acetylated derivative of trichothecenes, which are less toxic than their related metabolites [22]. Structural analysis of the NX-2 toxin indicated that a distinct variant of the TRII gene product (TRI1p) catalyzes C-7 hydroxylation, but not C-8 hydroxylation of calonectrin [23]. This lack of a C-8 carbonyl group allows NX-2 toxin to escape detection by HPLC-UV-based methods. While this underlines the value of genotyping at this initial stage of investigation, it also demonstrates the value of LC-MS or GC-MS methods of toxin detection. Initially, a low frequency of occurrence of NX-2 in northern US and in Canada was reported [20,21]. However, Lofgren et al. [24] more recently analyzed a large collection of *F. graminearum* strains from New York in the USA, and found that (i) the frequency of NX-2 genotype strains was up to 14 times higher than previously reported, (ii) NX-2 genotypes were detected in maize ears and stubble in addition to wheat heads, and (iii) 20% of the total *F. graminearum* population in the USA could be attributed to the NX-2 genotype. The detection of NX-2-producing strains in Canada, albeit at low frequency, reinforces the need for continued monitoring of *Fusarium* populations in wheat-growing regions of North America [21].

We present a study of 52 TRII peptide sequences (TRI1p) of *Fusarium* species including five *F. graminearum* (as NX-2 producers, FGNX-2) and four *F. graminearum* (as non-NX-2 producers, FG-
non-NX-2), in which systematic analysis of signature motifs of P450s and of protein sequence phylogeny are carried out in order to explain the functional difference of TRI1p of FGNX-2.

2. Results and Discussion

Note: In the figures, the five FGNX-2 and four FG-non-NX-2 sequences with 21 additional Fusarium sequences are shown for comparison, as the entire 52 TRI1p sequence dataset could not be depicted in any one figure.

2.1. TRI1 Peptide Primary Sequence Comparisons

Comparison of the TRI1p sequences revealed differences in the amino acid composition, in the primary predicted peptide structure, and in chemical characteristics, e.g., the hydrophobicity was higher for FGNX-2 and the number of hydroxyl groups was higher for FG-non-NX-2 (Figures 2 and 3A,B). A representative sequence of FGNX-2 (GenBank Accession No. AIU41071) and of FG-non-NX-2 (GenBank Accession No. AOC89125) are shown.

Figure 2. Comparison of primary structure TRI1 peptide sequences (TRI1p) showing the specific sequence length where differences in the structure are apparent; A: Representative FGNX-2 isolate (GenBank Accession No. AIU41071); B: Representative FG-non-NX-2 isolate (GenBank Accession No. AOC89125). Red circle indicates specific differences between the primary sequences of FGNX-2 and of FG-non-NX-2.
TRI1p sequences in five out of six prediction programs in TOPCONS2 (Figure 4). This finding is consistent with the data presented by Menke et al. [25], Kistler and Broz [26], and Boenisch et al. [27], in which systematic analysis of signature motifs of P450s and of protein sequence phylogeny was carried out in order to explain the functional difference of fungal CYP51s. Fusarium species had PRRW. Both FGNX-2 and FG-non-NX-2 isolates had the PRRW motif.

Figure 3. Comparison of TRI1 peptide sequences (TRI1p) showing differences in amino acid composition; A: Representative FGNX-2 isolate (GenBank Accession No. AIU41071); B: Representative FG-non-NX-2 isolate (GenBank Accession No. AOC89125).

2.2. Topology

Topology analysis indicated two transmembrane domains at the N- and C-terminus for all TRI1p sequences in five out of six prediction programs in TOPCONS2 (Figure 4). This finding is consistent with the data presented by Menke et al. [25], Kistler and Broz [26], and Boenisch et al. [27], in which trichothecene reaction products and intermediates are sub-cellularly compartmentalized and associated with the endoplasmic reticulum. Upon mycotoxin induction, the ER undergoes ultrastructural re-organization into proliferations of the organized smooth endoplasmic reticulum (SER) and TRI1p is associated with this modified ER membrane [27].

Figure 4. TOPCONS predicted topologies and ΔG values for FGNX-2 and FG-non-NX-2 TRI1p sequences.

2.3. PER/PxRW and ExxR Motifs

The alignment also revealed the conserved PER domain of P450s as the characteristic signature for fungi (PxRW) [28,29] (Figure 5). Two clusters were represented by PPRF while all other Fusarium species had PRRW. Both FGNX-2 and FG-non-NX-2 isolates had the PRRW motif.
Figure 5. Signature ‘PxRW’ and ‘ExxR’ motifs in the TRI1p sequence of Fusarium species.

Two clusters contained variations of this ExxR motif as Eggf and Eggm, while other Fusarium clusters contained EggR. The ExxR and PER motifs form “the E-R-R triad”, which functions to secure the heme pocket into its correct position, and thus, to ensure stabilization of the core structure of the enzyme [30]. Both FGNX-2 and non-NX-2 producers had the EggR motif (Figure 5). Sello et al. [31] analyzed the ExxR motif among oomycetes and found that the first and fourth residues, i.e., “E” and “R” are conserved in all P450 families, e.g., CYP5014, CYP5015, and CYP5017. There are a few exceptions; in CYP5017F8, the motif consisted of a “K” instead of “E” and in CYP5014N1 and CYP5015L, the motif consisted of “W” and “H”, respectively, instead of “R”. Variations of the “E” and “R” amino acids at the ExxR motif are uncommon [32].

2.4. Dileucine (LL) Motifs

WoLFPSORT detected four dileucine sequence motifs (LL) based on the alignment of 52 TRI1p sequences (Figure 6). The function of the LL sequence motif depends on the nature of the adjacent residues; however, there is no specific peptide sequence within which LL motif resides [33]. Several transmembrane proteins consist of LL motifs that function as sorting signals [34]. Although dileucine sequence motifs are not necessarily characteristic of P450s, their detection was still included here.
2.5. Heme Motif

P450s have a signature heme motif sequence $FXXGX_bXXCXG$, where $X_b$ is a basic amino acid and the cysteine residue serves as the catalytic ligand located axial to the heme moiety, i.e., the specific thiolate group of the cysteine amino acid structure occupies the axial coordination site of iron opposite to the bound oxygen [35,36]. Substrates, due to their hydrophobicity, bind in a cleft or pocket above heme.

Analysis of the 52-amino acid sequence alignment revealed that FGNX-2 producers have a variant amino acid sequence signature for heme compared to the 10-amino acid consensus sequence: $[FW]-[SGNH]-x-[GD]-[F]-[RKHPT]-[P]-C-[LIVMFAP]-[GAD]$, where C is cysteine residue that interacts with the heme iron ligand (Figure 7; Figure 8). This motif is located at the C-terminus of the P450 and this finding is supported by Menke et al. [25], who reported that the heme binding domains, and, therefore, the enzyme active sites are near the C-terminus.
**Figure 7.** Variation in heme motif of TRI1p amino acid sequences of the *Fusarium* species.

**Figure 8.** Catalytic cycle of cytochrome P450. The heme macrocycle is depicted to represent the resting enzyme which is in the ferric state and has a thiolate proximal ligand (cysteine thiolate, indicated as an S‐Cys atom linked to the iron) and a distal ligand (a water molecule, which changes to dioxygen as the cycle proceeds). (1) The substrate (RH) binds in proximity to the heme group displacing the distal water ligand leading to a conformational change in the active site of the P450 molecule and a shift in the ferric heme iron spin‐state equilibrium from low‐spin (S = 1/2) to a high‐spin (S = 5/2). (2) This leads to a shift in the redox potential that enables electron transfer from a redox partner to occur to reduce the heme iron to the ferrous state. The first electron transfer occurs which reduces the ferric iron (heme‐Fe(III)) to its ferrous form (heme‐Fe(II)). The ferric complex in the presence H₂O₂ or any organic peroxy compound (e.g., an alkyl hydroperoxide or peracid) can lead to the production of the ferric hydroperoxo intermediate (although inefficiently in most cases). (3) Oxygen then binds to the ferrous heme center to produce the ferrous‐oxy intermediate that is isoelectronic with the ferric superoxo form. (4) A second electron transfer occurs reducing the heme iron to the ferric peroxo intermediate. This electron transfer step is usually, but not always, the rate‐limiting step in the cytochrome P450 catalytic cycle. (5) Rapid protonation of the peroxo intermediate leads to the ferric hydroperoxo intermediate. (6) The hydroperoxo formed in step (5) is unstable and undergoes rapid protonation leading to scission of the dioxygen bond with the production of a water molecule and the generation of iron‐oxo‐Fe(IV) porphyrin π cation radical (ferryl species) also known as Compound I. This is considered to be the catalytically reactive substrate oxidant in most cytochrome P450 reactions. Alternative formulations of the ferryl intermediate shown are as (i) a protein radical cation Fe(IV) species or (ii) as an Fe(V) species. (7) Attack of the nearby substrate by the ferryl species effects its hydroxylation and metabolite/product (ROH) dissociation from the cytochrome P450 molecule via...
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Varga et al. [22] analyzed amino acid sequences of *F. graminearum* DON-producers and *F. graminearum* NX-2 producers and found that NX-2 producers had a distinct heme binding motif according to a 20 amino acid heme binding motif. Sello et al. [31] analyzed the heme motif across three P450 families and reported that amino acid residues "F", "G" and "C" located as the first, fourth, and eighth positions in the heme motif are conserved in among P450s across biological kingdoms [32], but there are some P450s with different amino acids at these positions [42].

P450-catalyzed hydroxylation is the most characteristic reaction catalyzed by P450 enzymes (Figure 9); however, substrate specificity is determined by three factors.
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(i) Substrate lipophilicity affects its compatibility with the P450 active site architecture and ultimately influences the affinity of the substrate for the P450 active site. The domain located above the heme group is relatively hydrophobic compared to other enzymes and interactions with substrates are driven entirely by lipophilic contacts [43].

(ii) In a case where oxidation of C-H bond is controlled by intrinsic reactivity rather than by steric constraints or positioning of the substrate within the active site, the C-H bond strength determines the reactivity of the substrate [43].

(iii) The size and shape of P450 active sites impose substrate selectivity [30]. Substrates are situated in catalytic pockets where the atom to be hydroxylated is oriented within a specific distance from the heme iron depending on the P450 with restricted mobility [43]. Regio- and stereoselective hydroxylation is enabled by specific active site-substrate interactions that position the substrate for oxidation [36]. P450s with high catalytic specificity have key residues involved in orientation and steric interactions between the substrate and the protein residues of the active site, and it is these imposed steric barriers that affect access of the ferryl species [44]. Regio-selectivity is mainly controlled by amino acid residues at the active site of P450s and specific amino acid substitutions within the active site have the potential to interact with an aromatic hydrocarbon substrate to induce selective hydroxylation of the ortho- or meta- or para-position despite having a native preference for

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**Figure 9.** A–D. Single amino acid substitutions of 30 representative TRI1 peptide sequences including those of FG-non-NX-2 isolates (numbers 1 to 4) and of FGNX-2 (numbers 5 to 9); 21 sequences belonged to the other *Fusarium* species; the entire 52-sequence alignment could not be shown here.
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Supplementary Figures S1 and S2 show the details of substrate binding relative to the heme moiety.

2.6. TRI1 DNA Polymorphism Profile and Evidence of Selection

Silent mutations are not manifested in an organism’s phenotype. Silent mutations in the nucleotide sequence of the TRI1 gene will not result in an amino acid change and there should be no alteration of enzyme function. When FGNX-2 TRI1 nucleotide sequences (N = 5) were compared with FG-non-NX-2 (N = 4) in DnaSP 6, there were minor differences in DNA polymorphism profiles between the two datasets (Table 1). This is in contrast to DNA polymorphism analysis of the dataset consisting of 52 nucleotide sequences of all Fusarium species which indicated that the TRI1 gene is highly polymorphic with the exception of significant nucleotide sequence conservation at nucleotide positions nt 1–36 [ATGGCTTTGATTACTTCATTGCAAGATGTTAGATTG] (p = 0.0013).

Table 1. Comparative DNA polymorphism profile for FGNX-2 and FG-non-NX-2 isolates based on an alignment of 1533 nucleotides of the partial TRI1 gene.

<table>
<thead>
<tr>
<th>DNA Polymorphism Parameter</th>
<th>FGNX-2</th>
<th>FG-Non-NX-2</th>
<th>52 Fusarium Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5</td>
<td>4</td>
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</tr>
<tr>
<td>h</td>
<td>3</td>
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<td>0.00065</td>
<td>0.30227</td>
</tr>
<tr>
<td>k</td>
<td>0.80000</td>
<td>1.00000</td>
<td>463.382</td>
</tr>
<tr>
<td>Ct</td>
<td>0.14</td>
<td>0.14</td>
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</tr>
<tr>
<td>C</td>
<td>0.049</td>
<td>0.049</td>
<td>0.049</td>
</tr>
</tbody>
</table>

N—number of sequences in dataset; h—number of haplotypes; Hd—haplotype differences; Pi—nucleotide diversity; k—nucleotide differences; Ct—conservation threshold; C—sequence conservation.
Conversely, synonymous mutations result in changes to the amino acid sequence that can affect transcription, post-transcriptional modifications, mRNA export, and translation, and which result in alterations to the structure and function of the protein. Analysis of 52 TRI1 nucleotide sequences in DnaSP 6 revealed that the ratio of non-synonymous to synonymous substitutions (dN/dS ratio) was 3.54, which is >1. Fu and Li’s D* statistic was also positive and significant (2.05433; p < 0.02), and thus, it is inferred that TRII gene sequences are under positive selection and under selective functional constraints. However, Kelly et al. [49] reported that tests of positive selection were not significant and the TRII gene sequences of the FGNX-2 were identical except for one nucleotide. In a subsequent F. graminearum genome study by Kelly and Ward [50], it was reported that among several genomic regions, TRI genes exhibit the strongest signals of selection. These non-synonymous substitutions that are restricted to FGNX-2 TRII sequences suggest that the emergence of the NX-2 genotype may have been driven by changes in selection pressure on this gene [49] and perhaps this genotype is a transient P450 conformation [51].

2.7. TRI1p Residue Substitutions

TRI1p sequences of the five FGNX-2 isolates were highly conserved. When compared with other Fusarium species, however, there were approximately six amino acid substitutions specific only to FGNX-2 sequences from all other Fusarium TRI1p sequences (Figure 9A–D). Amino acid substitutions specific to FGNX-2 sequences were: at alignment position 252 A/R/N>S; at alignment position 254 L>M; at alignment position 349 F/K>I; at alignment position 421 Q/E/L>K; at alignment position 435 T>P; at alignment position 455 A > V. Kelly et al. [49] reported that there were no differences in the predicted amino acid sequences of FGNX-2 but, detected 14 amino acid differences specifically between the TRI1 gene product of FGNX-2 strains and F. graminearum strain PH-1 (non-NX-2-producer) due analysis of a larger dataset.

2.8. Phylogeny of TRI1p Sequences of Fusarium

This difference in TRI1p amino acid substitution among Fusarium species is also shown in the phylogenetic tree produced in RaXML (Figure 10). Species-specific clustering was indicated with moderate to high bootstrap support (>75% and >90%, respectively) for most taxa. It was also apparent that residue substitutions in the PxRW and ExxR motifs accumulated in three clusters of Fusarium species which excluded F. graminearum TRI1p sequences. In addition, there was also distinct separation of FGNX-2 from FG-non-NX-2 isolates with high bootstrap support (>99%).
Figure 10. Maximum likelihood phylogenetic tree based on an alignment of 52 amino acid sequences of TRI1p of Fusarium species.
2.9. N-Linked Glycosylation

Based on NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) predictions, only the TRI1p sequences of FGNX-2-producers have a definite N-X-S/T sequon motif; this motif is absent in non-NX-2-producers (Figure 11). This N-X-S/T sequon is conserved in N-linked glycosylation. N-linked glycosylation is a critical post-translational modification of proteins that are synthesized and folded in the endoplasmic reticulum [52].

Figure 11. Comparative NetNGlyc prediction of N-linked glycosylation of asparagine amino acid in the TRI1p sequence of FGNX-2 and FG-non-NX-2 isolates. A: Representative FGNX-2 sequence, B: Representative FG-non-NX-2 sequence. Red frame indicates the location of a N-X-S/T sequon found only in FGNX-2 sequences. Purple circle indicates common location of dileucine repeat in both FGNX-2 and FG-non-NX-2 sequences.

There are >900 P450 structures available in the Protein Data Bank (http://www.wwpdb.org/), many of which contain bound ligands. Structural flexibility enables an expansion of the substrate spectrum of a P450 due to flexibility of the active site where substrate docking occurs. Most P450s have a conserved basic P450 structural fold, but their substrate-binding pockets demonstrate high structural plasticity which enables these enzymes to significantly vary the dimensions of their active sites according to the chemical structure of the substrates and this drives catalytic selectivity [36,43]. Thus, the existence of multiple conformations of P450s is reflected in multiple docking models [53]. It follows that a given substrate can bind in multiple orientations depending on regio- and/or stereo-selectivity of the active site. In extreme cases, a single amino acid substitution may be enough to change an enzyme’s regio-specificity and catalytic efficiency [54]. TRI1p can apparently utilize calonectrin, 3, 15-DAS and, to a lesser extent, isotrichodermin as substrates in the production of trichothecene mycotoxins, which suggests that the TRI1p active site has a relaxed substrate specificity [14,55].

In view of the role of the heme-Fe complex and the conformational interactions of specific amino acid residues in P450-driven oxidation, it is hypothesized that the TRI1p variant heme sequence and select amino acid substitutions of NX-2-producers may affect regio- and stereo-selectivity for substrate (calonectrin) docking, orientation and position for oxidation relative to the heme-Fe complex. Substitution of key residues would affect the ability of the substrate to make multiple orientations in the active site relative to the heme-Fe complex. Furthermore, the N-X-S/T sequon, which is tagged for N-linked glycosylation that appears to be unique to FGNX-2, would impact upon folding of the
protein compared to non-NX-2- producers for which this sequon is absent. It is an advantage to P450 chemistry if the correct tertiary structure is retained as P450s also repositions their active site residues upon substrate binding [51].

According to Kimura et al. [14], closely-related *Fusarium* species (i) produce trichothecenes as different structural variants due to substitution patterns of functional groups at C-3, C-4, C-7, C-8, and C-15, (ii) the late stages of trichothecene biosynthesis differs for Type A and Type B trichothecene production as a result of substrate specificity, and (iii) trichothecene biosynthesis operates along “metabolic grids” rather than linear pathways. As such, 7-hydroxycalonectrin and 8- hydroxycalonectrin can both be used as substrates for generation of DON. Although there is no specific data on the ratio of 7, hydroxycalonectrin to 8, hydroxycalonectrin produced, their production is sequential and not random [14,56]. Furthermore, structural diversity that results in altered TRI gene function (acetylation, acylation, and hydroxylation) in different fungal genera that produce these mycotoxins and the current TRI gene functions can be either ancestral, derived, or demonstrate retained, but attenuated ancestral gene function [4].

Although the folded structure and catalytic competence of P450s must be maintained, the active sites can tolerate certain mutations and still retain its function. It is perhaps this mutational robustness that reflects the diversity of the P450 family, the key role of the iron-heme prosthetic group in catalysis, the hydrophobicity of the active site, and the conformational variability upon substrate binding [51]. Although most random mutations are either neutral or deleterious, mutation fixation in certain *F. graminearum* genomes indicates that these heme motif mutations may confer an adaptive advantage that is not yet defined [57–60].

3. Conclusions

*TRI1* gene sequences are more divergent among different *Fusarium* species than among closely related species [14]. It is important to understand the molecular mechanisms that drive the different substitution patterns at the later stages of trichothecone biosynthesis because the relative toxicity of trichothecenes is determined by the pattern of oxygenation, acetylation, and/or esterification of different substrates [14]. Furthermore, if a significant change in selective pressure enabled motif and amino acid variation in NX-2-producers, monitoring of the NX-2 genotype according to host and geographical range in an effort to identify potential source and range expansions should be carried out [49]. It is also relevant to understand whether the NX-2 genotype confers a selective advantage over other TRI genotypes [60].

4. Materials and Methods

The bioinformatics pipeline developed for analysis of *TRI1* nucleotide and protein sequences are outlined in Figure 12.
**Figure 12.** Bioinformatics pipeline developed for analysis of TRI1 nucleotide and protein sequences.

### 4.1. Protein Sequence Selection and Alignment

TRI1 nucleotide sequences of different *Fusarium* species were accessed as PopSets in GenBank: PopSet: 699128280 [20] and PopSet: 1052473830 [49]. These sequences were selected, as they included *F. graminearum* NX-2 producers from two different studies [20,49], other *F. graminearum* strains as a non-NX-2 producers as well as TRI1 sequences of other *Fusarium* species to be used as additional references. The sequence identities of all 52 sequences were verified in NCBI Multiple Sequence Alignment Viewer (MSAViewer: https://www.ncbi.nlm.nih.gov/projects/msaviewer/). Nucleotide sequences were translated (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) and the amino acid sequences were aligned in CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/) and trimmed to a common length and analyzed for motifs and amino acid substitutions in BioEdit.

### 4.2. Comparative Primary Peptide Sequence Analysis

Primary sequence structure of the TRI1 peptide of FGNX-2 and FG-non-NX-2 sequences were predicted and drawn on pepdraw (http://pepdraw.com/). The FGNX-2 protein sequences are AOC89153, AIU41071, AIU41072, AIU41073, and AOC89167. The FG-non-NX-2 protein sequences are AOC89125, AOC89149, AOC89272, and XP_011315667. The proteins’ theoretical properties were also calculated in NPS-Network Protein Sequence Analysis (https://npsa-prabi.ibcp.fr/) and ProtParam-Protein Identification and Analysis Tools on the ExPASy Server (https://web.expasy.org/protparam/) [61].

Alignment of TRI1p of FGNX-2 and a representative FG-non-NX-2 was compared in the Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/color_align_prop.html). The color represents the biochemical properties of a particular residue.

### 4.3. Topology Analysis

Topology analysis was carried out in TOPCONS2-Consensus prediction of membrane protein topology and signal peptides (http://topcons.cbr.su.se/pred/result/rst_gW7Qq0/prediction) [62] and in WoLFPSORT (https://wolfpsort.hgc.jp/). Topologies for FGNX-2 and a representative FG-non-NX-2 were based on structure prediction and modeling against macromolecule data in the Protein Data Bank (PDB). Phobius (http://phobius.sbc.su.se; http://phobius.sbc.su.se/poly) [63] and SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/) [64] were used to determine presence/absence and location(s) of signal peptide sequences.
4.4. Signature Motifs of P450s

The alignment of 52 TRI1p sequences was examined for characteristic signature motifs of fungal P450s as identified by Syed and Mashele [42]. These motifs have been identified as conserved among P450 tertiary structure and enzyme functions [30]. Therefore, our specific objective was to detect TRI1p motif differences between FGNX-2 producers and non-NX-2 producers.

4.5. Selection Bias

Alignment of nucleotide sequences was carried out in MAFFT (Multiple Alignment using Fast Fourier Transform) server (https://www.ebi.ac.uk/Tools/msa/mafft/) and aligned sequences were then edited to a common length prior to analysis. DnaSP 6 [65] was used out to determine if TRI1 gene sequences were under positive selection by assessing the ratio of non-synonymous to synonymous substitutions (dN/dS) [66] by using Fu and Li’s D* test statistic at p < 0.02 [67,68].

4.6. Phylogeny of TRI1 Nucleotide and Protein Sequences

Inferred TRI1 phylogenetic relationships among Fusarium species were analyzed using the Maximum Likelihood (ML) algorithm. Phylogenetic inference of TRI1p sequences was estimated in RAxML [69] v0.9.0 (https://raxml-ng.vital-it.ch) using the maximum likelihood optimality criterion. The 75% consensus trees were retained and re-drawn in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Clusters for which motif variation were detected are also indicated as blue- and orange-colored icons on the tree.

4.7. Post-Translational Modification of TRI1p

NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) predicts the number and location of N-Glycosylation sites using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons (where Xaa is not proline). GPMAW lite (https://alphalyse.com/gpmaiw/) was also used to detect N-glycosylation sites in the protein sequences.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/11/12/689/s1, Figure S1: Examples of heme interactions in a fungal cytochrome P450 monoxygenase, Figure S2: Example of substrate/ligand binding relative to heme moiety.


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