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New tricks of an old enemy: isolates of *Fusarium graminearum* produce a type A trichothecene mycotoxin

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Summary

The ubiquitous filamentous fungus *Fusarium graminearum* causes the important disease *Fusarium* head blight on various species of cereals, leading to contamination of grains with mycotoxins. In a survey of *F. graminearum* (sensu stricto) on wheat in North America several novel strains were isolated, which produced none of the known trichothecene mycotoxins despite causing normal disease symptoms. In rice cultures, a new trichothecene mycotoxin (named NX-2) was characterized by liquid chromatography-tandem mass spectrometry. Nuclear magnetic resonance measurements identified NX-2 as 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-ene. Compared with the well-known 3-acetyl-deoxynivalenol (3-ADON), it lacks the keto group at C-8 and hence is a type A trichothecene. Wheat ears inoculated with the isolated strains revealed a 10-fold higher contamination with its deacetylated form, named NX-3, (up to 540 mg kg⁻¹) compared with NX-2. The toxicities of the novel mycotoxins were evaluated utilizing two *in vitro* translation assays and the alga *Chlamydomonas reinhardtii*. NX-3 inhibits protein biosynthesis to almost the same extent as the prominent mycotoxin deoxynivalenol, while NX-2 is far less toxic, similar to 3-ADON. Genetic analysis revealed a different TRI1 allele in the N-isolates, which was verified to be responsible for the difference in hydroxylation at C-8.

Introduction

The growth of fungi on agricultural goods is a worldwide problem, limiting available food and feed supplies. The Food and Agriculture Organization of the United Nations (FAO) estimates that about a quarter of the world’s food crops are significantly contaminated with mycotoxins (Smith et al., 1994). Mycotoxins are secondary fungal metabolites of low molecular weight, which are toxic to humans and animals (Bennett and Klich, 2003; Marin et al., 2013). Deleterious health effects caused by different mycotoxins include nephropathy, infertility, cancer or death. One of the main classes of mycotoxins are trichothecenes (McCormick et al., 2011). This family encompasses around 200 different toxins, which inhibit eukaryotic protein synthesis (Cundliffe and Davies, 1977). They are therefore also potent phytotoxins and act as virulence factors of pathogenic fungi on sensitive host plants, e.g. of *Fusarium graminearum* in wheat (Bai et al., 2002; Jansen et al., 2005). Although all trichothecenes share a tricyclic 12,13-epoxytrichothec-9-ene structure...
(Fig. 1), they can be further classified according to their substituents on C-8. Type A trichothecenes are characterized by a hydroxyl group, an ester or no substituent at all at C-8, whereas type B trichothecenes carry a keto group at this position (McCormick et al., 2011).

Although several fungi can form trichothecenes, the by far most relevant genus regarding pathogenicity on cereals is Fusarium, with F. graminearum being the dominant species causing Fusarium head blight disease (scab) (reviewed in Goswami and Kistler, 2004; Kazan et al., 2012). This disease occurs worldwide, but its dramatic reappearance in North America during the 1990s had a particularly severe impact on US farming communities (McMullen et al., 1997). Because of reduced yields and market prices for mycotoxin contaminated grain, growers had to give up planting wheat and brewing barley in severely affected areas (McMullen et al., 2012).

The most frequently occurring mycotoxin due to F. graminearum infection is the type B trichothecene deoxynivalenol (DON, Fig. 1A), along with its biosynthetic precursors 3-acetyl-DON (3-ADON, Fig. 1B) and 15-acetyl-DON (15-ADON, Fig. 1C). The currently known mechanisms of action and the toxicological relevance of DON are reviewed (Pestka, 2010). It causes immunosuppressive, emetic and anorexic effects after ingestion. Occurrence data for DON, collected by 21 European countries between 2007 and 2012, and population exposure was recently summarized by the European Food Safety Authority (2013). Several dozen countries, including Canada, China, the European Union, India, Japan and Russia, set maximum limits for DON in different foodstuffs (FAO, 2006). In the European Union, the maximum level (European Commission Regulation, 2006) for DON in unprocessed cereals is set at 1.25 mg kg⁻¹, while cereals intended for direct human consumption may contain up to 0.75 mg kg⁻¹. The US Food and Drug Administration set advisory levels for DON in finished wheat products for human consumption (1 mg kg⁻¹) as well as for grains and grain by-products used for animal feed (US Food and Drug Administration, 2010).

Fusarium isolates can be classified according to their chemotype (Moss and Thrane, 2004) based on which toxin accumulates in axenic cultures. Different TRI8 alleles result either in the deacetylation of the biosynthetic precursor 3,15-diacyetyl-DON at C-15 to yield 3-ADON or at C-3 to yield 15-ADON (Alexander et al., 2011). While the 15-ADON chemotype of F. graminearum used to be more prevalent in North America, the frequency of the 3-ADON chemotype in western Canada increased 14-fold between 1998 and 2004 (Ward et al., 2008). The biosynthetic enzymes required for trichothecene production are encoded by TRI genes at three different loci (TRI101 locus, TRI1 and TRI16 locus and the 12 gene core TRI cluster) (McCormick et al., 2011). TRI13 is responsible for the introduction of a hydroxyl group at C-4 of the trichothecene skeleton, e.g. leading to the type B trichothecene nivalenol (NIV), which is more prevalent in Asia.

Strains of a newly identified population of F. graminearum (sensu stricto), collected from wheat in Minnesota, were genotyped to be 3-ADON producers, but chemical analysis showed that they produced neither DON nor NIV nor acetylated derivatives thereof (Gale et al., 2010). Rice cultures of the isolated strains were screened with a multi-mycotoxin method (Vishwanath et al., 2009), confirming the absence of all trichothecenes included in the method. These ‘no trichothecene’ producers were previously investigated as potential biocontrol strains, as co-inoculation of wheat with a DON producer reduced the DON content (Yuen et al., 2010).

The aim of our work was to investigate whether the isolated F. graminearum strains can produce novel trichothecene toxins because clear disease symptoms could be seen on wheat. Here we report on the identification and characterization of two novel mycotoxins (NX-3 Fig. 1D, NX-2 Fig. 1E) produced by these strains. The toxicity of these compounds renders our work potentially important for food safety.

Results

Identification of new trichothecenes

Gas chromatography-mass spectrometry (GC-MS) headspace (HS) analysis of isolates previously identified as ‘Northland’ (Gale et al., 2010) or ‘No trichothecene’ (N)

Fig. 1. Structures of (A) deoxynivalenol, (B) 3-acetyl-deoxynivalenol, (C) 15-acetyl-deoxynivalenol as well as of the novel metabolites (D) NX-3, (E) NX-2 and (F) NX-4.
showed that they produce the volatile trichothecene precursor trichodiene, suggesting that unknown trichothecenes might be produced by the N-strains.

Full-scan liquid chromatographic-mass spectrometric (LC-MS) measurements of the extracts from rice cultures of an N-isolate and a control strain, PH-1, identified two candidate compounds with significant intensity (Fig. 2). The first eluting peak of \( m/z \) 342 was slightly more polar than DON and was termed NX-1. The second one (\( m/z \) 324) was about as polar as 3-ADON and was named NX-2. The substances were purified from 24-day-old rice cultures of isolate 06-156 via normal phase and subsequent reversed phase chromatography. Further liquid chromatographic high-resolution mass spectrometric (LC-HRMS) measurements revealed a sum formula of \( C_{17}H_{24}O_6 \) for NX-2 (\( [M+H]^+ \) calculated. for \( C_{17}H_{24}O_6 \), 325.1646; found, 325.1647; \( [M+NH_4]^+ \) calculated. 342.1911; found, 342.1915; \( [M+Na]^+ \) calculated. 347.1465; found, 347.1467). The obtained HRMS/MS spectra indicated the presence of an acetyl group and at least two hydroxyl groups in NX-2 (Fig. S1A). NX-1 was heavier than NX-2 by two protons and one oxygen atom.

Structure elucidation and signal assignment was carried out based on 1D nuclear magnetic resonance (NMR) (\( 1^H, 13^C-CPD \) or \( 13^C-APT \)) and 2D NMR (\( 1^H-1^H \) correlation spectroscopy, \( 1^H-13^C \) heteronuclear single quantum correlation and \( 1^H-13^C \) hetero-nuclear multiple bond correlation). 1D nuclear Overhauser effect (NOE) difference or 2D-nuclear Overhauser effect spectroscopy (NOESY) spectra were recorded to elucidate stereochemistry. The chemical structure was elucidated as 3\( \alpha \)-acetoxy-7\( \alpha \),15-dihydroxy-12,13-epoxytrichothec-9-en (Fig. 1E, 7-hydroxy-15-decalonectrin) and hence showed the characteristic backbone structure of trichothecenes. NMR data are summarized in Table S1 for \( 1^H \) and in Table S2 for \( 13^C \). Compared with 3-ADON, NX-2 lacks the keto-group at C-8 characteristic for type B trichothecenes and hence is classified as a type A trichothecene. NX-1 is a derivative of NX-2 (addition of water) very similar to the previously described DAS-M1 (Shams et al., 2011) and therefore assumed to be a preparation artefact.

3-ADON and 15-ADON can be rapidly hydrolysed to DON \textit{in planta}, and DON is the major form found in inoculated wheat. Suspecting a similar reaction for NX-2, its deacetylated product (named NX-3, Fig. 1D) was produced by alkaline hydrolysis and subsequent purification via preparative high-performance liquid chromatography (HPLC). The structure of NX-3 was also confirmed by HRMS (\( [M+H]^+ \) calculated. for \( C_{15}H_{22}O_6 \), 283.1540; found, 283.1540; \( [M+Na]^+ \) calculated. 305.1359; found, 305.1365) and NMR (see Table S1 for \( 1^H \)-NMR and in Table S2 for \( 13^C \)-NMR).

Formation of the new trichothecenes \textit{in planta}

The highly sensitive wheat cultivar ‘Apogee’ (Mackintosh et al., 2006) was grown in a growth chamber (19 h light per day, 20°C, rel. humidity 55%), and the ears were spray inoculated with suspensions of \textit{F. graminearum} PH-1 (as control) and different N-isolates (02-264, 06-146, 06-156 and 06-171). Samples were harvested after 11, 16 and 22 days. Liquid chromatographic-tandem mass spectrometric (LC-MS/MS) analyses revealed that DON was present in the control sample in high amounts (> 30 mg kg\(^{-1}\)), and none of the novel trichothecenes were detected. In the wheat ears treated with the N-isolates, approximately 10 times more NX-3 than NX-2 was detected (Table 1) and no DON was found. Similar results were obtained with these and further isolates on wheat cv. ‘Norm’ in a glasshouse experiment. Again the major compound was NX-3, with NX-2 occurring around 10 times less. No NX-1 was detected in any of the analysed wheat samples. Because GC-MS instruments are often used for the determination of trichothecenes in cereals, wheat heads inoculated with the \textit{F. graminearum} isolates were also analysed using this technique. A dominant peak at 6.08 min was observed in GC-MS, matching the retention

| Table 1. Concentration of NX-2 and NX-3 in wheat ears inoculated with different N-isolates. |
|---------------------------------------------|
| 02-264 | 06-146 | 06-156 | 06-171 |
| 11d | 16d | 20d | 11d | 16d | 20d | 11d | 16d | 20d | 11d | 16d | 20d |
| NX-2 (mg kg\(^{-1}\)) | 55 | 25 | 46 | 30 | 21 | 62 | 45 | 38 | 42 | 30 | 34 | 34 |
| NX-3 (mg kg\(^{-1}\)) | 480 | 190 | 260 | 220 | 260 | 540 | 330 | 350 | 460 | 270 | 380 | 370 |

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The mass spectrum of TMS-NX-3 is shown in Fig. S2. The compound produces a weak molecular ion at \( m/z \) 498 (\( M^+ \)) (Fig. S2, insert), which is confirmed by an intense \([M+H]^+\) ion at \( m/z \) 499.2720 by positive chemical ionization (PCI) high-resolution GC-MS. The molecular formula of TMS-NX-3 is verified as \( C_{24}H_{46}O_5Si_3 \). The existence of three hydroxyl groups is confirmed by the formation of fragment ions at 408.2152 \( m/z \), 318.1649 \( m/z \), and 305.1568 \( m/z \) (loss of one TMSOH and one \([\text{CH}_2=\text{OSi} (\text{CH}_3)_3]\) group). The mass spectrum of TMS-NX-3 also shows intense peaks at \( m/z \) 103, 147 and 181, which are common features for DON and its trichothecene analogues (Rodrigues-Fo et al., 2002). While a second peak at a retention time of 6.66 min was observed for TMS-NX-2, no NX-1 was found in the wheat samples.

**In vitro translation inhibition and phytotoxicity assays**

Trichothecenes are potent inhibitors of eukaryotic protein synthesis. However, derivatization at C-3 alleviates toxicity of otherwise inhibitory trichothecenes. To prevent self-intoxication of the producing fungi, the C3-OH of trichothecenes is protected by Tri101 mediated acetylation during biosynthesis and strongly reduced toxicity of 3-ADON to mammalian ribosomes compared with DON has been documented (Kimura et al., 1998). The inhibitory activity of the novel substances on plant and mammalian ribosomes were tested using *in vitro* translation systems based on wheat germ extracts or rabbit reticulocyte lysates. NX-3 showed translation inhibitor activity similar to DON in both assays (Fig. 3). NX-2 did not inhibit ribosomes of the rabbit reticulocyte lysate, but seemed to be inhibitory for wheat ribosomes to a considerable extent. This might be due to different susceptibilities of plant and mammalian ribosomes towards NX-2 or might be an artifact caused by de-acetylation during the assay. Therefore, we determined the NX-2 and NX-3 concentrations in the translation assays. In wheat germ assays, 31% of NX-2 was converted to NX-3 during the incubation time, while only marginal deacetylation (<1%) was observed with the rabbit reticulocyte lysate. From these observations, we conclude that NX-2 is not inhibitory for both rabbit reticulocyte and wheat ribosomes, and the apparent toxicity for wheat ribosomes is fully explained by formation of NX-3 by the wheat germ extract. Furthermore, the phytotoxicity of NX-2 and NX-3 using a *Chlamydomonas reinhardtii* growth assay was tested. Average generation times over 4 days using 100 \( \mu \)M of the trichothecene, were 21.5 h (NX-2), 23.9 h (3-ADON), 64.8 h (NX-3) and over 96 h (DON) compared with 20 h for an acetone control (Fig. S3).

**Genetic basis of the NX chemotype**

We intended to elucidate the cause for biosynthesis of NX-2 instead of 3-ADON. The *TRI1* genes of different *Fusarium* species encode rather distantly related cytochrome P450 monoxygenases (CYP), which are responsible for oxidation at C-7 and/or C-8 of the trichothecene scaffold (Alexander et al., 2009). While the Tri1 protein of *F. graminearum* was shown to hydroxylate both carbon atoms, the *F. sporotrichioides TRI1* gene product catalyses oxidation at C-8 only. Thus, we hypothesized that the N-isolates either contain a variant of *TRI1*, which is able to introduce the hydroxyl group at C-7 only, or this gene is defective and another of the numerous CYP genes in the *Fusarium* genome has taken over this function. To test this, we first determined the sequences of 20 N-isolates. A multilocus genotyping (MLGT) assay was used to confirm the identity of these isolates as *F. graminearum*. Furthermore, each of these isolates had single-nucleotide polymorphisms within the main trichothecene biosynthetic gene cluster (*TRI3* and *TRI12*), predictive of the 3-ADON trichothecene chemotype. The *TRI1* sequences of the N-isolates were compared with
those of *F. graminearum* PH-1. Eighteen of the 20 N-isolates shared identical *TRI1* sequences and the two remaining isolates (00-556 and 06-204) each differed from the major N-isolate sequence type by only one unique nucleotide. There were no differences in the predicted amino acid sequences of the 20 N-isolates. However, we identified 14 amino acid differences between the *TRI1* gene product of N-strains and PH-1 (Table 2).

To test whether the *TRI1* variant of the N-isolates is indeed responsible for specific oxidation at C-7, we constructed strains where the *TRI1* coding regions of PH-1 and one N-isolate are swapped (Fig. 4). In brief, we first deleted the *TRI1* genes in PH-1 and isolate 02-264 (lab designation WG-9 for ‘wild grass’) by replacing a large part of their coding regions with the hygromycin resistance gene; the resulting strains were named IAPT (isogenic to PH-1) and IAWT (isogenic to 02-264, WG-9). In the *tri1Δ* strains, neither DON, ADON nor NX-2 could be detected, but they accumulated calonectrin, which is an intermediate of trichothecene biosynthesis and the substrate of Tri1p. This result is inconsistent with the hypothesis that another gene may be responsible for the C7-hydroxylation. In the second step, we complemented the *TRI1* deletions with constructs carrying the other *TRI1* version. To this end, one *tri1Δ* strain of each genotype (IAPT10 and IAWT2) was transformed with a construct carrying a hybrid gene consisting of the core part of the *TRI1* from the opposite strain flanked by promoter and terminator regions of the receptor strain.

Five independent transformants were obtained for the PH-1 background carrying the N-version of *TRI1* (IAWP48, IAWP49, IAWP84, IAWP88, IAWP140). The heterologous complementation was confirmed by polymerase chain reaction (PCR) and restriction analysis (Fig. S4). The complementary experiment yielded one transformant (IAPW13), which, as expected, produced 3-ADON and DON instead of NX-2. This is consistent with the fact that the N-strains have single-nucleotide polymorphisms in the core *TRI* cluster that are predictive of the 3-ADON chemotype. As expected for a 15-ADON chemotype strain, PH-1 produced 15-ADON and DON, while in the WG-9 control samples formation of NX-2 was confirmed. One of the PH-1 derived strains carrying the N-*TRI1* allele (IAWP88) yielded a similar metabolite pattern as the parental *tri1* knockout strain IAPT10, indicating lack of expression for unknown reasons. Yet, in four out of the five PH-1 derived strains containing the *TRI1* from WG-9 (IAWP48, IAWP49, IAWP84 and IAWP140), two novel compounds were observed. One metabolite (named NX-4, Fig. 1F) was predicted to be the 15-acetyl equivalent of NX-2 because PH-1 is a 15-ADON producer. While the MS/MS pattern of NX-4 (Fig. S1C) was very similar to NX-2, the retention times were different (9.5 min instead of 10.4 min). The other compound presumably is the 15-acetyl-equivalent of NX-1. The candidate compound NX-4 was purified from strain IAWP84 by reversed phase chromatography and was characterized by HRMS and NMR (see Table S1 for 1H-NMR and in Table S2 for 13C-NMR). NX-4 was confirmed to be 15-acetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-ene and the HRMS results were [M+H]+ calculated for C17H24O6, 325.1646; found, 325.1643; [M+NH4]+ calculated 342.1911; found, 342.1914; [M+Na]+ calculated 347.1465; found, 347.1466. Hence, with the *TRI1* swap between PH-1 and the NX-isolate, we verified that the N-variant of Tri1p is responsible for the specific oxidation at position C-7 alone.

**Table 2.** Amino acid differences between N-isolates and PH-1.

| Amino acid position | N-isolate | PH-1 |
|---------------------|-----------|------|
| 33                  | T         | A    |
| 100                 | N         | S    |
| 115                 | L         | F    |
| 210                 | T         | S    |
| 252                 | S         | R    |
| 254                 | M         | L    |
| 256                 | N         | T    |
| 346                 | I         | F    |
| 361                 | F         | I    |
| 363                 | I         | V    |
| 373                 | E         | D    |
| 418                 | K         | Q    |
| 430                 | P         | T    |
| 450                 | V         | A    |

**Discussion**

We could demonstrate that the *F. graminearum* N-strains, seemingly lacking toxin production and previously even considered as potential biocontrol strains (Yuen et al., 2010), in fact produce a new type A trichothecene. The new metabolite, named NX-2, is formed in axenic cultures. It is similar to 3-ADON but lacks the C-8 keto group, which together with the conjugated C-9,10 double bond
creates the UV signal that is widely used by HPLC methods for detection of type B-trichothecene contamination. Hence, NX-2 escapes the analysis by HPLC-UV based methods.

In planta NX-2 is deacetylated to NX-3, which is the equivalent of the well-known mycotoxin DON without the C-8 keto group. We demonstrated that in kernels of infected wheat much higher levels of the deacetylated toxin, NX-3, compared with NX-2, are found, similar to the situation with DON, where the acetylated derivatives are also typically present in levels less than 10% of that of DON.

Regarding toxicity, we could demonstrate that at the level of the ribosomal target, NX-3 and DON have similar potency to inhibit protein synthesis. NX-2 is not inhibitory in the tested concentration range, similar to the situation for its counterpart 3-ADON. As the acetylated forms of DON are rapidly deacetylated after oral uptake in animals and humans, they are considered to be toxicologically equivalent to DON and were included in a group provisional maximum tolerable daily intake of 1 μg kg⁻¹ body weight together with DON (World Health Organization, 2011). It is currently unknown to which extent the new compounds occur in naturally contaminated grain and whether they are therefore of potential toxicological relevance. It is also still unknown, whether or to which extent the new toxins are covered already due to cross-reactivity by antibody-based detection devices, which are widely used to screen for DON contamination of grain.

In a survey of strains conducted in the northern part of the US wheat-growing areas highly affected by F. graminearum (Minnesota, North and South Dakota) NX-2 producing strains were isolated in only low frequency (approximately 3% of samples) (Liang et al., 2014). Some isolates, for instance the strain 02-264, were isolated from non-agricultural grasses. NX-2 producers therefore may have a reservoir in plants other than wheat. At present, it is completely unclear how prevalent N-strains are in other parts of the United States and whether they occur, for instance, also in Europe. The identified changes in the TRI1 gene can be used to develop specific PCR-based assays. Anecdotal evidence of grain with high levels of F. graminearum damaged kernels, but low DON content might be explained by the presence of such strains, which so far escaped detection.

It will be important to monitor whether the frequency of N-strains is changing, and most importantly to test whether they have a selective advantage on certain crop plants or genotypes. In the Northern United States and Canada, a shift in the chemotype composition of F. graminearum was observed and directly tied to the spread of a novel, and potentially introduced, population of F. graminearum (Ward et al., 2008). Prior to 2000, strains from the United States and Canada were almost exclusively 15-ADON producers; however, they have been increasingly replaced by 3-ADON producers in some major wheat-growing regions. The fact that all N-strains characterized to date have core TRI clusters of the 3-ADON type may indicate that the appearance of NX-2 producing strains is connected to the recent expansion of the 3-ADON population in North America.

NX-2 and 3-ADON producing strains primarily yield 3-acetylated trichothecenes, which have reduced toxicity. It is possible that this allows for higher toxin production, as was observed among the 3-ADON population in North America (Ward et al., 2008). We also observed a higher level of contamination with NX-3 of the spray inoculated ‘Apogee’ compared with DON levels produced by the genetically unrelated PH-1.

An intriguing hypothesis is that the fungi may benefit from protecting the C3-OH of the toxin against plant detoxification enzymes, such as uridine diphosphate-glucosyltransferases, residing in the plant cytosol. In the highly F. graminearum-affect ed spring wheat regions, wheat cultivars incorporating the Fhb1 resistance quantitative trait locus (QTL) are increasingly common (McMullen et al., 2012). It has been shown that the Fhb1 locus colocalizes with the ability to efficiently convert DON into DON-3-O-glucoside (Lemmens et al., 2005), which is far less inhibitory to wheat ribosomes (Poppenberger et al., 2003).

The new toxin NX-2 in addition to potentially escaping glycosylation may provide another advantage for the fungus. It has been shown that a glutathione adduct of DON can be formed (Gardiner et al., 2010), and that this reaction actually occurs in planta (Kluger et al., 2013). Presumably, the bulky glutathione residue prevents interaction with the ribosomal target (Fruhmann et al., 2014). At present, it is unknown whether glutathione-mediated detoxification of the fungal virulence factor DON is relevant for Fusarium resistance and selected by breeders. For the formation of the Michael adduct, it is necessary that the thiol group of glutathione can react with the α,β-unsaturated ketone. The lack of the keto group therefore may allow to circumvent this detoxification pathway, which could provide a selective advantage to the fungus. The construction of near isogenic strains only differing in the TRI1 (and TRI18) alleles is an important step towards further work aiming to test these hypotheses on wheat cultivars differing in defined QTL regions.

In the TRI1 swapping experiment, we have also generated a new toxin, NX-4, corresponding to 15-ADON lacking the keto group. This compound at present is not known to occur naturally. Yet, because the TRI1 locus is not linked with the core TRI cluster, such segregants should arise during crossing of NX-2 producers and
15-ADON producers. More intensive screening may reveal that such strains occur in nature. Likewise, it seems possible that outcrossing with NIV producers, which have an intact TRI13 gene encoding a C4-hydroxylation enzyme in the core cluster and are prevalent in Asian countries, could lead to formation of the analogues of NIV and fusarenon X (4-acetyl-NIV) lacking the keto group.

In summary, we have discovered that a fungus that is a major threat to cereal production can make previously unknown toxins. The most relevant questions for future research are whether these compounds occur already in amounts relevant for public health and, in the worst case, whether NX-2 producers possess an adaptive advantage that may permit them to overcome progress made by plant breeders in the last decades.

Experimental procedures

Fungal strains

The following *F. graminearum* strains were used in this study: PH-1 (NRRL 31084, FGSC 9075), 00-556 (NRRL 66047), 02-264 (WG-9, NRRL 66037), 06-146 (NRRL 66030), 06-156 (NRRL 66038), 06-171 (NRRL 66036) and 06-204 (NRRL 66033). Details regarding their origin and construction of derived strains can be found in Table S3.

Trichodiene measurements by GC-MS

To verify an active Tri5 protein, required for trichothecene synthesis, the presence of trichodiene, the volatile precursor of trichothecene mycotoxins, was monitored in the HS of cultures from N-isolates of *F. graminearum* (02-264, 06-146, 06-156 and 06-171). Because of lack of an authentic standard, *F. graminearum* PH-1 (Gaffoor et al., 2005) in which trichodiene was already previously detected and annotated (Jélen et al., 1995) was used as a reference. For each culture, 8000 spores were pipetted on 2 ml potato dextrose agar in HS vials and cultivated at 22°C in the dark for 60, 70, 90, 100, 120 and 140 h. They were inoculated at different time points to allow consecutive measurements of all fungal cultures in 1 day. The fungal cultures were flushed with synthetic air 6 hours prior to extraction. HS solid phase microextraction (SPME) GC-MS measurements were performed using a Gerstel MPS2XL autosampler and Gerstel Maestro 1.3.20.41 (Mühlheim, Germany) for HS SPME and an Agilent GC 6890N coupled to 5975B inert XL MSD with Agilent MSD ChemStation G1701EA E.02.00.493 (all Agilent Technologies, Waldbronn, Germany) for GC-MS. HS SPME was performed at 22°C for 52 min using a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 23 gauge, 2 cm stable flex fibres (Gerstel). The analytes were separated on an Agilent DB5-ms column (30 m × 0.25 mm, 0.25 μm) and introduced to the MS interface via a non-coated guard column (0.5 μm × 0.1 mm, Agilent Technologies). The abundances of the detected sesquiterpenes were determined using METABOLITEDETECTOR (Hiller et al., 2009).

Cultivation of *F. graminearum* on rice cultures and sample preparation for LC-MS measurements

*Fusarium graminearum* PH-1 (as control) and four N-isolates (02-264, 06-146, 06-156, and 06-171) were routinely grown on Fusarium minimal medium (Leslie and Summerell, 2006). When appropriate, the following antibiotics were added: hygromycin B at 100 μg ml⁻¹ and G418 at 40 mg l⁻¹. Strains were sporulated in mung bean broth (10 g mung beans per litre of water), and conidiospores were quantified by counting them in a Fuchs-Rosenthal chamber. For mycotoxin production, strains were cultivated on solid rice media. Baby food jars (200 ml), containing 10 g rice from a local store and 10 ml reverse osmosis water, were incubated at room temperature for 1 h, autoclaved for 60 min at 121°C and incubated with 10⁵ spores. Incubation was performed for 1, 2 or 3 weeks at 20°C, 55% humidity at constant illumination. Thereafter, the samples were homogenized using 40 ml of acetonitrile : water : acetic acid (79:20:1, v:v:v) by an Ultra-Turrax T25 from IKA-Werke (Staufen, Germany) and extracted for 90 min at room temperature on a GFL rotary shaker (Burgwedel, Germany). After separation by centrifugation (10 min, 3220 g), 1 ml of the supernatant was transferred to an HPLC vial for analysis.

Screening for novel metabolite with LC-MS

Screening was performed on an Agilent Technologies 1100 series HPLC system coupled to a QTrap LC-MS/MS system from Applied Biosystems (Foster City, CA, USA). Using atmospheric pressure chemical ionization, a full scan from 200 to 800 Da (cycle time 1 s) using the third quadrupole in positive ionization was conducted. Two microlitres of the fungal extracts were injected. Chromatographic separation was achieved at 25°C with a flow rate of 0.75 ml min⁻¹ on an Agilent Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5 μm). The eluents were composed of water : methanol (A: 20:80, v:v; B: 10:90, v:v), and both contained 5 mM ammonium acetate. After an initial holding time of 2 min at 0% B, a linear gradient to 100% B within the next 10 min was applied, followed by a holding period of 3 min at 100%, a quick switch to 0% B at 15 min and a column equilibration for 5 min at 0% B. The following MS settings were used: source temperature 450°C, curtain gas 35 psi, sheath gas (GS1) 60 psi, drying gas (GS2) 15 psi, interface heater on, corona discharge current 2 μA, declustering potential 20 V. Data were acquired and analysed using Analyst 1.5.2. software from AB Sciex.

Purification of NX-2

For purification of NX-2 rice cultures of isolate 06-156 were cultivated for 24 days as described above and stored at −20°C until further processing. Each rice culture was homogenized with 80 ml ethyl acetate using an Ultra-Turrax and then extracted for 90 min at room temperature on a rotary shaker. The extracts were pooled and evaporated to dryness on silica gel using a rotary evaporator at room temperature to avoid formation of NX-1. Normal phase chromatography was performed using a 40 × ca. 800 mm self-packed silica gel
Production and purification of NX-3

Part of the purified NX-2 was dissolved in methanol : water (50:50, v:v) and mixed with the equal volume of 1% aqueous Na2CO3. Every 20 min, the conversion was checked by LC-MS measurements on the QTrap-LC/MS system, and after 200 min the reaction was stopped by the addition of acetic acid. Preparative HPLC purification was performed immediately afterwards using a Gemini NX C18 column (150 × 21.5 mm, 5 μm) and a guard column of the same material from Phenomenex (Aschaffenburg, Germany). A linear methanol-water gradient comprising of an initial 1 min hold time of 44% methanol and reaching 100% within further 5 min was applied. NX-2 was collccted with a peak-based threshold at around 5 min. Chemstation B04.03 was used for acquisition. The fractions containing NX-2 were pooled and freeze dried after removal of methanol using a rotary evaporator at room temperature. Twenty-five milligrams of a white powder (purity > 95% according to LC-UV at 200 nm) were obtained from 42 rice cultures.

Formation of new trichothecenes in planta

The highly sensitive wheat line ‘Apogee’ was spray inoculated with spore suspensions of F. graminearum PH-1 and four different N-isolates (02-264, 06-146, 06-156 and 06-171). After incubation at 25°C for 11, 16 and 22 days the wheat heads were removed from the stems and frozen at −80°C until further processing. The samples were ground using a Retsch ball mill (Haan, Germany) for 1 min and were subsequently extracted with the fourfold amount of acetonitrile : water : acetic acid (79:20:1, v:v:v) for 60 min at 20°C on a rotary shaker for LC-MS/MS analysis. They were centrifuged for 10 min at 20°C and 4000 g, the supernatant was transferred to an Eppendorf tube and centrifuged again for 5 min at 10 000 g before being transferred to an HPLC vial. The samples were stored at −20°C until analysis.

LC-MS/MS measurements of inoculated wheat

With the obtained standards for NX-2 and NX-3, the compounds were optimized on a 4000 QTrap LC-MS system from AB Sciex (Foster City, CA, USA) using positive and negative electrospray ionization. A selected reaction monitoring (SRM) method in negative ionization mode was developed and used for the analysis of the infected wheat ears. Chromatographic separation was achieved using an Agilent Technologies 1290 UHPLC system and a Gemini C18 column (150 × 4.6 mm, 5 μm, Phenomenex) at 25°C and a flow rate of 800 μl min⁻¹. The eluents were composed of water : methanol : ammonium acetate (A: 80:19:1, v:v:v) both containing 5 mM ammonium acetate. After an initial holding period of 0% B for 1 min, the gradient was linearly increased to 100% B within the next 10 min. The column was washed for 2.5 min at 100%, followed by a fast switching back to the initial conditions and holding these for 2.5 min; hence, the total run time was 16 min. For the SRM transitions (summarized in Table S4), the applied dwell time was 25 ms. The following MS settings were used: source temperature 550°C, curtain gas 30 psi, sheath gas (GS1) 50 psi, drying gas (GS2) 50 psi, interface heater on. Data were acquired and analysed using ANALYST 1.5.2. software from AB Sciex.

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GC-MS characterization of inoculated wheat

A wheat spikelet inoculated with strain 02-264 was weighed, 2 ml volume of acetonitrile : water (84:16, v:v) were added and the vial was shaken on an Eberbach reciprocal shaker (Ann Arbor, MI, USA) at room temperature for 24 h. The extract was passed through a minicolumn packed with C18 and aluminum oxide (1:3; w:w). An aliquot (1.5 ml) of the filtrate were evaporated to dryness under nitrogen and 25 μl derivatization reagent (trimethylsilylimidazole: TMS chloride = 100:1, v:v) were added. After 10 min of shaking, 200 μl isooctane containing 4 mg l−1 of mirex (internal standard) were added and shaken gently, after which 200 μl of HPLC water was added. The vial was vortexed and the clear upper isooctane layer was transferred to a GC vial with a 200 μl glass insert. The GC-MS analysis was performed on a Shimadzu GCMS-QP2010 (Kyoto, Japan). Perfluorotributylamine was used to tune the mass spectrometer, and an Agilent DB-5ms capillary column (30 m × 0.25 mm, 0.25 μm) was used in the GC system. A high-pressure injection method (300.0 kPa, 1.00 min) was used in the splitless injection system. The injector, ion source and interface temperatures were kept at the following oven temperature programme: 150°C for 1 min, increase with 30°C min−1 to 280°C and holding for 5 min. Injection, ion source and interface temperatures were kept at 260, 250 and 280°C respectively. Injection volume was 1 μl. The data were collected from m/z 50 to 550 in positive ion electron ionization (EI) mode with EI energy of 70 eV. High-resolution accurate mass measurements were performed in positive ion EI and PCI modes using a Finnigan MAT 95 double-focusing instrument (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a HP5890 Series II gas chromatograph (Agilent Technologies) with the same capillary column. The instrument resolution was set at 4000 (10% valley). Perfluorokerosene was used as internal reference for EI. The reagent gas for PCI was 4% ammonia in methane.

In vitro translation assays

Coupled in vitro transcription/translation systems [TnT® T7 coupled Rabbit Reticulocyte Lysate System and Tnt® T7 Coupled Wheat Germ Extract System both from Promega (Madison, WI, USA)] were used for the assays. Standard transcription/translation reactions were performed in a total volume of 15 μl according to the manufacturer’s instructions in the presence of the respective compounds in 0.2% methanol (final concentration). First, the ribosomes were pre-incubated with buffer, amino acids, DNA and inhibitor for 7 min, and T7-RNA polymerase was added to start the coupled in vitro transcription/translation. The reactions were stopped by addition of 1 μl of a 1 mM cycloheximide solution after 24 min in case of reticulocyte lysate or after 30 min for the wheat germ assays respectively. Efficiency of translation was determined by measuring activity of the firefly luciferase reporter using the Promega Steady-Glo® Luciferase Assay System and the 2300 EnSpire®Multimode Plate Reader from Perkin-Elmer. At least three independent experiments were conducted for each compound and each treatment. The amount of luciferase was determined by using the Promega Steady-Glo® Luciferase Assay System.

Chlamydomonas reinhardtii toxicity assays

Chlamydomonas reinhardtii wild-type strain CC-125 mating type + was obtained from the Chlamydomonas Genetics Center (Department of Botany, Duke University, Durham, NC, USA). The phytotoxicity of individual trichothecenes was tested as described previously (Alexander et al., 1999). Triplicate 10 ml liquid cultures were initiated with 1×10^5 cells ml−1 on a high-salt, high-acetate medium (Harris, 1989) containing 100 μM concentration of an individual trichothecene and grown for 4 days at room temperature, with shaking at 200 r.p.m. Trichothecenes were dissolved in acetone and the final concentration of acetone was less than 1%. Generation times were calculated from the number of culture doublings (log of the final density – log of the initial cell density) (log) 2 over 4 days.

Genetic basis of the NX chemotype

Fusarium graminearum DNA was extracted as published (Umpierrez-Failache et al., 2013). Species identity and the genotype profile of the main trichothecene biosynthetic gene cluster were determined simultaneously via MLGT as described previously (Ward et al., 2008). Amplification of the TRI1 region was performed in 25 μl volumes with 1× High Fidelity PCR buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), 2 mM MgSO₄, 0.2 mM concentration of each deoxynucleoside triphosphate, 0.6 μM concentrations of primers Tri16-IF1 and Fg_Tri1-R1, 1.0 units of Platinum Taq DNA Polymerase High Fidelity (Invitrogen Life Technologies) and 200 ng of genomic DNA. PCR consisted of an initial denaturation of 120 s at 96°C, followed by 35 cycles of 30 s at 94°C, 30 s at 53°C and 180 s at 68°C. PCR products were purified using Montage PCR96 Cleanup filter plates from Millipore (Billerica, MA, USA). Sequencing reactions were carried out according to the method of Platt and colleagues (2007) using internal primers. Sequencing reaction products were purified using BigDye XTerminator (Applied Biosystems) and analysed with an Applied Biosystems ABI 3730 DNA Analyzer. DNA sequences were assembled and edited with Sequencher (version 4.10, Gene Codes, Ann Arbor, MI, USA), and were aligned with the TRI1 locus from the whole genome shotgun sequence of PH-1 (locus tag FG00071.1) using the MUSCLE algorithm implemented in MEGA (Tamura et al., 2013). Amino-acid sequences were inferred based on the predicted TRI1 coding DNA sequence of PH-1.

Disruption and heterologous complementation of TRI1 in PH-1 and 02-264 (WG-9)

Fusarium graminearum strains PH-1 and 02-264 (WG-9) were routinely grown and sporulated as described above. Transformation of F. graminearum was essentially performed...
as described (Gaffoor et al., 2005) with the following minor modifications: 100 ml yeast extract peptone dextrose were inoculated with 2.5 to 5 x 10⁸ conidia from mung bean broth and incubated with shaking (180 r.p.m.) at 20°C; after separation from remaining mycelium protoplasts were pelleted and washed once with STC buffer (containing sorbital, Tris-HCl and CaCl₂); 10⁷ protoplasts were transformed with a total of 10 μg DNA in a total volume of 6 ml, and 600 μl portions were transferred to 15 ml aliquots of regeneration medium and poured into Petri dishes. After 2 h of regeneration, each culture was overlayed with 15 ml of regeneration medium containing twofold concentration of antibiotic (200 mg l⁻¹ hygromycin B or 80 mg l⁻¹ G418 respectively). After 4–8 days, resistant strains were transferred to Fusarium minimal medium plates containing the appropriate amount of antibiotic. Each transformant was sporulated and plated to single colonies on selective media for two times to ensure genetically pure clones. For PCR of genomic DNA, small amounts of freshly grown mycelium were re-suspended in 50 μl Tris pH 7.5, heated at full power in a microwave oven for 45 s. Two microlitres were used for PCR.

Genomic DNA was prepared from both strains and the TRI1 coding region (including introns) and 3′ flanking region was amplified using primers Xba-FgTRI1-fw and FgTRI1.Kpn-rv and cloned into pAB86. The resulting plasmids were termed pAB206 and pRS21. Tables S5 and S6 provide detailed information about the oligonucleotides used.

For disruption of the TRI1 genes in PH-1 and WG-9, the flanking regions of both TRI1 alleles were first cloned into plasmid pASB42, derived from pUG5 (Güldener et al., 1996). Approximately 750 bp from the TRI1 promoter regions of PH-1 or WG-9 were amplified with primer pair TRI1(PH-1)Δ down_SalI-fw and TRI1Δ up_SfiI-fw or TRI1(PH-1)Δ up_SfiI-rv and down_SalI-rv, respectively, for amplification of DNA from PH-1 and WG-9. The resulting fragments were digested with SfiI and SalI to release fragments containing only the 5′ flanking region from PH-1 and the coding region from WG-9 (Fig. S4). Transformants were selected on 40 mg l⁻¹ hygromycin resistance marker. A total of 170 G418 resistant PH-1 transformants yielded five hygS strains (named IAWP48, IAWP49, IAWP84, IAWP88 and IAWP140), while one hygS transformant (IAPW13) was found among 26 WG-9 transformants. To confirm that the hph marker was indeed replaced by the hybrid genes, PCRs were performed with the same primer combinations as described above for TRI1 deletion. Furthermore, the TRI1 genes were amplified from all transformants and from the parental strains PH-1 and WG-9 using primers Xba-FgTRI1-fw and FgTRI1.Kpn-rv, and the resulting fragments were digested with ApoI (Fig. S4). Constructs and genomic regions relevant for disruption of TRI1 and heterologous complementation are summarized in Fig. S5.

Purification of NX-4

Three 21-day-old rice cultures of the strain IAWP84 with swapped TRI1 (Table S3) were extracted with ethyl acetate as described above. The extracts were pooled and evaporated to dryness at room temperature. After dissolving in acetonitrile, the extract was defatted two times with the 2.5 times amount of hexane. The acetonitrile layer was then directly injected into the preparative HPLC system. Separation was achieved on a SunFire OBD C18 column (100 x 19 mm, 5 μm, Waters, Eschborn, Germany) and a C18 guard column using a methanol water gradient. After an initial hold time of 1 min with 25% methanol, the methanol content was linearly increased to 70% methanol within the next 4 min. From 5.1 to 8 min, the column was flushed with...
100% methanol before the column was equilibrated using the starting conditions. NX-4 was collected by time-based fractioning around 4.9 min. The fractions containing NX-4 were pooled and freeze dried after removal of methanol using a rotary evaporator at room temperature. From three rice cultures, 7.5 mg of a white powder (purity > 90% according to LC-UV at 200 nm) were obtained.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher’s web-site:
Fig. S4. PCR – restriction fragment length polymorphism (PCR RFLP) for confirmation of swapping of TRI1 alleles. Left side: schematic representation of TRI1 locus with primers used for amplification and the Apol restriction sites. Right side: Restriction analysis of PCR fragments amplified from wild-type controls and transformants.

Fig. S5. Constructs and genomic regions relevant for disruption of TRI1 and heterologous complementation. (A) Schematic representation of plasmids used for production of DNA used for disruption and for construction of hybrid genes for heterologous complementation. (B) Genomic region of TRI1 wild-type and (C) genomic region of tri1 deletion. Fusarium graminearum genomic DNA is represented in grey, plasmid sequences and selection markers are given in black, small black boxes represent primers for PCR amplification (Table S2). PCR products are shown in orange (TRI1 flanking regions), blue (for gene disruption) and red (for confirmation of tri1 deletion).

Table S1. ¹H NMR data (δ, p.p.m.; multiplicity; J, Hz).
Table S2. ¹³C NMR data (δ, p.p.m.).
Table S3. Fusarium graminearum strains used in this work including the genetic background and the source.
Table S4. Optimized MS and MS/MS parameters.
Table S5. List of oligonucleotides used in this work including the sequence and purpose.
Table S6. List of plasmids used in this work including the relevant characteristics, backbone, purpose and source.