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ORIGINAL PAPER

Retrotransposon and gene activation in wheat in response to mycotoxigenic and non-mycotoxigenic-associated *Fusarium* stress

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Abstract Despite inhibition of protein synthesis being its mode of action, the trichothecene mycotoxin deoxynivalenol (DON) induced accumulation of transcripts encoding translation elongation factor 1α (*EF-1α*), class III plant peroxidase (*POX*), structure specific recognition protein, basic leucine zipper protein transcription factor (*bZIP*), retrotransposon-like homologs and genes of unknown function in the roots of wheat cultivars CM82036 and Remus. Fusarium head blight (FHB) studies using *Fusarium graminearum* and its trichothecene-minus (*Tri5*$^-$) mutant derivative and adult plant DON tests showed that these transcripts were responsive to both mycotoxigenic- and non-mycotoxigenic-associated *Fusarium* stress. In tests using the parents 'CM82036', 'Remus' and 14 double-haploid progeny that segregated for quantitative trait locus (QTL) *Fhb1* on chromosome 3BS (syn. *Qfhs.ndsu-3BS*) (from 'CM82036' that confers DON tolerance), *bZIP* expression was significantly more DON-up-regulated in lines that inherited this QTL. Basal accumulation of the *bZIP* transcript in spikelets treated with Tween20 (control), DON and in DON-relative to Tween20-treated spikelets was negatively correlated with DON-induced bleaching above (but not below) the treated spikelets ($AUDPC_{DON}$) ($r = -0.41$, -0.75 and -0.72, respectively; $P \leq 0.010$). *bZIP*-specific PCR analysis of 'Chinese spring' and its 3BS deletion derivatives indicated that *bZIP* is located in chromosomal region(s) other than 3BS. These results, and the fact that a homologous cold-regulated wheat *bZIP* (*wLIP19*) maps to group 1 chromosomes suggests that wheat bZIP may participate in defence response cascades associated with *Fhb1* and that there is a cross-talk between biotic and abiotic stress signalling pathways.

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Abbreviations

BZIP	Basic leucine zipper protein
cv. or cvs	Cultivar(s)
DDRT-PCR	Differential display reverse transcriptase-polymerase chain reaction
DON	Deoxynivalenol
EF-1α	Translation elongation factor 1 alpha
FHB	Fusarium head blight
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDNA	Genomic DNA
LTR	Long terminal repeat
PCR	Polymerase chain reaction
POX	Peroxidase
QTL	Quantitative trait loci
RT-PCR	Reverse transcriptase-PCR
SSRP	Structure-specific recognition protein

Introduction

Fusarium head blight (FHB) is an important disease of wheat and has worldwide distribution, which results in yield loss and mycotoxin contamination of grain when the disease is caused by a mycotoxin-producing *Fusarium* species. *Fusarium graminearum* and *F. culmorum* are the *Fusarium* species that most commonly cause FHB of wheat and both can produce trichothecene mycotoxins, predominantly deoxynivalenol (DON, also known as vomitoxin) (Parry et al. 1995). *Fusarium* fungi attack wheat heads at mid-anthesis, but do not invade host cells until they die, and Kang and Buchenauer (2000) speculated that this pathological change may be associated with toxins produced by the fungus. DON accumulation in infected wheat spikes showed a very close relationship to pathogenic changes in the host cells, symptom appearance and pathogen colonisation in the host tissues (Kang and Buchenauer 1999). The reduced aggressiveness of a trichothecene-minus mutant of *F. graminearum*, compared to that of the wild type DON-producing isolate, and the inability of the former to spread beyond the point of infection, confirmed the role of DON as a virulence factor (Bai et al. 2002).

Wheat cultivars (cvs) differ in their susceptibility to FHB disease and to DON. In susceptible cultivars, *Fusarium* infection causes premature bleaching of wheat heads, resulting in shrivelled grain (Parry et al. 1995). A series of alterations occur in *Fusarium*-infected host tissues, including degeneration of host cytoplasm and organelles, collapse of parenchyma cells, disintegration or digestion of host cell walls and appearance of electron-dense coating materials on vessel walls (Kang and Buchenauer 2000). DON also induces premature bleaching of wheat heads (Lemmens et al. 2005) and localises in the cytoplasm, plasmalemma and chloroplasts and is sometimes associated with endoplasmic reticula and ribosomes (Kang and Buchenauer 1999). DON alters plasma membrane permeability; the plasma membrane is more sensitive to such alterations in the dark and in the presence of calcium (Bushnell et al. 2004). Calcium triggers vacuole collapse and plays a key role in plant programmed cell death (PCD) (Jones 2001). Interestingly, DON treatment retarded the development of typical PCD cell morphology induced by heat shock in Arabidopsis (Diamond et al., submitted for publication). In Arabidopsis, UDP-glycosyltransferase detoxifies DON to DON-3-glucoside (Poppenberger et al. 2003); DON-3-glucoside has also been isolated from DON-treated spikelets of 52 different wheat cultivars (Berthiller et al. 2005).

Resistant genotypes may possess one or more types of FHB resistance. Many researchers describe resistance to initial infection (type I resistance) and resistance to spread of the fungus within host tissue (type II resistance) (Schroeder and Christensen 1963) as FHB resistance components. The ability to degrade the mycotoxin DON (Miller and Arnison 1986) is one of several other proposed components of resistance. Mapping studies have identified several quantitative trait loci (QTL) which are either associated with a specific FHB resistance component and/or general FHB resistance. QTL on the short arms of chromosomes 3B (i.e. *Fhb1*; syn. *Qfhs.ndsu-3BS*) and 6B have been detected in different mapping populations segregating for 'Sumai 3'-derived resistance genes (reviewed by Yang et al. 2005); whether these QTL represent a single gene or a cluster of linked genes remains to be elucidated. Lemmens et al. (2005) recently showed that the 'Sumai 3'-derived QTL *Fhb1* in 'CM82036' is also associated with DON tolerance.

Functional genomics studies have revealed that a range of host genes are activated in FHB resistant wheat spikelets in response to DON-producing *Fusaria*, including biotic and abiotic stress-related genes, house-keeping genes and several genes of unknown function (Pritsch et al. 2001; Kruger et al. 2002; Han et al. 2005; Kong et al. 2005). Han et al. (2005) and Kong et al. (2005) identified several *Fusarium*-responsive genes in wheat cvs Frontana and Ning7840, respectively, and determined the chromosomal location of several such genes. Some defence-related transcripts, such as actin-depolymerising factor, were activated in a genotype-dependent manner (Kong et al. 2005). A recent study identified that *F. graminearum* inoculation of spikelets of the FHB resistant cv. Ning7840 up-regulated a 14-3-3-protein and a β-1,3 glucanase and up-regulated or induced proteins with an antioxidant function (i.e. superoxide dismutase, dehydroascorbate reductase, and glutathione *S*-transferases) (Zhou et al. 2005),

As yet, no studies have determined what genes in the wheat genome are trichothecene-responsive. A preliminary study indicated that a novel wheat transcript was up-regulated in wheat in a non-tissue-specific manner in response to DON and *F. graminearum*; this transcript is currently being characterised (O. Rocha, K. I. Ansari and F. M. Doohan, unpublished data). The objective of this study was to identify transcripts in wheat roots that were DON-responsive and to analyse the accumulation of these transcripts in heads of wheat cvs Remus (FHB-susceptible) and CM82036 (FHB-resistant) in response to both a wild type DON-producing *F. graminearum* strain and its

trichothecene-minus (*Tri5*$^{-}$) mutant derivative. We also determined the relationship between transcript accumulation and DON tolerance using a population of double haploid progeny derived from 'CM82036' × 'Remus' cross. The double-haploid progenies contained either a QTL on chromosome 5A (i.e. *Qfhs.ifa-5A*) that is associated with FHB resistance, or chromosome 3BS (i.e. *Fhb1*) that is associated with FHB resistance and DON tolerance, or both the QTL or none (Buerstmayr et al. 2003; Lemmens et al. 2005).

Materials and methods

Plant material

Seeds of *Triticum aestivum* cvs CM82036 and Remus, and a population of 14 recombinant F_1-derived double haploid (DH) lines originating from a cross between CM82036 (FHB resistant)/Remus (FHB susceptible) (Buerstmayr et al. 2003) were kindly supplied by Dr. Hermann Buerstmayr (IFA-Tulln, Austria). 'CM82036' carries two major QTL for FHB resistance, one on the short arm of chromosome 3B associated with FHB resistance and DON tolerance (i.e. *Fhb1*; syn. *Qfhs.ndsu-3BS*) and one on chromosome 5A associated with FHB resistance (i.e. *Qfhs.ifa-5A*) (Buerstmayr et al. 2003; Lemmens et al. 2005). Seeds of 'Chinese spring' (accession No. Cltr 14108) and its four chromosome 3BS deletion mutants derivatives TA4524L1, TA4524L2, TA4524L4 and TA4524L7 were obtained from Wheat Genetics Resource Center of Kansas State University (Manhattan, KS, USA). These mutant derivatives lack the distal region of wheat chromosome 3BS; the fragment length (FL) of the retained part of chromosome 3BS is 0.33, 0.56, 0.55 and 0.75, respectively (Endo and Gill 1996; Liu and Anderson 2003). The wheat QTL Qfhs.ndsu-3BS was fine-mapped to the chromosome bin 3BS 0.78–0.87 (Liu and Anderson 2003).

Seedling experiments

Seeds were germinated on moist Whatman No. 1 filter paper (Whatman, UK) (at 20°C, for 48 h), vacuum infiltrated for 20 min (VP3 pump, KNF Neuberger, Germany) with either water, or 20 μg ml^{-1} DON and further germinated at 10°C in Petri dishes containing Whatman No. 1 filter paper (Whatman, UK) moistened with the same solution (12 seedlings per dish and two dishes per treatment per wheat cultivar). Roots were harvested 24 h post-treatment, flash-frozen in liquid N_2, freeze-dried and stored at −70°C prior to RNA extraction. This experiment was conducted twice for differential display analysis and twice for RT-PCR analysis.

Glasshouse head blight trials

The FHB glasshouse trials were conducted as described by Doohan et al. (1999), using conidial inoculum (10^6 conidia ml^{-1} 0.02% Tween 20) of *F. graminearum* wild type strain GZ3639 and its trichothecene-minus mutant GZT40 (kindly supplied by from Robert Proctor, USDA Agricultural Research Service, Peoria, IL, USA) (Proctor et al. 1995). Fungal maintenance and conidial inoculum production conditions were as described by Doohan et al. (1999). Heads were spray-inoculated with 4 ml of conidial inoculum; control heads were treated with 0.02% Tween 20 (Doohan et al. 1999). Visual disease symptoms (percentage bleached spikelets per head) were scored at 7 and 14 days post-inoculation (dpi). At 7 and 14 dpi, heads were harvested, flash-frozen in liquid N_2, freeze-dried and stored at −70°C prior to RNA extraction. The head blight experiment included four plants (two heads per plant) per treatment per time point and was conducted twice.

Adult plant DON tolerance trials

Plants were grown (two per pot) as described by Doohan et al. (1999) and the florets of four central spikelets per head were treated with 15 μl of either 5 mg ml^{-1} 0.2% Tween 20 DON or 0.2% Tween 20 (controls) at mid-anthesis, applied between the palea and the lemma, as described by Lemmens et al. (2005). Treated spikelets were harvested 24 h post-treatment, flash-frozen in liquid N_2, freeze-dried and stored at −70°C prior to RNA extraction. The number of bleached spikelets/total number of spikelets both below and above the removed treated spikelets was determined at 13 and 21 days post-treatment and these values were used to calculate the area under the disease progress curve ($AUDPC_{DON}$). The DON tolerance trial included two heads (one per plant) per treatment per wheat cultivar and was conducted twice.

DNA and RNA extraction

DNA was extracted from wheat leaves as previously described (Doyle and Doyle 1987). For RNA extraction, freeze-dried root, spikelet or head samples were placed in tubes containing 3.3 mm sterile stainless steel balls and ground to fine powder using an 8000 mixer/mill (Spex CentriPrep, Glen Creston Ltd., UK). Total

RNA was extracted from the resulting powder using the protocol described by Chang et al. (1993) and DNase1-treated according to the manufacturer's instructions (Invitrogen, UK).

Differential display analyses

Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) analysis was performed using a modification of the protocol described by Liang and Pardee (1992) (duplicate reactions per sample). Reverse transcription reactions were performed in a total volume of 25 µl containing 1 µg of total RNA, 2.4 µM of oligo dTNC or dTNG (Invitrogen, UK), 100 U of MMLV reverse transcriptase, 1× first strand buffer (Promega, USA), 100 µM each of dATP, dGTP, dCTP and dTTP (Gibco BRL, UK), 1 mM dithiothreitol and 20 U of RNaseOut (Invitrogen, UK). Two microlitre of cDNA was PCR-amplified in a 25 µl reaction volume containing 1 U of Taq DNA polymerase and 1× PCR buffer (Invitrogen, UK), 1.5 mM $MgCl_2$, 150 µM each of dATP, dGTP, dCTP and dTTP, and 0.8 µM each of dTNC/dTNG and 1 µM 10-mer random primers (Operon Technologies, USA). PCR reactions were conducted in a Peltier thermal cycler DNA engine (MJ Research, UK) and the programme constituted 40 cycles of 94°C for 30 s, 42°C for 30 s and 72°C for 45 s, with a final extension at 72°C for 5 min. The resulting DDRT-PCR products were separated using 5% polyacrylamide gel electrophoresis (PAGE) and visualised by silver staining as described by Doyle (1996). Bands in PAGE gels representing transcripts differentially expressed with respect to DON treatment were eluted and re-amplified by PCR as described above.

Cloning and sequencing

Differentially expressed bands were cloned (pGEM-T® cloning kit, Promega, UK), sequenced (duplicate reactions per sample) (MWG Biotech, Germany), and their consensus sequences were subjected to BLAST analysis (Altschul et al. 1997) using the NCBI and TIGR wheat blast search engine.

PCR and RT-PCR analyses

Sequence data was used to design gene-specific primers for PCR and RT-PCR analysis (duplicate reactions per DNA or RNA sample) (Table 1). PCR analysis of gDNA determined that primers showed no cultivar-biased amplification. PCR reactions were as described above for DDRT-PCR except that they contained 20 ng gDNA, 1 µM each of forward and reverse gene-specific primer and the programme constituted 40 cycles of 94°C for 30 s, 60°C for 20 s and 72°C for 45 s, with a final extension at 72°C for 5 min. PCR products were electrophoresed through 2% (w v^{-1}) agarose gels containing 0.5 µg ml^{-1} ethidium bromide and visualised using Imagemaster VDS and Liscap software (Pharmacia Biotech, USA).

Transcript accumulation was confirmed and further analysed by RT-PCR analysis. Both the genes of interest and actin were amplified (separately) using gene-specific and actin-specific primers, respectively; actin served as a control gene that was constitutively expressed in roots and heads. Reverse transcription was conducted as described above for DDRT-PCR analysis. Reverse transcription products were diluted to 100 and 3 µl was PCR-amplified as described above using appropriate primers (Table 1). Products were visualised as described above and the relative accumulation of the transcripts of interest in comparison to that of actin was calculated using Image Master ID 4.10 software (Amersham Pharmacia, Sweden). Data represent the RT-PCR transcript/actin ratios of samples.

Data analysis

Non-normally distributed visual disease scores, premature bleaching data and RT-PCR data were analysed using the Mann–Whitney Rank sum test in Minitab® release 14 (Minitab Inc., USA). The correlation between $AUDPC_{DON}$ and transcript accumulation

Table 1 Nucleotide sequence of primers used in RT-PCR

Transcript	Forward primer (5′→3′)	Reverse primer (5′→ 3′)
Erika LTR	[a]GCTGCAGCTCCACATCATAC	[a]CACTCAATGTCCGACTACGG
Romani PP	[a]CTTCTGGGGTGGTAAGCAAA	[a]AGCCTATGGTGTGATCGGTTA
EF-1α	[a]TCTCCACACCCTTGATGACA	[a]CAAGTTTGCTGAGCTGGTGA
POX	[a]AGGCTAAAGATTGCCGTCAC	[a]GCGTCTAGCTCCAGGACAGT
SSRP1	[a]TGTCGGTGTAGTGCTGAATGTT	[a]TAGCAACTAATCGGCCTTGG
bZIP	[a]AATGACATTGTTCTTATCCA	[a]GATAAGGTTGATGATTACTGCTGCT
C4D	[a]CTTCGGAGGCGAGGTTGATA	[a]GCCACTACATAGCAACACCAGA
C4C4	[a]ACCGCATAAGGGAGAGTGTG	[a]GGTTTGGAGTCGACCAAGGAA
Actin	[b]GCCCTTGATTATGAGCAGGA	[b]GCAATTCCAGGAAACATGGTA

Designed based on: [a] sequence of DDRT-PCR products, and [b] the partial conserved domains of a wheat actin gene (GeneBank accession No. AB181991)

data was determined by calculating the Spearman Correlation Coefficient using SPSS© release 11.0.1 (SPSS Inc., USA).

Results

DON-induced accumulation of retrotransposon-like, *EF-1α*, *POX*, *SSRP1* and functionally uncharacterised transcript homologs in wheat root tissue

DDRT-PCR results suggested that 70 transcripts were DON-up-regulated in roots of at least one of two wheat cultivars ('CM82036' and 'Remus') by 24 h post-treatment, as compared to in water-treated roots (results not shown). Eight bands were selected, based on the fact that in DDRT-PCR, they appeared more DON-inducible (relative to transcript accumulation in Tween20-treated roots) in 'CM82036' than in 'Remus', and they were re-amplified, cloned and sequenced (Table 2). RT-PCR assays were designed to confirm the DON-inducible accumulation of these transcripts; results are expressed as the transcript/Actin ratios (Fig. 1). In control water-treated roots, there were significant cultivar-dependent differences in the accumulation of several transcripts. In DON treated roots, accumulation of retrotransposon (*Erika* LTR and *Romani* PP), *POX*, *bZIP* and of *C4D* was significantly higher (≥160%) in 'CM82036' than in 'Remus' ($P \leq 0.003$). DON treatment resulted in up-regulation (DON/water values) of all the transcripts in 'CM82036' (146–263% relative to water controls), and of *Erika* LTR, *Romani* PP, *EF-1α*, *bZIP* and *C4D* in 'Remus' (124–175% relative to water controls). All except *EF-1α*, were 46–114% more up-regulated in 'CM82036' than in 'Remus' in response to DON ($P \leq 0.05$).

Up-regulation of DON-responsive genes in wheat heads in response to *Fusarium* infection

Preliminary data showed that the transcripts listed in Table 2 were *Fusarium*-responsive in wheat roots (results not shown). FHB experiments were performed (using the FHB-resistant 'CM82036' and the FHB-susceptible 'Remus') to determine if these transcripts were over-expressed in wheat spikelets in response to a trichothecene-producing *F. graminearum* isolate (GZ3639) and its trichothecene-minus mutant derivative (GZT40). Disease symptoms (Fig. 2a) and transcript accumulation (relative to actin) (Fig. 2b–d) were analysed at both 7 and 14 days post-treatment, because the first symptoms appeared on 'CM82036' at day 7 and by day 14 the majority of 'Remus' spikelets showed FHB symptoms in response to both fungi. The wild-type isolate caused >18% more spikelet bleaching on 'Remus' than did GZT40 ($P < 0.01$) (Fig. 2a). Both isolates caused few visual FHB symptoms on 'CM82036' (≤4%). No bleaching was observed on the Tween 20-treated control heads.

All transcript accumulation levels decreased from 7 to 14 dpi (data not shown). Transcript accumulation in

Table 2 Transcripts differentially expressed in wheat cultivars CM82036 and/or Remus roots due to DON (20 μg ml^{-1}) treatment (24 h post-treatment), as determined by differential display analysis

Transcript code	Nucleic acid homologs[a]	Total transcript sequence length (nt) and percentage homology in a given overlap (nt)
Erika LTR	Long terminal repeat (LTR) of wild wheat (*Triticum monococcum*) *Erika* retrotransposon (AF459639)	353 nt, 79% homology in a 316 nt overlap
Romani PP	Polyprotein of wild wheat *Romani* retrotransposon (Gypsy family) (AY188331)	275 nt, 86% homology in a 269 nt overlap
EF-1α	Wheat translation elongation factor-1 alpha (M90077)	658 nt, 93% homology in 532 nt overlap
POX	Class III plant peroxidases (TC143190 TIGR)	1,017 nt, 78% homology in 763 nt overlap
SSRP1	Chromosomal HMG domain protein SSRP1 (AJ244017)	899 nt, 97% homology in 577 nt overlap
bZIP	Wheat WLIP19 basic leucine zipper transcription factor (*bZIP*) (AB193552)	173 nt, 100% homology in a 173 nt overlap with the 3′ end of an EST (CD908870); the 5′ end of this 531 nt EST shows 97% homology to *Triticum aestivum bZIP* WLIP19 in a 337 nt overlap
C4D	cDNA of wheat (CD898702)	283 nt, 89% homology in a 244 nt overlap
C4C4	cDNA of wheat (BE442569)	399 nt, 94% in a 206 nt overlap

nt Nucleotides

[a] Nucleic acid homology determined by blast (Altschul et al. 1997) [(number in the parenthesis is the accession no. of the closest homologs; all are in the National Centre for Biotechnology Information (NCBI) Genbank database (http://www.ncbi.nlm.nih.gov/) except *POX* that is in The Institute for Genomic Research (TIGR) database (http://www.tigr.org)]

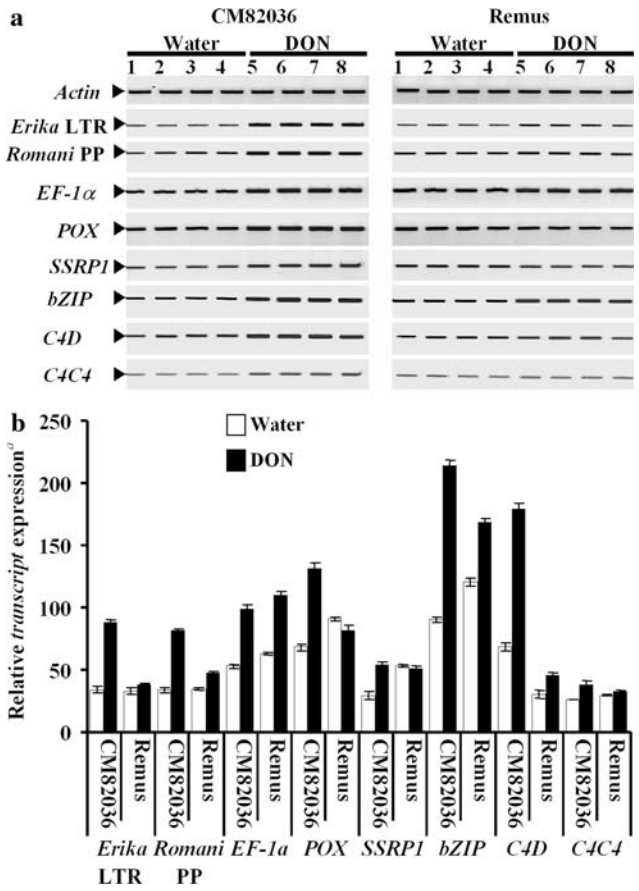

Fig. 1 Effect of deoxynivalenol (*DON*) on transcript accumulation in the roots of wheat cultivars CM82036 and Remus harvested 24 h post-treatment. **a** Visualisation of the RT-PCR products. Lanes represent RNA extracts from samples treated with: *1–4* water, *5–8* DON. *Arrows* indicate: *Actin*, actin gene (control constitutively expressed gene; 271 bp); *Erika* LTR, long terminal repeat of an Erika retrotransposon (210 bp); *Romani* PP, poly protein of a Romani retrotransposon (250 bp); *EF-1α*, translation elongation factor 1 alpha (220 bp); *POX*, class III plant peroxidase (200 bp); *SSRP1*, structure-specific recognition protein 1 (210 bp); *bZIP*, basic leucine zipper protein (174 bp); *C4D* and *C4C4*, transcripts of unknown function (240 and 225 bp, respectively). **b** [a]Transcript accumulation was expressed as the specific transcript/actin ratios. *Bars* indicate standard errors

control (0.2% Tween 20-treated)- and in GZ3639- or GZT40-treated heads was often cultivar-dependent (Fig. 2c, d). Considering that inoculation by both *F. graminearum* strains caused >95% less disease symptoms on 'CM82036' than on 'Remus', transcript up-regulation (over disease level) in 'CM82036' in response to both *F. graminearum* strains was higher, relative to in 'Remus' (Fig. 2b–d). Transcripts significantly up-regulated (by up to 1.3×) in both cultivars in response to the mutant GZT40 included *Erika* LTR, *EF-1α*, *bZIP* at 7 dpi. All transcripts except the *Romani* PP, *SSRP1* homologs and *C4C4* were up-regulated at 14 dpi ($P < 0.03$). This up-regulation was most pronounced for *EF-1α*, and *bZIP* (1.3–1.9 times in GZT40-, relative to Tween 20-treated heads). The transcripts *Romani* PP and *bZIP* were significantly more up-regulated in 'CM82036' than in 'Remus' by the mutant, at 7 and 14 dpi, respectively ($P < 0.05$). In contrast, mutant-induced up-regulation of *EF-1α* was significantly higher in 'Remus' than in 'CM82036' at both 7 and 14 dpi ($P = 0.002$).

As with disease symptoms, transcript accumulation in wheat heads was generally higher in response to wild-type GZ3639 than to mutant GZT40 (up to 1.7 times higher). This was true for the *Romani* PP at both time points in both cultivars (1.6–1.7 times higher) and for all other transcripts except *bZIP* in at least one cultivar and at least at one time point ($P < 0.05$). Transcript accumulation in wild-type-treated heads in compared to controls (Tween 20-treated), ranged from 1.1 to 2.3 times. The *Romani* PP and *bZIP* were the only transcripts whose accumulation in GZ3639-treated heads of 'CM82036' was statistically higher and more up-regulated (relative to Tween20-treated heads) than in 'Remus' (1.7–2.3 times higher and 1.2–1.5 times more up-regulated) ($P = 0.03$) (at 14 dpi).

Relationship between transcript accumulation and inheritance of DON tolerance

Adult plant DON tolerance trials were conducted to determine if there was any relationship between transcript accumulation and the DON tolerance associated with QTL Fhb1 of 'CM82036' (Lemmens et al. 2005). These tests were carried out using DH lines or parent cultivars that inherited no QTL, chromosome 3BS QTL Fhb1 (associated with Fusarium resistance and DON tolerance), chromosome 5A QTL Qfhs.ifa-5A (associated with Fusarium resistance but not DON tolerance) (Buerstmayr et al. 2003; Lemmens et al. 2005) or both QTL (four lines per QTL class). DON-induced bleaching was assessed 13 and 21 days post-treatment and used to calculate the $AUDPC_{DON}$ values for spikelets below and above those treated (Fig. 3a). Above the treated spikelets, DON caused a significant amount of premature spikelet bleaching (11–100%; mean = 51%) by 21 days post-treatment in lines not carrying QTL Fhb1. Lemmens et al. (2005) observed much more DON-induced bleaching in the basipetal rather than the antipetal direction in the same wheat lines that lacked Fhb1. But, in these experiments, DON did not cause any significant bleaching in the direction basipetal to the treatment point in any line tested (mean ≤6% in all lines tested). In lines carrying the 3BS QTL Fhb1, DON had not caused any bleaching of spikelets above or below the treatment point at this time point.

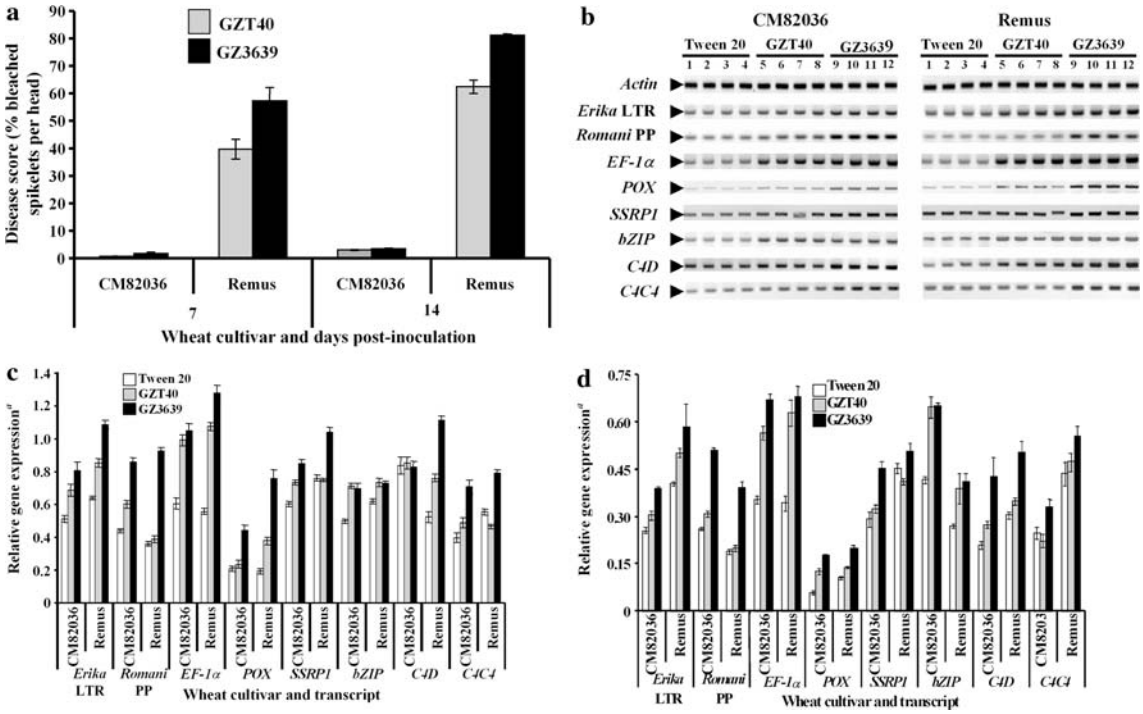

Fig. 2 Disease symptoms and transcript accumulation in heads of wheat cultivars CM82036 and Remus in response to both the wild type trichothecene producing *Fusarium graminearum* strain GZ3639 and its non-trichothecene producing mutant derivative GZT40. Wheat heads were inoculated at mid-anthesis with conidial inoculum or treated with 0.02% Tween 20 (negative controls). **a** Visual disease symptoms (percentage bleached spikelets per head) at 7 and 14 days post-inoculation (dpi). No bleached spikelets were observed on Tween 20-treated control heads. **b** Visualisation of transcript accumulation levels in samples harvested 7 dpi. Lanes represent samples treated with: *1–4* Tween 20, *5–8* GZT40, *9–12* GZ3639. *Arrows*: *Actin*, actin (270 bp); *Erika* LTR, long terminal repeat of an Erika retrotransposon (210 bp); *Romani* PP, poly protein of a Romani retrotransposon (250 bp); *EF-1α*, translation elongation factor 1 alpha (220 bp); *POX*, class III plant peroxidase (200 bp); *SSRP1*, structure-specific recognition protein 1 (210 bp); *bZIP*, basic leucine zipper protein (174 bp); C4D and C4C4, genes of unknown function (240 and 225 bp, respectively). **c**, **d** Transcript accumulation at 7 and 14 dpi, respectively. [a]Transcript accumulation was expressed as the specific transcript/actin ratio. *Bars* indicate standard errors

We analysed the accumulation of the transcripts listed in Table 2, relative to that of the housekeeping gene actin, in the treated spikelets that were harvested 24 h post-treatment (a time point at which preliminary studies indicated high transcript up-regulation in response to DON). While all genes were up-regulated in response to DON (as compared to Tween 20) treatment in most lines tested (data not shown), only *bZIP* showed any distinct QTL-associated transcript accumulation pattern. DON treatment caused ≥140% more *bZIP* transcript accumulation ($P = 0.000$) and ≥150% more up-regulation (relative to Tween 20; $P = 0.00$) in lines containing the QTL *Fhb1* than in lines that did not (Fig. 3b). In Tween 20-treated samples there was no QTL-associated pattern of *bZIP* accumulation ($P > 0.05$). The 'CM82036' and 'Remus' *bZIP* fragments (174 bp) amplified by RT-PCR were identical in DNA sequence (results not shown). *bZIP* gene-specific PCR analysis of genomic DNA extracts from 'Chinese spring' (accession No. Cltr 14108) and its 3BS deletion mutant derivatives amplified a product of expected size (174 bp) and of similar intensity from all DNA extracts. Kobayashi and Takumi (personal communication) recently assigned the homologous wheat *lip19* gene (Table 2) to homoeologous group 1 chromosomes by Southern blot analysis with nulli-tetrasomic wheat lines. Therefore, it appears that *bZIP* is not located on 3BS (results not shown).

bZIP transcript accumulation in DON-treated spikelets better correlated with the $AUDPC_{DON}$ values for spikelets above the treatment point than did *bZIP* accumulation in Tween 20-treated samples, albeit both correlations were significant ($r = -0.75$ and -0.41, $P = 0.000$ and 0.010, respectively). Also, there was a strong correlation between these $AUDPC_{DON}$ values and the increase in *bZIP* transcript accumulation in DON-treated, relative to Tween 20-treated samples ($r = -0.72$, $P = 0.000$). Our *bZIP* expression data in DON-treated spikelets and in DON relative to Tween20-treated spikelets also correlated with the downward DON-induced bleaching observed by Lemmens et al. (2005).

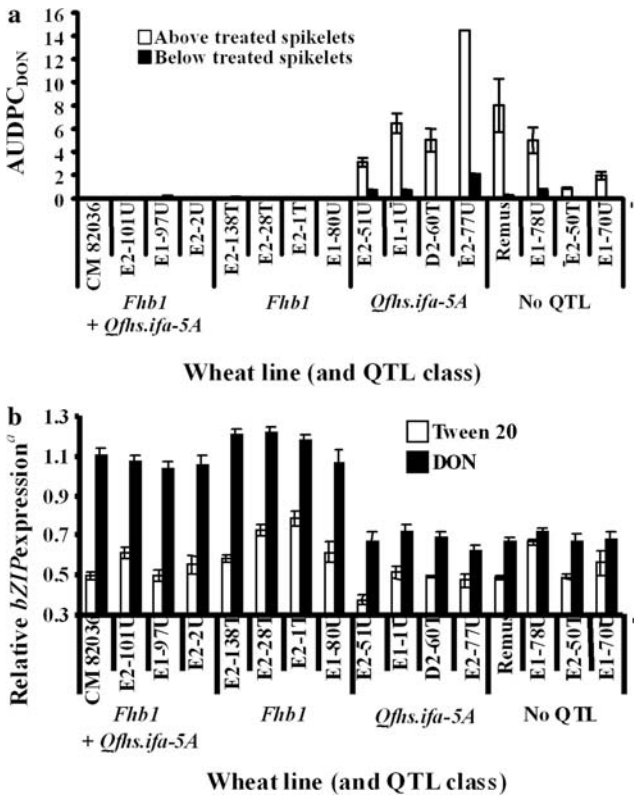

Fig. 3 Premature bleaching and transcript accumulation in response to deoxynivalenol (*DON*) treatment in spikelets of wheat cultivars CM82036 and Remus and double-haploids (*DH*) derived from a cross between these two genotypes. DH had inherited both, one or neither of chromosome 5A QTL *Qfhs.ifa-5A* and 3BS QTL *Fhb1* (syn. *Qfhs.ndsu-3BS*) from 'CM82036'. Central spikelets (4) were treated with DON (5 mg ml^{-1} 0.2% Tween 20) or 0.2% Tween 20 at mid-anthesis; RNA extracted from treated spikelets 24 h post-treatment was used for RT-PCR analysis and the percentage bleached spikelets below and above the inoculation point at 13 and 21 days post-treatment were used to calculate respective AUDPC$_{DON}$ values. **a** DON-induced damage observed above and below the treated spikelets (AUDPC$_{DON}$). **b** Basic leucine zipper protein (*bZIP*) transcript accumulation in 0.2% Tween 20 and DON-treated spikelets harvested 24 h post-treatment. [a]Transcript accumulation was expressed as the specific transcript/actin ratio. *Bars* indicate standard errors

Discussion

To our knowledge, this is the first report on the effect of a trichothecene mycotoxin on the wheat transcriptome. Transcripts were identified as being DON-responsive based on transcript accumulation studies in roots of two wheat cultivars of distinct genetic background. 'CM82036' is a FHB resistant CIMMYT line derived from 'Sumai 3' and several Brazilian sources. 'Remus' is a German spring wheat and is susceptible to FHB disease. DDRT-PCR probably over-estimated the number of DON-responsive transcripts (70), as more than one band may originate from the same transcript and results should be confirmed by RT-PCR. Nevertheless, the results presented here do highlight the complexity of the wheat-mycotoxin interaction.

Eight transcripts were chosen for further study, on the basis that, according to DDRT-PCR results, they appeared more DON-responsive (i.e. compared to water) in roots of "CM82036" than of "Remus". RT-PCR analyses confirmed that, with the exception of elongation factor-1α (*EF-1α*), this was the case. Retrotransposons (*Erika* LTR and *Romani* PP), *EF-1α*, *bZIP*, a class III *POX, SSRP1* and transcripts of unknown function (*C4C* and *C4D*) were up-regulated in wheat heads in response to wild type *F. graminearum*, its trichothecene-minus mutant and DON. While transcripts sometimes showed higher accumulation in wild type- as opposed to mutant-infected heads, this probably reflects the higher infection levels on heads inoculated with the former isolate. This suggests that they form part of the general plant stress response, rather than being part of a specific response to mycotoxin application, at least at the time points assessed and in wheat heads. Trichothecenes are known to enhance the virulence of *F. graminearum* towards wheat (Bai et al. 2002) and therefore it is logical that they increase the stress levels experienced by this host during *Fusarium* pathogenesis. In this and other research (Lemmens et al. 2005), DON induced premature spikelet bleaching in toxin-susceptible wheat lines. The DON-induced bleaching we observed above the treatment point could be due to either damage caused by upwardly translocated DON or the inhibition of water and nutrient translocation due to DON-induced death of rachis tissue. Such bleaching was not observed in Tween20-treated control samples and therefore was not due to spikelet removal. In our experiments, there was little DON-induced bleaching in the direction basipetal to the treated spikelets. This was in contrast to the observations of Lemmens et al. (2005); they observed much more DON-induced bleaching in the basipetal rather than the antipetal direction in the same wheat lines that lacked *Fhb1*. These discrepancies may be due to environmental conditions, or our removal of the spikelets at 24 h may have preceded downward translocation of DON and thus prevented DON-induced damage in the basipetal direction. But, we recently observed a similar phenomona (i.e. DON-induced antipetal, but not basipetal bleaching) in the DON-susceptible cv. Remus in an experiment where treated spikelets were not removed; J. M. Brennan, K. I. Ansari and F. M. Doohan, unpublished data; therefore it seems likely that environmental conditions might significantly influence DON transport within head tissue.

EF-1α was both DON- and *Fusarium*-responsive in heads of 'CM82036' and 'Remus'. Han et al. (2005) and Kruger et al. (2002) identified elongation factor 1α (*EF-1α*) as being *Fusarium*-responsive in the wheat cvs Sumai 3 and Frontana. This is not surprising as *EF-1α* is a multifunctional protein and, in various eukaryotes, *EF-1α* activity correlated with several biological processes including senescence and longevity of tissue (Silar and Picard 1994).

Class III plant peroxidases have been implicated in cross linking of phenolic compounds to proteins and polysaccharides, pathogen resistance, wounding responses, oxidative degradation of the major endogenous auxin and deposition of polyphenols, lignin, etc. (Andres et al. 2001; Hiraga et al. 2001, Jansen et al. 2001). Cross-linking of cell wall components reduces susceptibility to wall-degrading enzymes and restricts diffusion of pathogen-derived toxins and penetration of pathogens inside the cell (Brisson et al. 1994; Iiyama et al. 1994). Peroxidase and other defence transcript accumulation (e.g. β-1,3-glucanase, chitinase, thaumatin-like protein) has previously been shown to be part of the general wheat and barley defence response during *F. graminearum* infection of spikelets (Pritsch et al. 2000; Boddu et al. 2006). In rice more than 70 class III plant peroxidase isozymes have been found, of which 10 were induced in response to fungal infection (Sasaki et al. 2004).

Our study highlights several new candidate genes involved in the wheat response to both DON and *F. graminearum* [retrotransposons (*Erika* LTR and *Romani* PP), a structure-specific recognition protein (*SSRP1*) and a basic leucine zipper transcription factor (*bZIP*)]. Retrotransposons are mobile genetic elements; most are largely quiescent during development, becoming active under stress conditions, and transcriptional activation of retrotransposons can alter the expression of adjacent genes in wheat (Kashkush et al. 2003). It would be interesting to conduct a more in depth analysis of the effect of trichothecene producers and/or trichothecene treatment on epigenetic regulation and retrotransposon activity in different wheat genotypes. Other research implied that the degree of genetic or epigenetic transposon activation in plant cells in response to stress may be genotype-specific (Ivashuta et al. 2002). SSRPs are non-histone proteins that bind DNA of distorted conformation and these proteins are implicated in DNA replication, basal and regulated transcription and DNA repair, and interact with a larger network of proteins (including transcription factors) (Grasser 2003; Grasser et al. 2004).

bZIP comprises a large family of transcription factors. The closest homologs of the DON-responsive *bZIP* are wheat and rice *lip19* genes. These encode proteins that are involved in low temperature stress responses, and, like the FOS protein in mammals, the rice homolog lacks the usual ability of bZIP proteins to homodimerize and to bind DNA (Shimizu et al. 2005). The rice LIP19 forms a heterodimer with a new bZIP protein (OsOBF1) but the physiological role of this complex is unknown. Recent studies have revealed that maize and tobacco members of this family are expressed during leaf senescence and tobacco genes are also expressed during flower senescence (Berberich et al. 1999; Yang et al. 2001). In tobacco, the LIP19 proteins localize in the guard cells of senescing leaves and Yang et al. (2001) postulated that they may activate unidentified genes that function to retain cellular activity in senescing and cold-stressed guard cells. This is the first report of a pathogen and mycotoxin-inducible *lip19* transcript. It seems that LIP19 proteins may play a role in both biotic and abiotic stress responses and that there is a cross talk between cold-regulated and biotic stress signalling pathways.

Other defence-related bZIP transcription factors include PPI1 and CabZIP1 (Lee et al. 2002, 2006). *PPI1* transcript accumulated in pepper (*Capsicum chinense*) during an incompatible, but not during a compatible interaction with *Xanthomonas campestris* pv. *Vesicatoria* race 3 (as determined using near-isogenic susceptible and resistant cultivars) (Lee et al. 2002). CabZIP1 from hot pepper (*Capsicum annuum*) binds to the G-box region of pathogenesis-related protein 1 (*PR-1*) promoter and transient overexpression of this transcription factor resulted in increased *PR-1* transcript accumulation in tobacco (*Nicotiana benthamiana*) (Lee et al. 2006). Although we have shown that the wheat *bZIP* is not located within the *Fhb1* locus on wheat chromosome 3BS, we did observe enhanced accumulation of this transcript in 'CM82036' × 'Remus' progeny that inherited this QTL, as compared to in those that did not. It seems most likely that the wheat bZIP participates in defence response cascades associated with *Fhb1*. A more direct link is less likely, but possible: a heterodimerisation partner of this bZIP, or a transcriptional regulator of bZIP or its heterodimerisation partner may map to *Fhb1*. Determining the physiological role of this wheat bZIP protein in responding to DON-induced stress will be the focus of future work.

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