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Identification of genes induced by *Fusarium graminearum* inoculation in the resistant durum wheat line Langdon(Dic-3A)10 and the susceptible parental line Langdon

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ABSTRACT

The wheat recombinant chromosome inbred line LDN(Dic-3A)10, obtained through introgression of a *Triticum dicoccoides* disomic chromosome 3A fragment into *Triticum turgidum* spp. *durum* var. Langdon, is resistant to fusarium head blight (FHB) caused by *Fusarium graminearum*. To identify genes involved in FHB resistance, we used a cDNA-AFLP approach to compare gene expression between LDN(Dic-3A)10 and the susceptible parental line LDN at different time points post-inoculation. In total, 85 out of the ~500 transcript-derived fragments (TDFs) were found to be differentially expressed: 36 and 19% were upregulated in LDN(Dic-3A)10 and LDN, respectively, whereas 45% were induced in both genotypes. Several of the cloned TDFs showed similarity to proteins involved in specific recognition of plant pathogens or associated with early responses to infection. Some TDFs specific to the inoculation response did not show similarity to characterized proteins. The availability of *T. aestivum* genome sequences allowed the in silico mapping of 28 TDFs and the acquirement of the corresponding gene sequences and, in some cases, their regulatory regions. Analysis of promoter regions revealed the potential existence of shared transcription regulation mechanisms. For instance, three TDF-associated genes contained binding sites for WRKY transcription factors, which have been implicated in the regulation of genes associated with pathogen defense, and three for abscisic acid-responsive element (ABRE). Collectively, our results revealed specific pathogen recognition in the interactions of LDN and LDN(Dic-3A)10 with *F. graminearum*. Such recognition leads to changes in the expression of several transcripts, attributable to the presence of the wheat QTL *Qfhs.ndsu-3AS*.

1. Introduction

Fusarium head blight (FHB), also known as ear blight disease or head scab disease, is mainly caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch]. This is a re-emerging and devastating disease that affects all classes of wheat (*Triticum* spp.) and barley (*Hordeum vulgare* L.; McMullen et al. 1997; Stack 2000) and is promoted by warm conditions with frequent rainfall and high humidity during flowering. FHB has a great economic impact on the cereal industry, mainly by significantly reducing grain yield, test weight, grain quality and milling yield. Grains infected by *F. graminearum* are often shriveled, with lower kernel weight, and can be blown away with the chaff during threshing (Bai and Shaner 2004). Grains from infected plants can also be contaminated with mycotoxins, such as deoxynivalenol (DON) and zearalenone, produced by *F. graminearum* (Snijders 1990). Mycotoxins are a serious threat to human and animal health (Pestka 2010).

FHB infection is initiated when ascospores (sexual spores) or macroconidia (asexual spores) released from soil-borne debris and infected hosts are deposited on or inside flowering spikelets at conducive temperature and humidity. Fungal hyphae develop on the exterior surfaces of florets and glumes, rather than by direct penetration through the epidermis, prior to colonization of anthers, stigmas, and lodicules (Bushnell et al. 2003). The fungus spreads in wheat from spikelet to spikelet through the vascular tissue in the rachis and rachilla (Trail 2009). FHB exhibits a brief biotrophic phase before switching to the necrotrophic phase, when the rate of fungal colonization increases and the plant cells eventually die (Trail 2009).

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Fungicide application is one approach to reduce the risk of infection; however, this has not produced highly effective or consistent results for FHB control (Mesterházy 2003). One of the most economical, environmentally safe, and effective strategies for disease control is the development of resistant cultivars; in this case, resistant cultivars are particularly needed for warm and humid wheat-growing regions. FHB resistance sources within durum wheat germplasm (*Triticum turgidum* L. ssp. *durum*, $2n = 4x = 28$ AABB) are scarce, but wild species offer some opportunity to improve resistance to FHB in durum wheat. Langdon(Dic-3A)10 is a substitution line derived from crosses between *T. turgidum* spp. *durum* var. Langdon and the wild emmer *T. turgidum* spp. *dicoccoides* ($2n = 4x =$ AABB) that contains a QTL, *Qfhs.ndsu-3AS*, associated with Type II resistance to FHB (resistance to spread of pathogen within heads) on chromosome 3AS (Otto et al. 2002). However, the molecular events during the early stages of the infection process related to the presence of the QTL *Qfhs.ndsu-3AS* that confers resistance to the Langdon(Dic-3A)10 durum wheat line are still not understood.

Wheat responds to *Fusarium* infection by inducing various defense mechanisms (Ding et al. 2011; Geddes et al. 2008; Golkari et al. 2007; Jia et al. 2009; Li et al. 2001; Li and Yen 2008; Pritsch et al. 2000). Differential expression analysis of wheat spikes during infection by *F. graminearum* will help us to understand the mechanism underlying FHB resistance and identify genes related to FHB resistance in Langdon(Dic-3A)10. Here, we adopted a cDNA-amplified fragment length polymorphism (cDNA-AFLP) method (Bachem et al. 1996) to identify differentially expressed genes because this technique is robust, reproducible, and able to amplify low-abundance transcripts, without requiring prior assumptions about which genes might be induced or repressed (Lievens et al. 2001). cDNA-AFLP allows parallel analysis of expression patterns over time and can reveal changes in expression for both rare and common mRNAs (Durrant et al. 2000). Using this approach, we successfully identified genes that were upregulated during early *F. graminearum* infection in either the Langdon(Dic-3A)10 (FHB-resistant) or Langdon (susceptible) genotype and, more interestingly, genes that were differentially expressed between the two genotypes. Among the identified genes were several previously reported to be associated with early responses to infection and with pathogen avirulence determinant recognition (reviewed in Li et al. 2015). Additionally, there was a group of transcripts that, in spite of being specific to the inoculation response, did not show similarity to characterized proteins and thus represent newly identified candidate pathogen-response proteins. Analysis of the promoter regions revealed that a number of the TDF-associated genes contained binding sites for WRKY transcription factors, which have been implicated in the regulation of genes associated with pathogen defense (Buscaill and Rivas 2014). Collectively, our results suggest that gene-for-gene relationships are involved in Langdon and Langdon(Dic-3A)10 interactions with *F. graminearum* involving changes in the expression of several transcripts.

2. Materials and methods

2.1. Plant materials and growth conditions

Two durum wheat genotypes with contrasting phenotypes for FHB resistance were used in this study: the susceptible cultivar Langdon (LDN) and its derived line LDN(Dic-3A)10. The LDN(Dic-3A)10 is recombinant inbred chromosome line (RICL) obtained from the cross between Langdon and LDN(Dic-3A), selected based on the presence of the QTL *Qfhs.ndsu-3AS* that confers FHB resistance (Otto et al. 2002). LDN(Dic-3A) in turn was obtained via LDN-*T. dicoccoides* chromosome 3A substitution (Stack et al. 2002).

Seeds of the two lines were germinated in pots (17 cm diameter, 20 cm height) containing a mixture of compost and sand and placed in the greenhouse. The temperature in the greenhouse was $24 \pm 1\,^{\circ}C$ with 16 h light. Several replications were planted every ~15 days, to obtain spikes in the same developmental stage at the time of inoculation. The inoculum infectivity was tested using two additional durum varieties, the susceptible line Ciccio and the resistant line Bonaerense INTA Facón (BI Facón), which were grown as described for LDN and LDN(Dic-3A)10.

2.2. Preparation of fungal cultures

A macroconidial suspension of *F. graminearum* strain KBC7 (kindly provided by Dr. Cecilia Fernochi, Universidad Nacional de Río Cuarto, Argentina) was cultured in a liquid mung bean medium for 7 days at 25 °C as described by Bai et al. (2000). The medium was then removed by two centrifugation steps in distilled water. The conidia concentration was directly quantified under an optical microscope with a Neubauer chamber and diluted to the desired concentration.

2.3. Ear inoculation, tissue sampling, and disease severity analysis

Spikes were point inoculated with a micropipette during early anthesis, by injecting 10 μL of 1×10^5 conidia/mL suspension of *F. graminearum* into the two basal florets of two central spikelets per spike. The plants used for control purposes were injected with sterile medium. After inoculation, the *F. graminearum* heads were sprayed with water and covered with plastic bags for 3 days to provide high humidity. The greenhouse temperature was $24 \pm 1\,^{\circ}C$ with 16 h light.

Inoculated heads from each line were harvested at four sampling times: immediately after inoculation (0 h) and at 6, 48 and 72 h after inoculation (hai). Samples were immediately frozen in liquid nitrogen and stored at −80 °C. This experiment was performed twice (Experiments 1 and 2). Several *F. graminearum*-inoculated heads of each genotype were maintained to check disease symptoms. Disease severity was expressed based on a 0–100% severity scale, quantified as the percentage of symptomatic spikelets/spike at 21 days post-inoculation (Schroeder and Christensen 1963). The mean values for inoculated spikes were compared between genotypes using Student's *t*-test for independent samples. All statistical analyses were performed using Infostat software version 2010 (Di Rienzo et al., 2010).

2.4. Molecular methods

The presence of the donor fragment was monitored in LDN and LDN(Dic-3A)10 wheat lines through PCR amplification of the linked SSR locus *Xgwm2*, localized near the *Qfhs*.ndsu-3AS resistance region (Otto et al. 2002). DNA was extracted from leaf tissue following a modified CTAB method (Saghai-Maroof et al. 1984).

PCR reactions were performed in a total volume of 25 μL containing 0.25 μM each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1× Taq buffer, 1 U *Taq* polymerase (Invitrogen, Brazil), and 75 ng of the DNA template. The sequences of the forward and reverse primers were 5′-CTGCAAGCCTGTGATCAACT-3′ and 5′-CATTCTCAAATGATCGAACA-3′ (Röder et al. 1998), respectively. The PCR program consisted of an initial denaturation step of 3 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C, and a final extension step of 10 min at 72 °C. Amplification reactions were performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR amplification products were separated by 2% (w/v) agarose gel electrophoresis, visualized after staining with ethidium bromide under UV light, and imaged with a Kodak Easy share Z7590 zoom digital camera.

Table 1
cDNA-AFLP adaptors and adaptor-based primer sequences.

Primers		Sequences
Adaptors	PstI-A1	5′-TGT ACG CAG TCT AC-3′
	PstI-A2	5′-CTC GTA GAC TGC GTA CAT GCA-3′
	MseI-A1	5′-TAC TCA GGA CTC AT-3′
	MseI-A2	5′-GAC GAT GAG TCC TGA G-3′
Pre-selective primers	PstI + 0	5′-GAC TGC GTA CAT GCA G-3′
	MseI + 0	5′-GAT GAG TCC TGA GTA A-3′
Selective primers	PstI + 2a	5′-GAC TGC GTA CAT GCA GAT-3′
	PstI + 2b	5′-GAC TGC GTA CAT GCA GCG-3′
	PstI + 2c	5′-GAC TGC GTA CAT GCA GAC-3′
	MseI + 2a	5′-GAT GAG TCC TGA GTA ACG-3′
	MseI + 2b	5′-GAT GAG TCC TGA GTA AAC-3′
	MseI + 2c	5′-GAT GAG TCC TGA GTA AAT-3′

The sequences listed correspond to the AFLP adaptors, the pre-selective primers and the selective primers used in the present study.

2.5. RNA preparation and cDNA-AFLP analysis

The middle spikelets from treated heads of LDN and LDN(Dic-3A)10 lines at four sampling times were separated from the subtending section of rachis and ground into fine powder in liquid nitrogen using a mortar and pestle, resulting in eight samples from each of the two replicates at each time point. Total RNA was extracted from 30 mg of tissue using the SV Total RNA Isolation Kit (Promega) according to the manufacturer's instructions. The RNA quality and quantity were determined on agarose gels and by spectrophotometry.

cDNA was synthesized using the single-strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). Reverse transcription was performed with 1 μg of the total RNA in 20 μL of reaction mixtures containing 1 mM each dNTP, 20 U RiboLock RNase Inhibitor, 40 U M-MuLV Reverse Transcriptase, 5× reaction buffer and 0.5 μg of Oligo(dT)18 primer. Then, double-strand replacement synthesis was carried out using 30 U *Escherichia coli* DNA polymerase I, 10× reaction buffer and 1 U *E. coli* ribonuclease H (Fermentas, St Leon-Rot, Germany). Double-stranded cDNA was precipitated in isopropanol, resuspended, and checked for DNA quality based on constitutive *tef-1α* (translation elongation factor 1α subunit) expression, using the primers tef-1αF: 5′-GCCCTCCTTGCTTTCACTCT-3′ and tef-1αR: 5′-ACGCGCCTTTGAGTACTTG-3′ (Distelfeld and Dubcovsky 2010).

The cDNA-AFLP analysis was carried out according to the method described by Vos et al. (1995), except that the cDNA samples were digested with *Pst*I and *Mse*I. Briefly, 500 ng of cDNA was incubated for 3 h at 37 °C with 5 U *Pst*I (Promega, EE.UU.) and 5 U *Mse*I (Promega, EE.UU.) in a 25-μL reaction volume containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM Tris–HCl pH 7.9, and 50 ng/μL BSA. Enzymes were inactivated at 70 °C for 15 min. The success of the digestion was checked by running aliquots of the reactions on 1.5% (w/v) agarose gels. Then, adapters were annealed (PstI-A1/PstI-A2 and MseI-A1/MseI-A2; Table 1) by incubation at 95 °C for 5 min, 65 °C for 10 min, and 37 °C for 10 min in TE 1× buffer. Ligation was performed in a 25 μL final volume containing 10 μL digested cDNA, 5 pmol *Pst*I adapters, 50 pmol *Mse*I adapters, 1 U T4 DNA ligase (Promega), and 1× T4 ligase buffer, with incubation for 3 h at 20 °C. After ligation, the reaction mixture was diluted to 500 μL with 10 mM Tris–HCl, 0.1 mM EDTA pH 8.0, and stored at −20 °C. A pre-amplification step was conducted by PCR amplification of 100 ng cDNA fragments ligated to adaptors with 0.56 μM specific primers based on the adaptor sequences PstI + 0 and MseI + 0 (Table 1) in the presence of 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 2 U *Taq* polymerase, 1× *Taq* polymerase buffer in 50 μL of final volume. The PCR reaction consisted of 25 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. The final amplification was carried out through a PCR reaction using 2 μL 1:6 dilution (in TE) of the pre-amplification product, with 0.25 μM primers including two additional nucleotides located at the 3′ end (PstI + 2a, PstI + 2b, PstI + 2c, MseI + 2a, MseI + 2b and MseI + 2c; Table 1) in the presence of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.75 U *Taq* polymerase, 1× *Taq* polymerase buffer in 20 μL of final volume. The PCR program consisted of 13 touchdown cycles (0.7 °C each step) of 30 s at 94 °C, 30 s at 65–56 °C, and 1 min at 72 °C followed by 27 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. Seven of the nine possible primer combinations were included in the study since they lead to defined and reproducible bands.

Denatured PCR fragment separation was performed on 6% (w/v) polyacrylamide gels [acrylamide:bis-acrylamide (19:1), 0.5× TBE, 7.5 M urea] at 40 W for 1.5 h with 0.5× and 1× TBE buffer (pH 8.0) in the top and bottom reservoirs, respectively. Gels were stained with silver nitrate using the DNA Silver Staining System (Promega, EE.UU.) and scanned on an Astra 3400 (UMAX) flat bead scanner. Gel images were visually analyzed using standard imaging software.

2.6. Excision, cloning, sequencing, and annotation of TDFs

The transcript-derived fragments (TDFs) that showed differences among genotypes and/or experimental time points were excised from gels with a scalpel, eluted, and reamplified by PCR with selective primers (Table 1). Elution was performed overnight at 37 °C in 50 μL of 0.5 M ammonium acetate and 1 mM EDTA (pH 8.0). Precipitation was conducted during 30 min at −80 °C in 0.05 vol. of 5 M NaCl and 2.5 vol. of absolute ethanol followed by 10 min centrifugation at 13,000 rpm. Pellet was washed twice in ethanol 70%, dried at 37 °C, and resuspended in 15 μL of sterile water. The presence of the expected PCR products was verified by running the PCR products on 2% (w/v) agarose gels. Such products were further purified using the Wizard SV Gel and PCR CleanUp Kit (Promega, Mannheim, Germany) and cloned into the *pGEM®-T Easy Vector System* (Promega) according to the manufacturer's instructions. Competent *E. coli* cells (strain DH5α) were transformed with the recombinant vector and plated onto LB-agar ampicillin XGal–IPTG plates. White colonies were picked and amplified in liquid LB with ampicillin, and plasmids were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega). The incorporation of the PCR fragments into the cloning vectors was checked based on restriction profiles with the enzyme *Eco*RI, visualized on 2% (w/v) agarose gels stained with ethidium bromide. The TDFs were sequenced at SIGYSA (INTA Castelar, Argentina). The VecScreen software was used to eliminate vectors fragments from the obtained sequences (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html).

Homology searches were conducted using the algorithm BLASTX against the non-redundant protein database deposited at NCBI (http://www.ncbi.nlm.nih.gov/; version 2.2.29) to assign putative functions to the TDFs. BLASTN searches against the EST (expressed sequence tag) and TSA_nr (transcriptome shotgun assembly) databases at NCBI were further conducted with the TDF sequences for which there were no protein matches. For BLASTX and BLASTN analyses, matches were considered positive when they met the criteria of having an *e*-value $\leq 10^{-5}$. The highest similarity score was considered the best match for the putative identity of the corresponding protein, EST, or TSA sequence. To determine whether any of the identified TDFs originated from fungal transcripts, they were also BLAST queried against the *F. graminearum* Genome DataBase (http://mips.gsf.de/genre/proj/fusarium). The sequence data reported are available in the dbEST database (JZ719264–JZ719297).

Table 2
RT-PCR primer sequences based on 21 TDFs identified by cDNA-AFLP.

TDF	Forward primer	Reverse primer
TDF#4	5′-AGATGCACGATCACCCAGAAGG-3′	5′-GGAGACGAGGACGACGAGTTTG-3′
TDF#5	5′-CTCAAAGCCTCAGGTGATGTC-3′	5′-TCGACCTTGATCTTCGATTTGG-3′
TDF#6	5′-GACCCAAGGGAGCATAGTCG-3′	5′-AAGGCAGCCAACCTCAACAA-3′
TDF#26	5′-GAGGCTGCTTCAGTGATGATAC-3′	5′-ACGCCTGAATGGTTTGCT-3′
TDF#31	5′-TCGGAATCGAACCCACATTT-3′	5′-AGCTGATCCTCTGTACCTTAGT-3′
TDF#40	5′-GTGATGAAGAAGGATACCGAGAAG-3′	5′-GAAAGCTGGGATGCTAGAACA-3′
TDF#44	5′-AGCTCATGTCACCGATGATTCTT-3′	5′-TTGTCCCCAAGATCTCGGACT-3′
TDF#47	5′-TGTTCTCTCCTTGTGTTTCACT-3′	5′-CCTAACACAGTTGACATTTCCTTT-3′
TDF#49	5′-GCTGCACCGTCAATGAAATC-3′	5′-CTCAACCGATGCTAGGCTAAA-3′
TDF#50	5′-GATCTCAAACCCAGCAACATTC-3′	5′-GTCGAGCCATCAAAGGATGA-3′
TDF#54	5′-ACTGTTCTATTGATACCTGCTGAA-3′	5′-TATGCAATGCAACCCAAAGAAG-3′
TDF#55	5′-GCCATCAATCATCTTCCAGGAG-3′	5′-GGCTAGTCATTATCATGCACCTTA-3′
TDF#58	5′-TGACACGCAACGCTGAATA-3′	5′-GCTTGGTCTCTCATGCTCAA-3′
TDF#68	5′-TCTGGCTACAGGGATAGAGTAG-3′	5′-AACAAGATACGTCCTCGACTTC-3′
TDF#70	5′-TGGTGATGCTCGGCAATATAC-3′	5′-GCTTTACTCGCCAACAAGTTTC-3′
TDF#73	5′-AACAACCGAGCAAGTAGGTGA-3′	5′-TTGGCAGCAACCAGTCAAAT-3′
TDF#77	5′-TCGATGAGAAAGATGTCACGATG-3′	5′-AAGTTTCCAATTATGTCAGCAAGAG-3′
TDF#79	5′-CGTTCGAGTCGCTGAAGTT-3′	5′-TGGACGAGGTTCTCTACAAATTAC-3′
TDF#81	5′-GCAATCCTAATGAACGTCTGCC-3′	5′-TCAGAAATCCTGCTACCCAACA-3′
TDF#83	5′-CGGGAGTAACAGATCCAATGCT-3′	5′-GCTGTACAAATAGTGATCTTCGACA-3′
TDF#84	5′-GCTTCCAGGCGATCTTTTTCC-3′	5′-GGAGAGGCTTTTGAAGGACC-3′

The first column shows the TDF name, whereas the two subsequent columns show the forward and the reverse primer sequences designed based on the indicated TDF.

2.7. Reverse transcriptase (RT)-PCR

Primers for RT-PCR were designed using the program Primerquest (http://www.idtdna.com/Scitools/Applications/Primerquest/) based on the targeted cDNA sequence (Table 2) and synthesized by IDT (Integrated DNA Technologies, Coralville, Iowa, USA). RT-PCR was performed using 2 μL single-strand cDNA prepared as described above. PCR reactions consisted of 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$, 1× Taq buffer, 0.75 U *Taq* polymerase (Invitrogen, Brazil), and 0.25 μM of each primer in a final volume of 20 μL. PCR amplification conditions were as follows: 3 min at 94 °C; 8 touchdown cycles (1 °C each step) of 30 s at 94 °C, 30 s at 58–50 °C and 30 s at 72 °C; and after that, 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C followed by a final extension step of 10 min at 72 °C. Ten microliters of the reaction product were electrophoresed on a 3% (w/v) agarose gel, ethidium-bromide stained, visualized under UV light, and imaged. The expression of the constitutive gene *tef* was used to normalize the transcript level in different samples (Distelfeld and Dubcovsky 2010).

2.8. In silico mapping and identification of coding and regulatory gene regions

The in silico mapping of the identified TDFs was carried out with the *T. aestivum* chromosome arm databases deposited at URGI using the TDFs as probes (IWGSC; https://urgi.versailles.inra.fr/blast/blast.php). The positive hits were carefully manually analyzed and a TDF was assigned to a particular chromosome arm contig after confirmation that: (1) showed a single hit; (2) showed two or more hits with really distinguishable *e*-value and/or identity; (3) when hits appeared in homologous chromosomes, there were assigned to one of them, following the criteria mentioned in (2). As a result, all the mapped TFDs presented at least 85% identity and an *e*-value lower than 10^{-30}.

The coding sequences were identified in the genomic sequences through BLASTX searches against the NCBI protein databases (nr). When available, the 1500 bp upstream of the transcriptional start sites of the TDF-corresponding genes were extracted and considered as promoters for analysis of transcription factor binding sites (TFBSs). The upstream regions of the genes were analyzed using PLANTPAN (http://plantpan.mbc.nctu.edu.tw) to predict TFBSs and the corresponding transcription factors that could activate the differentially expressed genes (Chang et al. 2008).

The homology of the TDFs with 2000 common wheat DArT (*Diversity Arrays Technology*) markers (downloaded from http://www.diversityarrays.com/dart-map-sequences) was determined through BLASTN search.

The expression of the genes identified in the present study was compared to 74,740 ESTs comprising 5 libraries from common wheat spikes at different developmental stages (dbESTs #19546, #10467, #10481, #10480, and #10470) and 22,901 ESTs from 15 libraries of common wheat spikes, *F. graminearum* inoculated (dbESTs #19527, #19526, #19521, #19524, #19525, and #19528) or sprayed (dbESTs #9984, #9788, #11142, #11143, #11144, #19522, #18753, #5584, and #13766), downloaded from NCBI. Two virtual libraries were generated from these data, a control and an inoculated library, which were uploaded as local databases in the software BioEdit (Hall 1999; version 7.0.9.0).

3. Results

3.1. Molecular evaluation of the genotypes and the response to FHB

We began by confirming the presence of the DNA marker associated with increased FHB resistance in the original and introgressed genotypes (Fig. 1a). The microsatellite locus *Xgwm2* (Röder et al. 1998) was found to be tightly linked to the QTL *Qfhs.ndsu-3A* (Otto et al. 2002). Here, PCR reactions performed based on the SSR *Xgwm2* resulted in the expected ~200 bp amplification band indicative of the presence of the allele that confers resistance (Otto et al. 2002) in LDN(Dic-3A)10, whereas a ~220 bp band was observed in the susceptible parent LDN (Fig. 1a).

Severity assays were conducted to verify the effectiveness of the *F. graminearum* inoculation procedure via inoculation of two cultivars previously characterized in our laboratory as resistant and susceptible, respectively (Soresi et al. 2015). Disease severity was expressed as the percentage of symptomatic spikelets/spike at 21 days after inoculation (Schroeder and Christensen 1963). The *F. graminearum* inoculum cultivated in our laboratory caused visible infection symptoms in both cultivars, with the susceptible Ciccio (88.4%, $n = 19$) exhibiting significantly higher disease severity than the resistant BI Facón (54.2%, $n = 25$; $p \leq 0.001$; Fig. 1b). Finally, the resistant derivative LDN(Dic-3A)10 (39.7%, $n = 21$) was confirmed to have statistically better performance against *F. graminearum*

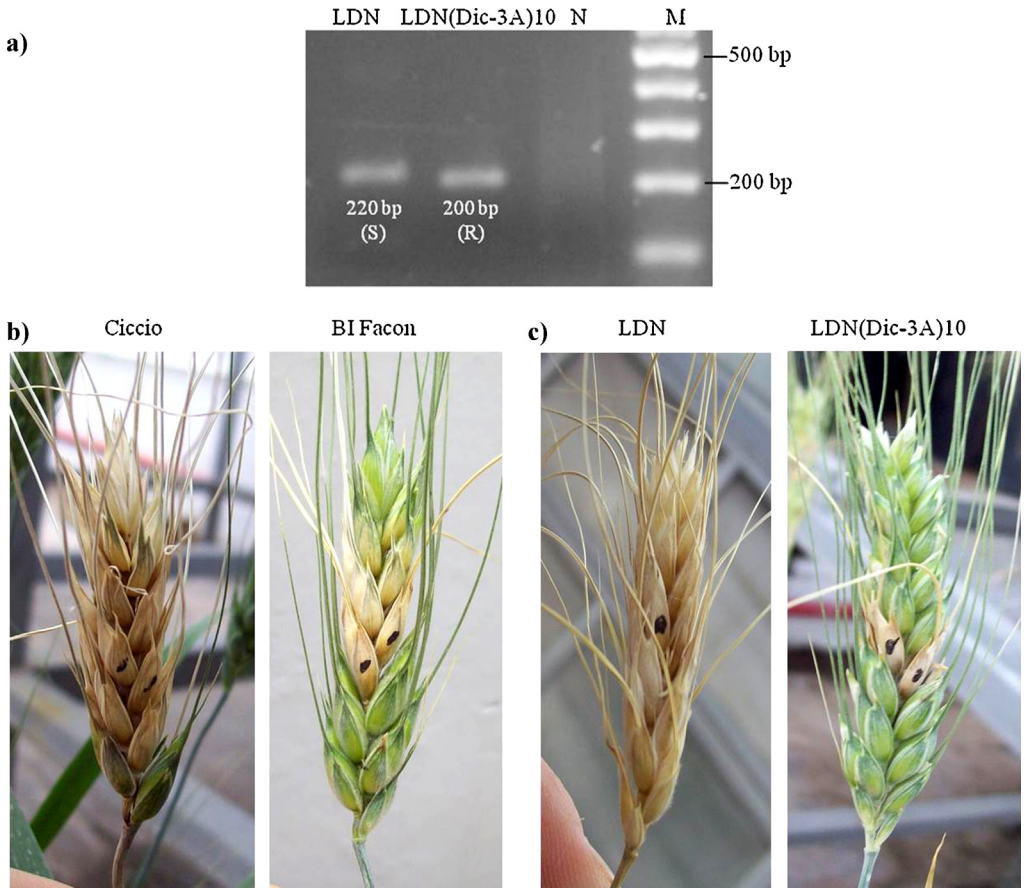

Fig. 1. Molecular and phenotypic evaluation of FHB resistance of durum wheat cultivars. (a) PCR amplification of the SSR marker *Xgwm2* in LDN and LDN(Dic-3A)10 genotypes. The introgressed genotype is recognized by allele size as the primers produce a 220-bp amplification band (S) in the susceptible genotype LDN and a 200-bp amplification band (R) in the resistant LDN(Dic-3A)10. M: molecular marker. N: negative control. (b) Disease severity assay at 21 days post-inoculation in cultivars previously characterized as susceptible (Ciccio, left) and resistant (BI Facón, right). (c) Disease severity assay at 21 days post-inoculation of the LDN susceptible genotype (left) and the LDN(Dic-3A)10 resistant genotype (right). Inoculated spikelets were marked using a colored marker.

infection than the original susceptible cultivar LDN (98.4%, $n = 21$; $p \leq 0.001$; Fig. 1c).

3.2. cDNA-AFLP analysis and validation assays

Seven AFLP primer combinations were used to amplify the eight cDNA samples obtained from the four times points analyzed for each genotype, resulting in a total of ~500 evaluated transcript-derived fragments (TDFs), ranging from 50 to 100 per primer combination. TDFs observed in the cDNA-AFLP acrylamide gels ranged in size from 50 to 600 bp (Fig. 2). Of these, 85 TDFs showed differential expression between genotypes and/or time points. As a first approach, the TDF banding patterns were classified into two groups in order to qualitatively describe the global changes in gene expression that occurred under *F. graminearum* inoculation. Group A included ~45% of the TDFs and consisted of pathogen-responsive TDFs, i.e., the TDFs that showed altered expression patterns induced by *F. graminearum* inoculation in both susceptible and resistant genotypes. Since differences were observed in some cases between genotypes regarding the timing or duration and intensity of expression, Group A was further sub-classified as: (A1) TDFs for which pathogen-responsiveness was undistinguishable between genotypes; (A2) TDFs that differed in the expression start time between genotypes; (A3) TDFs with differences in the duration of expression; (A4) TDFs with differences in signal intensity over time (Table 3). Group B consisted of the TDFs that displayed differential expression after fungal attack that depended on the genotype, and included 19% and 36% out of the 85 TDFs for LDN (B1) and LDN(Dic-3A)10 (B2), respectively. This latter group is the most interesting one, since these fragments represent genotype-specific genes with potential to be involved in the response to the pathogen.

Forty out of the 85 TDFs could be cloned and sequenced. For the others, technical limitations such as the lack of amplification or amplification of more than one band when using the cDNA-AFLP selective primers impeded the acquisition of the whole sequences. Based on the TDF sequences, 21 specific primers pairs were designed (Table 2) to validate the reliability of cDNA-AFLP in our experimental system by means of RT-PCR performed in biological replicates. The expression patterns determined by the two methodologies were in agreement for most of the primer pairs tested (Fig. 3). However, there were three TDFs (TDF#26, TDF#49, and TDF#79) that showed expression in additional or earlier time points by RT-PCR as compared to cDNA-AFLP (Fig. 3). Taking into account the low intensity of such bands, it could be assumed that such differences between methodologies could be attributed to the greater resolution achieved by amplification of specific genes in RT-PCR. Other specific primers failed to amplify from cDNA samples or led to non-specific amplification. Finally, the *F. graminearum* specific induction of gene expression was demonstrated by the absence of RT-PCR amplification of six random TDFs on cDNA samples from LDN and LDN(Dic-3A)10 spikes inoculated with sterile medium, as compared to *F. graminearum*-inoculated samples (Fig. 4). TFD-specific primers were further tested on genomic DNA. Amplification bands were detected in both genotypes for all the TDFs targeted, including those grouped in B1 and B2, thus suggesting

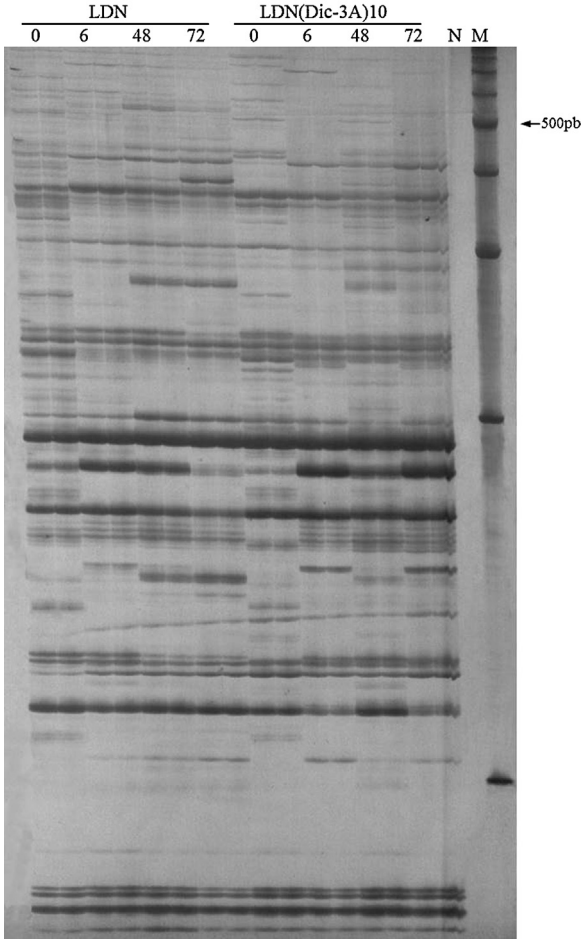

Fig. 2. cDNA-AFLP patterns of wheat transcripts upon inoculation with *F. graminearum*. Image shows polyacrylamide gel (6%, w/v) of TDFs obtained using the primer combination MseI + 2a/PstI + 2a on cDNA samples from LDN and the introgressed line LDN(Dic-3A)10 at 0, 6, 48 and 72 hai with *F. graminearum*. N: negative control, M: molecular marker.

that none of the isolated TDFs originated from the introgressed fragment. Genomic amplifications are illustrated in Fig. 4b, for TDFs from B2 (TDF#54, #77, and #81), B1 (TDF#47) and A1 (TDF#47 and #58).

TDF sequences were compared to two in silico-generated EST libraries. The first one, taken as the control condition, consisted of 74,740 ESTs obtained from 5 libraries deposited in NCBI, constructed from spikes at different developmental stages. The second library was composed of 22,901 ESTs from 15 libraries of *F. graminearum*-inoculated spikes. Out of the 40 sequenced TDFs, 34 (85%) produced no significant matches when compared to control or inoculated libraries. The remaining TDFs showed identity to ESTs from the inoculated libraries, including TDF#73 from Group B2, with one TDF also matching ESTs from the control library (Table 4). Taking into account that a greater number of TDFs showed identity to ESTs from the inoculated libraries and that the inoculated library size is near one third of the control one (74,740 vs. 22,901 ESTs), it can be assumed that the TDF expression was mainly induced by *F. graminearum* inoculation, providing an additional validation of our experimental system.

3.3. TDF annotation

The TDFs were probed against the nr protein database from NCBI though a BLASTX search, revealing that 17 TDFs showed identity to characterized proteins and 2 were proteins with unknown function (Table 3). Additionally, five TDFs showed identity to sequences from *F. graminearum* (Table 3). The 16 sequences for which no matches were found were subjected to BLASTN analysis against other databases, revealing that six TDFs shared identity with sequences from ESTs and six TDFs with sequences from TSA_nt databases, indicating that most of the TDFs had matches with previously identified sequences, even when no function could be attributed to some of them (Table 3). In total, ~90% of the TDFs were found to have strong homology to previously reported transcripts, including all the TDFs from group B2 and four from Group B1 (Table 3), whereas four TDFs showed no homology. It is interesting to remark that no redundancy in the TDFs sequenced was detected, mainly due to technical reasons as the use of a rare cutter enzyme with 6-base recognition that favors the visualization of a single cDNA fragment for each mRNA present in the original sample (Breyne et al. 2003) combined with the use of two selective nucleotides in the final PCR amplification step.

Among the proteins similar to TDF sequences from Group A were plant disease resistance proteins (TDFs #44, 49, 50, 58 and 70), a germin-like protein (TDF#6), a cell wall protein (TDF#4), transcription factors (TDFs #5 and #26), cell-cycle proteins (TDFs #40 and #83) and DNA processing-related proteins (TDFs #69). A pre-mRNA processing factor probably involved in stress-responsive gene regulation (TDF#33) was the only sequence with an annotated function in Group B1, whereas Group B2 included sequences similar to protein transport protein SEC23 (TDF#66), Myb family transcription factor (TDF#73) and a calcium-activated outward-rectifying K+ channel involved in the activation of pathogen-induced signal transduction cascades (TDF#77).

The five TDFs similar to *Fusarium* sp. sequences (Table 3) may correspond to genes expressed at the fungus infection stage, since they were not detected on the acrylamide gels when a fungus suspension was sampled. Interestingly, two of these TDFs belong to group B1, i.e., were exclusively expressed in samples from the susceptible genotype, whereas the other three were either expressed earlier in LDN (TDF#14, Group A2) or during more sampled time points (TDFs #56 and #78, Group A3). These data suggest that due to the existence of compatible interactions, the pathogen developed more fungal tissue mass in the susceptible LDN genotype than in the resistant LDN(Dic-3A)10, where incompatible interactions exists.

3.4. In silico mapping and gene isolation

The International Wheat Genome Sequencing Consortium (IWGSC; www.wheatgenome.org/) has adopted the flow-sorted chromosomal arms genomic approach to the analysis of the wheat genome, achieving a great reduction in complexity (Doležel et al. 2009). As members of the IWGSC, we followed such a chromosome-based approach to search the database of separate chromosome arms using TDF sequences as probes for in silico mapping and to obtain genomic and regulatory sequences. For each identified sequence, we calculated the identity to the probe, i.e., the number of aligned nucleotides that were the same in both sequences, and the coverage of the alignment, expressed as the quotient of the number of aligned base pairs and the total length of the probe. To avoid incorrectly assigning the locations of the TDFs, matches were considered as positive when they aligned with a unique sequence from the database or the quality parameters of the first hit significantly differed from that of the subsequent hits (Table 5). Near half of the mapped TDFs presented distinguishable gene copies in the three hexaploid wheat genomes. Due to the equal quality of the alignments, TDF#26 was included in the analysis in spite of showing positive matches with sequences from 2AS, 2BS and the non-homologous chromosome 6BS. The presence of a gene copy in a non-homologous chromosome is probably a consequence of the interchromosomal arrangements suffered by hexaploid wheat.

Table 3
Similarity searches of pathogen-induced and genotype-specific TDFs.

Group	TDF	GenBank accession[a]	Length (bp)	BLAST				
				Description	GenBank database	GenBank accession no.	Specie	*E*-value
A1	TDF#4	JZ719264	589	Cellulose synthase A catalytic subunit 9 (UDP-forming)	nr	EMT30130	*A. tauschii*	7×10^{-11}
	TDF#26	JZ719267	252	PHD finger transcription factor-like protein	nr	BAD31424	*O. sativa*	3×10^{-18}
	TDF#31	JZ719269	191	Coiled-coil domain-containing protein 115-like	nr	XP003580113	*B. distachyon*	8×10^{-21}
	TDF#49	JZ719276	271	Cysteine-rich receptor-like protein kinase 10	nr	EMT32307	*A. tauschii*	2×10^{-07}
	TDF#50	JZ719277	260	Putative LRR receptor-like serine/threonine-protein kinase	nr	EMT05017	*A. tauschii*	4×10^{-39}
	TDF#55	JZ719280	161	Wheat *Fusarium graminearum* infected spike cDNA library, mRNA sequence (dbEST 9984)	EST	CN009342	*T. aestivum*	9×10^{-68}
	TDF#58	JZ719281	581	Putative LRR receptor-like serine/threonine-protein kinase	nr	EMT22760	*A. tauschii*	2×10^{-74}
	TDF#68	JZ719284	292	UCW Tt-k45 contig 57237 transcribed RNA sequence	TSA_nt	GAKM01041090	*T. turgidum*	2×10^{-184}
	TDF#83	JZ719295	114	Lysine-specific demethylase 5A	nr	EMS65270	*T. urartu*	3×10^{-20}
A2	TDF#5	JZ719265	515	Transcription initiation factor IIE subunit beta	nr	EMS56352	*T. urartu*	2×10^{-55}
	TDF#6	JZ719266	486	Germin-like protein (GER4 h)	nr	ACT22757	*H. vulgare*	2×10^{-108}
	TDF#14	-	317	Hypothetical protein - ATP-grasp domain	nr	EYB22743	*F. graminearum*	6×10^{-59}
	TDF#69	JZ719285	160	RNA intron-encoded homing endonuclease	nr	XP003614385	*M. truncatula*	7×10^{-18}
	TDF#40	JZ719271	296	26S protease regulatory subunit 8 homolog A-like	nr	XP003563677	*B. distachyon*	2×10^{-49}
A3	TDF#56	-	158	Hypothetical protein—early set domain	nr	XP391273	*F. graminearum*	3×10^{-27}
	TDF#70	JZ719286	246	Disease resistance protein RPM1	nr	EMS53986	*T. urartu*	6×10^{-25}
	TDF#78	-	259	Transketolase–Transketolase domain	nr	EWY90787	*F. oxysporum*	7×10^{-51}
	TDF#79	JZ719292	206	cDNA clone G550116K15, mRNA sequence (dbEST 14017)	EST	CD913034	*T. aestivum*	3×10^{-102}
A4	TDF#44	JZ719274	129	kinase R-like protein	nr	AAL51077	*T. aestivum*	2×10^{-18}
	TDF#61	JZ719282	247	NS	–	–	–	–
	TDF#62	-	199	NS	–	–	–	–
B1	TDF#9	-	412	Hypothetical protein - RhoGAP domain	nr	XP388814	*F. graminearum*	2×10^{-59}
	TDF#33	JZ719270	104	Putative pre-mRNA processing factor	nr	AAY84874	*T. aestivum*	2×10^{-14}
	TDF#47	JZ719275	170	Y. Ogihara unpublished cDNA library whchul, mRNA sequence (dbEST 21051)	EST	CJ963093	*T. aestivum*	3×10^{-58}
	TDF#51	JZ719278	114	NS	–	–	–	–
	TDF#60	-	304	Hypothetical protein - DnaJ domain	nr	XP385309	*F. graminearum*	0
	TDF#82	JZ719294	87	NS	–	–	–	–
B2	TDF#30	JZ719268	189	TA RNA 281274 transcribed RNA sequence	TSA_nt	GAJL01022296	*T. aestivum*	1×10^{-30}
	TDF#41	JZ719272	122	Unknown protein	nr	EMS57082	*T. urartu*	9×10^{-07}
	TDF#42	JZ719273	202	cDNA clone G118107N03, mRNA sequence (dbEST 14013)	EST	CD888378	*T. aestivum*	1×10^{-36}
	TDF#54	JZ719279	226	Y. Ogihara unpublished cDNA library whsct, mRNA sequence (dbEST 21043)	EST	CJ800799	*T. aestivum*	2×10^{-113}
	TDF#66	JZ719283	414	Protein transport protein SEC23-like	nr	XP003578242	*B. distachyon*	4×10^{-27}
	TDF#71	JZ719287	246	UCW Tt-k35 contig 19978 transcribed RNA sequence	TSA_nt	GAKM01007089	*T. turgidum*	2×10^{-124}
	TDF#73	JZ719288	605	Myb family transcription factor APL	nr	EMT26247	*A. tauschii*	3×10^{-09}
	TDF#75	JZ719289	330	UCW Tt-k31 contig 47747 transcribed RNA sequence	TSA_nt	GAKM01038610	*T. turgidum*	3×10^{-171}
	TDF#76	JZ719290	75	Unknown protein TRIUR3 31578	nr	EMS52013	*T. urartu*	6×10^{-05}
	TDF#77	JZ719291	271	Putative calcium-activated outward-rectifying K+ channel 6	nr	EMS60717	*T. urartu*	2×10^{-26}
	TDF#81	JZ719293	161	UCW Tt-k55 contig 73562 transcribed RNA sequence	TSA_nt	GAKM01045949	*T. turgidum*	6×10^{-74}
	TDF#84	JZ719296	106	*Hordeum vulgare* subsp. vulgare cDNA clone HM09P17 5-PRIME, mRNA sequence (dbEST 010856)	EST	BU995353	*H. vulgare*	3×10^{-40}
	TDF#85	JZ719297	105	UCW Tt-k63 contig 75867 transcribed RNA sequence	TSA_nt	GAKM01123626	*T. turgidum*	2×10^{-44}

Expression patterns were grouped in (A1) pathogen-response undistinguishable between genotypes; (A2) TDFs that differed in the expression start time between genotypes; (A3) TDFs with differences in the duration of expression; (A4) TDFs with differences in signal intensity over time; (B1) TDFs with expression specific to susceptible genotype LDN; and (B2) TDFs with expression specific to resistant genotype LDN(Dic-3A)10. The TDF accession number, length and the matched sequence are shown in columns 3–5, whereas columns 6–9 show the database searched, the accession number of the matched sequence, the corresponding species and the alignment *e*-value. nr: GenBank non-redundant protein sequences; nt: GenBank nucleotide collection; EST: GenBank expressed sequence tags; TSA_nt: Transcriptome Shotgun Assembly; NS: no significant similarity found.

[a] Sequences with identity to *F. graminearum* were not submitted to GenBank.

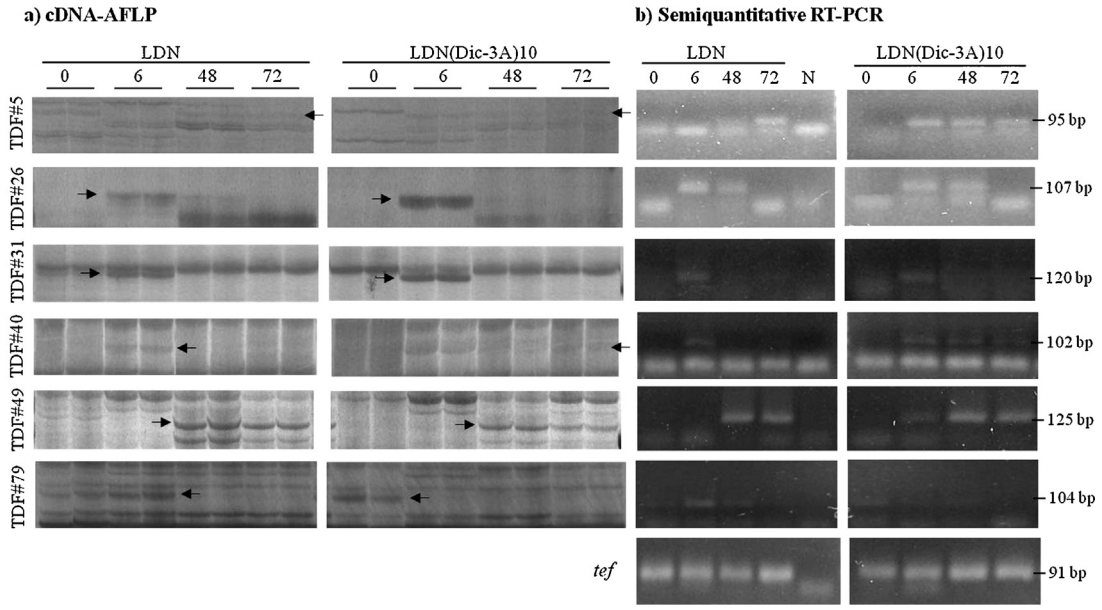

Fig. 3. Validation of cDNA-AFLP expression patterns by semi-quantitative RT-PCR. (a) cDNA-AFLP banding patterns on polyacrylamide gels. (b) PCR fragments amplified by semi-quantitative RT-PCR separated on agarose gels. Both experiments were conducted on LDN and LDN(Dic-3A)10 inoculated spikes at 0, 6, 48 and 72 hai. The primer pairs tested are shown at the left. The expression of the constitutive gene *tef* was used as the reference transcript level. N: negative control containing the same PCR mixture, but without template cDNA.

Interestingly, copies for TDFs#5 and #50 located in the homologous group 4 reflected the inversion suffered by chromosome 4A (Devos et al. 1995; Garbus et al. 2013). In total, 28 TDFs were mapped to chromosome arms (Table 5). Although TDFs were located throughout the chromosomal arms, 5BL contained the largest share (~18% of mapped TDFs) (Table 5). Concerning the genotype-specific TFDs, 11 and 3 associated to Groups B2 and B1, respectively, could be mapped but they did not show any preponderance in chromosome location (Table 5). Further alignments of the TDFs with individual genomic sequences from IWGSC followed by its alignment to the corresponding protein, allowed the identification of the complete coding regions for 14 TDFs and partial coding regions for 6.

3.5. Identification of transcription factor binding sites in the gene regulatory regions

Regulation of transcriptional activity often involves transcription factor binding sites (TFBSs or *cis*-regulatory elements) that are present in the non-coding sequence upstream of a gene (Deihimi et al. 2012). To identify potential regulatory motifs in the promoter regions of TDF-associated genes, we used the PlantPAN database (Plant Promoter Analysis Navigator, http://plantpan.mbc.nctu.edu.tw) to predict TFBSs and the corresponding transcription factors. Accordingly, ~1500 bp upstream the transcriptional start sites of the genes associated with TDFs from Group B2, i.e., #41, #66, #73, #77 and #84, established based on the alignment with the corresponding proteins, were extracted from *T. aestivum* genome and considered as promoters. The upstream the transcriptional start sites of genes associated with TDFs from Group A1 (#4, #26, #49, #50 and #58) and A2 (#5 and #6), A3 (#70), were additionally identified and included in the analysis. Due to the characteristics, the assembled sequences deposited in the chromosome arm sequence database, and we could not obtain the 5′ regions for some of TDFs, even when we searched on the homologous chromosomes. In other cases, it was not possible to define the beginning of the protein. Analysis of the promoter regions allowed the identification of several TFBSs, including AMYBOX1, AMYBOX2, −300ELEMENT, ABREOSRAB21, CACGTGMOTIF, EMBP1TAEM, WBBOXPCWRKY1, −300CORE (Table 6). We focused on the TFBSs WBBOXPCWRKY1 and ABREOSRAB21 since they have been previously related to defense mechanisms (Adie et al. 2007; Singh et al. 2002). The TFBS WBBOXPCWRKY1 is the target of WRKY transcription factor

Table 4
Comparison of TDF sequences to EST libraries.

TDF No.	Group	TDF length (bp)	EST	EST length (bp)	dbEST	Alignments		Score (bits)	*E*-value	I (%)	C (%)
a)											
#31	A1	191	EB514120	282	19525	Q: 116-191	S: 282-208	123	1×10^{-28}	96	40
#55	A1	161	CN009342	637	9984	Q: 1-154	S: 369-215	260	7×10^{-70}	97	96
#6	A2	486	CN008153	489	9984	Q: 206-481	S: 489-214	476	2×10^{-134}	97	57
#69	A2	160	BM172715	573	9984	Q: 1-160	S:19-178	289	5×10^{-83}	100	100
#40	A3	296	BM136136	275	9984	Q: 1-150	S: 126-275	298	6×10^{-81}	100	51
#73	B2	605	CD373966	613	13766	Q: 47-605	S: 613-55	1.069	0	99	92
b)											
#69	A2	160	CJ499517	536	19546	Q: 1-160	S: 135-294	318	5×10^{-83}		100

a) Comparison to *F. graminearum*-inoculated *T. aestivum* EST libraries. BLASTn searches were performed in a local database constructed from 15 EST libraries containing 22,901 ESTs from *T. aestivum* inoculated spikes (dbESTs #19527, #19526, #19521, #19524, #19525, #19528, #9984, #9788, #11142, #11143, #11144, #19522, #18753, #5584 and #13766). b) Comparison to *T. aestivum* EST libraries. BLASTn searches were performed in a local database constructed from 5 EST libraries containing 74,740 ESTs from *T. aestivum* spikes at different developmental stages. (dbESTs #19546, #10467, #10481, #10480 and #10470). dbEST: name of the EST database; Q and S represent the regions of the query and subject sequences, respectively, that align; I: Identity; C: Coverage.

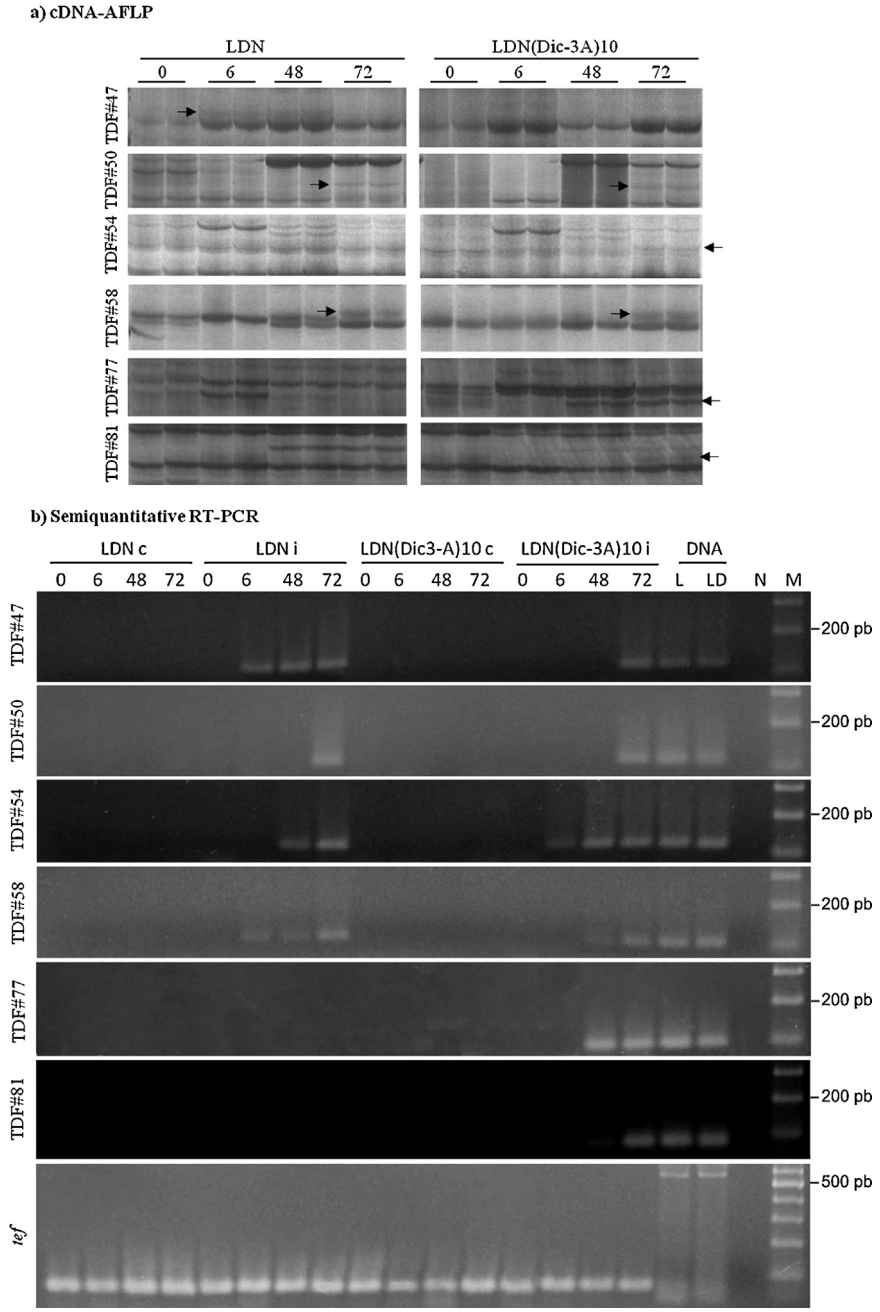

Fig. 4. Validation of *F. graminearum*-induced expression. (a) cDNA-AFLP banding patterns from *F. graminearum*-inoculated spikes separated on polyacrylamide gels. (b) PCR fragments amplified by semi-quantitative RT-PCR separated on agarose gels. LDN and LDN(Dic-3A)10 spikes were inoculated with a conidial suspension (i) or sterile medium (c). Amplification from genomic DNA for both genotypes is shown (L and LD) as a control, as is the negative control (N), performed with the same PCR mixture, but without template cDNA. The expression of the constitutive gene *tef* was used as the reference transcript level. Experiments were conducted on LDN and LDN(Dic-3A)10 at 0, 6, 48 and 72 hai. The primer pairs tested are shown at the left.

proteins that bind to the consensus sequence TTGACY (where Y is C or T) to regulate gene transcription. Most WRKY proteins are involved in responses to biotic or abiotic stresses, such as pathogenic infection (Eulgem et al. 1999). The TFBS ABREOSRAB21 is known as an abscisic acid-responsive element (ABRE) and was identified from the promoter analysis of ABA-regulated genes (Leung and Giraudat 1998). Abscisic acid (ABA) is one of the major plant hormones and plays an important role in plant immunity (Cao et al. 2011). Statistical analysis of the co-occurrence of TFBSs among the gene regulatory regions performed using the PlantPAN web application revealed the presence of TFBS WBBOXPCWRKY1 in the promoter regions (−) strands of the TDFs #6, #58 and #73 ($p < 6.1 \times 10^{-5}$) and the presence of TFBS ABREOSRAB21 in the promoter regions (+) or (−) strands of the TDFs #6, #58 and #77 ($p < 1.5 \times 10^{-5}$), suggesting that these sets of genes could be regulated by the same transcription factors.

3.6. Homology searches against molecular markers linked to resistance QTLs

Sequence homology searches were used to verify whether the identified genes were located close to reported QTLs for resistance to *F. graminearum*, such as *Qfhs.ndsu-3AS* or others. BLAST searches were performed between all TDFs previously identified and 2000

Table 5
In silico mapping of TDFs to chromosome arms of *T. aestivum using the IWGSC database.*

TDF No.	Group	TDF length (bp)	Chromosome	Hit length (bp)	Hits	Alignments		Score (bits)	*E*-value	Identities (%)	Coverage (%)
#4	A1	589	5BL	19,551	1	Q: 1-589	S: 7678-7090	1144	0	100	100
#5	A2	515	4BL	9581	1	Q: 1-515	S: 5179-4665	998	0	99	100
#6	A2	486	4BL	10,418	1	Q: 1-486	S:7916-8401	887	0	99	100
#26	A1	252	2AS	15,445	2	Q: 73-236	S: 12089-12252	292	2×10^{-82}	99	93
						Q: 1-71	S: 11918-11988	132	6×10^{-34}	100	
			6BS	669	2	Q: 71-234	S: 430-593	292	1×10^{-83}	99	92
						Q: 1-71	S: 262-332	132	2×10^{-35}	100	
			2BS	23,604	2	Q: 71-234	S: 9190-9353	292	4×10^{-82}	99	92
						Q: 1-71	S: 9022-9092	132	9×10^{-34}	100	
#30	B2	189	5BL	10,088	1	Q: 25-189	S: 8949-8784	306	6×10^{-82}	99	87
#31	A1	191	2BL	7378	2	Q: 13-139	S: 2434-2560	235	2×10^{-65}	100	93
						Q: 138-191	S: 2641-2694	95,3	3×10^{-23}	98	
#33	B1	104	5BL	11,695	1	Q:1-104	S: 9431-9534	193	8×10^{-53}	100	100
#40	A3	296	7AL	7121	2	Q: 1-122	S: 2227-2338	207	5×10^{-57}	100	99
						Q: 110-296	S: 2739-2925	340	5×10^{-97}	99	
#41	B2	211	4AS	18,725	1	Q: 1-122	S: 12187-12308	221	6×10^{-66}	100	100
#42	B2	202	1BS	12,824		Q: 1-127	S:3167-3041	221140	4e−551e^{-30}	98	100
						Q: 126-202	S: 1966-1890			100	
#44	A4	129	5BL	9514	1	Q: 1-129	S: 9105-9233	256	3×10^{-67}	100	100
#47	B1	170	6AL	44,446	1	Q: 1-170	S: 39301-39132	337	8×10^{-92}	100	100
#49	A1	271	3B	416	1	Q: 1- 271	S: 394-124	486	9×10^{-135}	99	100
#50	A1	260	4BS	13,669	2	Q: 1-232	S: 5951-5720	429	2×10^{-123}	100	89
						Q: 230-260	S: 5636-5606	58,4	9×10^{-12}	100	
#54	B2	226	3AL	6264	1	Q: 1-226	S: 1109-1334	449	5×10^{-125}	100	100
#55	A1	161	2BL	7856	1	Q: 1-161	S: 1262-1102	320	3×10^{-86}	100	100
#58	A1	581	4AL	2296	1	Q: 1-581	S: 1665-2238	780	0	93	100
#66	B2	414	5BL	14,639	1	Q: 1-414	S: 7801-8214	813	0	100	100
#68	A1	292	5BS	9735	1	Q: 1-292	S: 5043-4733	390	6×10^{-112}	90	100
#70	A3	246	3AS	5354	1	Q: 2-246	S: 3256-3022	177	$7 \times e^{-66}$	86	86
#73	B2	605	3B	17,435	2	Q: 1 - 120	S:7681- 7562	112877	7×10^{-52} 0	99	100
						Q: 117-605	S: 7462 - 6964			99	
#75	B2	330	4BL	1099	1	Q: 1-330	S: 391-62	655	0	100	100
#77	B2	271	3B	6192	1	Q: 25-271	S: 4571-4816	383	6×10^{-110}	95	91
#79	A3	206	2AL	23517	1	Q: 1-206	S: 23269-23064	409	5×10^{-113}	100	100
#81	B2	161	2BS	14232	1	Q: 1-161	S: 7712-7872	320	2×10^{-86}	100	100
#83	B1	114	1AL	12779	1	Q: 10-90	S: 8866-8946	153	2×10^{-36}	99	71
#84	B2	106	7AS	7067	1	Q: 1-106	S: 5230-5125	211	7×10^{-54}	100	100
#85	B2	105	1BL	6508	1	Q: 1-105	S: 3332-3228	121	$5 \times e^{-31}$	90	100

TDF: transcript-derived fragments. Q: Query sequence. S: Subject sequence from the database.

wheat sequences for diversity array technology (DArT) markers. These markers are commonly used for mapping, to evaluate genetic diversity, and for association mapping studies (Marone et al. 2012). TDF#30 (Group B2) was aligned with the Wpt-8125 marker (Identity = 98%, *e*-value = 2×10^{-83}), mapped to chromosome 5BL at 2.5 cM from the *QFhb.usw-5B3* QTL that is associated with FHB resistance (Ruan et al. 2012).

4. Discussion

In this work, we characterized gene expression induced after *F. graminearum* inoculation in the introgressed resistant durum wheat line LDN(Dic-3A)10 relative to that in the susceptible parental line LDN, including the identification of genes that were selectively induced in the genotype harboring the QTL *Qfhs.ndsu-3AS*. Differential gene expression was analyzed based on cDNA-AFLP (Bachem et al. 1996), which has been demonstrated to be a powerful technique to study genes involved in host–pathogen interactions (Durrant et al. 2000; Eckey et al. 2004; Polesani et al. 2008; Sestili et al. 2011; Wang et al. 2009; Zhang et al. 2003). This method has the important advantage of allowing genome-wide expression analysis without prior sequence knowledge so that both known and unknown genes can be analyzed (Bachem et al. 1996). The cDNA-AFLP approach has been successfully employed to identify TDFs that represent genes and alleles that are differentially expressed after *Fusarium* inoculation in the resistant bread wheat genotype CM82036 (Steiner et al. 2009). The number of identified bands per primer combination obtained in our study is in agreement with those obtained in the mentioned articles. Concerning the number of primer combinations used, although the wide range used (30–500), in most of the studies the percentages of the differentially expressed TDFs ranged from 1 to 17%, encompassing our results to the reported ranges.

Our experiments were conducted under controlled greenhouse conditions, with all wheat florets inoculated at the same developmental stage. The temporal window considered in this study includes the earlier fungal infection events, from conidial germination (6–12 hai) to host tissue penetration (36 hai), hyphal network formation on the inner surfaces of lemma, palea and glume (48 hai) and initiation of necrosis (72 hai; Brown et al. 2010; Kang and Buchenauer 2000). Near 500 TDFs were amplified using seven different primer combinations, representing ~1% of the ~70,000 functional protein-coding genes estimated for tetraploid wheat according to recent estimations (IWGSC 2014). Such percentage would increase if it could be considered the genes expressed in the particular tissue and developmental stage analyzed here. Then, 85 out of the 500 TDFs were differentially expressed. Even when the number of differential transcripts seems to be scarce, by one side resulted informative and by the other side the common genetic background between genotypes is promising that there are indeed modulated by the infection. Most of them (65%) were expressed at 48 and/or 72 hai, indicating that such point times were the most informative in our experimental system. Approximately 45% of TDFs were expressed both in the susceptible and resistant genotypes (Group A), reflecting a common response, which is expected given the shared LDN genetic background in the two genotypes.

Table 6
Identification of transcription factor binding sites (TFBSs) in the TDF regulatory regions.

Group	TDFs	TFBS	Sequence TFBS	Start	End	Strand
	#4	AMYBOX1	TTTGTTA	−927	−933	+
	#26	AMYBOX1	TAACAAA	−696	−702	–
			TCTGTTA	−569	−575	–
		OCTAMERMOTIFTAH3H4	GATCCGCG	−52	−59	–
	#49	−300ELEMENT	ATTTTACA	−210	−217	–
		AMYBOX2	ATGGATA	−229	−235	–
	#50	–	–			
	#58	−300ELEMENT	CCTTTGCA	−1478	−1485	–
			TGCAAAGT	−1467	−1474	+
A1			TGAAAAAT	−1106	−1113	+
			ATTTTACA	−709	−716	–
			ACTTTGCA	−119	−126	–
		ABREOSRAB21	GGCCACGT	−928	−935	–
		CACGTGMOTIF	CACGTG	−925	−930	–
			CACGTG	−920	−925	+
		EMBP1TAEM	GCCACGTG	−927	−934	–
		WBBOXPCWRKY1	GGTCAAA	−692	−698	–
			TTTGACC	−673	−679	+
	#5	−300ELEMENT	TGCAAAGT	−335	−342	+
		AMYBOX1	TTTGTTA	−191	−197	–
		NONAMERMOTIFTAH3H4	CGTTGGATG	−443	−451	–
		OCTAMERMOTIFTAH3H4	CGCGGATC	−1047	−1054	+
	#6	−300CORE	TGTAAAG	−221	−227	+
A2			TGTAAAG	−207	−213	+
		−300ELEMENT	TGTAAAGG	−220	−227	+
			TGTAAAGT	−206	−213	+
			TGCAAAAT	−195	−202	+
		ABREOSRAB21	GCCCACGT	−1180	−1187	–
		WBBOXPCWRKY1	TTTGACT	−535	−541	+
A3	#70	−300ELEMENT	ATTTTTCA	−684	−691	–
	#41	−300ELEMENT	CTTTTTCA	−1493	−1500	–
			ATTTTTCA	−895	−902	–
	#66	−300ELEMENT	CTTTTACA	−1481	−1488	–
			CTTTTGCA	−1233	−1240	–
	#73	AMYBOX1	TAACAAA	−1065	−1071	+
		AMYBOX2	ATGGATA	−631	−854	–
		WBBOXPCWRKY1	GGTCAAA	−631	−637	–
	#77	−300ELEMENT	TGCAAAGG	−1219	−1226	+
			CTTTTGCA	−1128	−1135	–
B2		ABREOSRAB21	GCGCACGT	−582	−589	–
		AMYBOX2	TATCCAT	−1509	−1515	+
		CACGTGMOTIF	CACGTG	−579	−584	–
			CACGTG	−574	−579	+
	#84	−300ELEMENT	CTTTTGCA	−550	−557	–
			ACTTTGCA	−391	−398	–
			TGCAAAAG	−380	−387	+
			TGCAAAAT	−64	−71	+
		AMYBOX1	TTTGTTA	−693	−699	–
			TCTGTTA	−595	−601	–

The table lists the name and sequence of the TFBSs identified in the promoter regions of the TDF related genes, together with their location and strand, taking the base positioned immediately 3′ to the transcription start site to be −1.

However, the most promising information arose from the TDFs that were expressed solely in the resistant (Group B2) and susceptible genotypes (Group B1), reaching the ∼36 and ∼19%, respectively, of the differentially expressed TDFs.

We used three different approaches to validate the cDNA-AFLP results. First, semi-quantitative RT-PCR experiments performed on biological replicates revealed expression profiles highly in agreement with the cDNA-AFLP patterns. However, RT-PCR produced some bands that were not observed by cDNA-AFLP, consistent with the higher sensitivity of RT-PCR conferred by the use of gene-specific primers. The specificity of the *F. graminearum*-induced expression was demonstrated by RT-PCR assays using cDNA samples obtained from spikes inoculated with sterile medium. All six tested TDFs, from groups A, B1 and B2, were confirmed to be expressed only in *F. graminearum*-inoculated samples. Finally, an in silico approach was undertaken using *T. aestivum* EST libraries obtained from inoculated (22,901 ESTs) and non-inoculated (74,740 ESTs) spikes. Recent studies estimates 106,000 functional protein-coding genes for hexaploid wheat, supporting previous calculations ranging between 32,000 and 38,000 for each diploid subgenome (IWGSC 2014). It is possible to assume that there are ∼70,000 genes in *T. turgidum*, even when not expressed continuously and in every tissue. So, even considering the possible redundancy of the virtual non-inoculated libraries, there are composed of a number of sequences compatible to that expressed of mature spikes, validating the subsequent inferences made. Thus, six TDFs had identity to ESTs from inoculated libraries, including TDF#73 from Group B2, and only one showed identity to an EST from control libraries, whereas the other TDFs showed no identity to any EST. The lack of ESTs corresponding to several of the TDFs likely reflects the relatively low number of ESTs available from inoculated samples and the fact that different response mechanisms might be activated among different species and/or genetic backgrounds. Collectively, these validation

experiments support the efficacy of cDNA-AFLP to identify differentially expressed transcripts at different post-inoculation times in LDN and LDN(Dic-3A)10 genotypes. Moreover, 90% of the TDFs aligned to sequences from various species deposited in protein, EST and/or TSA_nt databases, providing further support that they represent sequences that are likely to be expressed. Interestingly, all the TDFs from Group B2 and half from B1 showed identity to expressed sequences, thus reinforcing their biological significance.

The early resistance reaction has been monitored in other studies related to the *F. graminearum*–wheat interaction and may be a decisive factor in the successful defense response (Ding et al. 2011; Pritsch et al. 2000). Resistance to FHB is complex and is inherited as a quantitative trait governed by polygenes on different chromosomes (Buerstmayr et al. 2009). Genetic evidence suggests that genes functional in pathogen recognition, defense signaling and defense responses are, at least in part, involved in quantitative resistance (Poland et al. 2009). The expression of some genes was induced by the presence of the pathogen in both genotypes, in agreement with the idea that the induction of some genes is a general response to FHB but resistant and susceptible genotypes could differ in their chronological and/or quantitative responses (Pritsch et al. 2000). Nevertheless, ~36% of the TDFs identified in the present study corresponded to transcripts expressed only by the resistant LDN(Dic-3A)10 genotype (Group B2). However, even when these transcripts were expressed only in the resistant line LDN(Dic-3A)10, the corresponding genes were not located on the introgressed region since the specific primers lead to DNA amplification in both genotypes. Studies performed in near isogenic lines with contrasting alleles for the resistance QTL *Fhb1* identified several proteins that were associated with wheat FHB resistance, probably by degrading fungal cell walls and strengthening plant cell walls, detoxifying in the ethylene pathway and maintaining photosynthesis and energy metabolism (Zhang et al. 2013a,b). They postulate that *Fhb1* gene(s) on the chromosome 3BS could transregulate the expression of some of these genes in downstream to provide FHB resistance. Moreover, Zhuang et al. (2013) reported two genes in wheat that were functionally associated with a QTL from 6BL (*Fhb_6BL*) but physically mapped on chromosomes 7D and 5A, encoding a 13-lipoxygenase and a PR-4-like protein. In this context, it is not surprising that the introgressed fragment lead to specific defense responses in the resistant line that provoke changes in signal transduction or other processes involving genes present at other regions in the genome.

Among the resistant line-specific TDFs (Group B2), only three showed similarity to known proteins, whereas the others corresponded to non-characterized proteins, or to expressed sequences such as ESTs or TSAs. Moreover, some of these sequences were detected only in *F. graminearum*-inoculated *T. aestivum* EST libraries. Such TDFs may represent candidate genes implicated in FHB Type II resistance, even though their identity has not yet been established. In agreement with our findings, a high number of wheat genes specifically induced by *F. graminearum* infection in other studies were classified as unknown proteins (Golkari et al. 2007; Hill-Ambroz et al. 2006; Schweiger et al. 2013; Steiner et al. 2009), implying that the molecular basis for FHB resistance mechanism is far to be known and understood.

However, is it interesting that among the genes upregulated by the presence of the pathogen in both genotypes (Group A) were five TDFs related to known proteins that act in the pathogen recognition stage, including plant disease resistance (R) genes, involved in avirulence determinants of plant pathogen recognition (Li et al. 2015). These TDFs included resistance protein RPM1, mapped to chromosome 3AS, which has been reported to be activated after *F. graminearum* attack on wheat bread with the Type II resistance source of Sumai 3 (Kong et al. 2007; Kugler KG et al., 2013). Our results are consistent with the existence of an association between this QTL and the RPM1 gene, even when not co-located with the QTL. The induction of some PR genes, as RPM1, is a general response to FHB, but it has been observed that are up-regulated, earlier, faster and/or more in resistant genotypes than in susceptible genotypes (Pritsch et al. 2000). The differentially expressed genes also included two encoding kinase-domain proteins and two encoding LRR motifs often found disease resistance proteins related to pathogen recognition (Ellis et al. 2000), mapped within least at 15 cM of the peak for the QTL *Qfhs.ndsu-3AS*, associated with FHB resistance in LDN(Dic-3A)10 (Otto et al. 2002). Additionally, TDFs #6 and #4 showed similarity to germin proteins and the enzyme cellulose synthase (CesA9), respectively, reported to be involved in defense responses in plants. Germin-like proteins of barley have been described as pathogenesis-related proteins (PR; Christensen et al. 2004; Woo et al. 2000; Zimmermann et al. 2006), whereas CesA proteins are part of a surveillance system for cell wall integrity that senses the presence of a pathogen and transduces that signal into a rapid transcriptional reprogramming of the affected cell (Gus-Mayer et al. 1998; Schulze-Lefert 2004; Vorwerk et al. 2004). Although the expression of this group of genes likely did not account for the difference in susceptibility to *F. graminearum* infection between genotypes since they were expressed in both lines, they reflect a shared plant response to infection. It remains possible that they play a role in LDN(Dic-3A)10 resistance, perhaps as members of pathways in which the introgressed genes participate to confer resistance. Thus, the observed *F. graminearum* induction of gene expression is in agreement with the idea that wheat-*Fusarium* interaction is highly specific, similar to that reported previously by Golkari et al. (2007).

The location of the TDF-associated genes across the wheat genome was examined based on the homology with the bread wheat chromosome arm sequences. QTLs *Qfhs.ndsu-5BL* and *QFhb.usw-5B3* associated with FHB resistance have previously been reported to reside on 5BL (Ghavami et al. 2011; Ruan et al. 2012). Interestingly, although the mapped TDFs were located throughout the bread wheat chromosomes, ~17% were found on 5BL. Moreover, TDF#30 (Group B2) aligned with the Wpt-8125 marker, which mapped to chromosome 5BL at 2.5 cM from the *QFhb.usw-5B3* QTL, which is also associated with a visual rating index that combines the effects of disease incidence and severity (Ruan et al. 2012). TDF#70 (Group A3) was mapped to the chromosome 3AS arm where the QTL *Qfhs.ndsu-3AS* from *T. turgidum L.* var. *dicoccoides* (Otto et al. 2002) is located. The TDFs #49 (Group A1) and #73 (Group B2) were mapped to the homologous chromosome 3B, where the QTL *Qfhs.ndsu-3BS*, associated to *Fusarium* resistance in the resistant line Sumai 3 (Liu and Anderson 2003), was mapped.

A TTTGACT motif or W-Box was identified in the promoter regions of the genes associated with two TDFs that belong to the Group A, #58 and #6, and one from Group B2, #73. The W-Box is the binding site for the WRKY family of transcription factors, involved in regulating plant responses to pathogen attack (Buscaill and Rivas 2014; Eulgem and Somssich, 2007; Pandey and Somssich 2009). Notably, TDF#6 has identity with Germin (GER), which contains multiple W-boxes (Himmelbach et al. 2010). Moreover, the expression of *WRKY1* genes has been reported to be enhanced in *F. graminearum*-inoculated anthers (Golkari et al. 2007). In transgenic wheat plants, the overexpression of *TaWRKY45* induced a downstream pathway that is involved in defense signaling, resulting in inhibition of *Fusarium* spreading from the inoculated spikelet to others, i.e., type II resistance (Bahrini et al. 2011). Although not identified as having a W-box in this study, TDF#49 (Group A) showed identity to Cysteine-rich receptor-like protein kinase 10 (CRK10), which was first identified as a pathogen-response gene and has WRKY-binding sites in its promoter region (Chen et al. 2003; Du and Chen 2000), being responsive to both pathogen- and salicylic acid-induced WRKY transcription factors (Chen et al. 2003;

Du and Chen 2000). The finding of common TFBSs among TDFs from different groups, in agreement with our previous observations, indicates that several defense pathways genes are activated by *F. graminearum* inoculation in both genotypes while the difference in susceptibility probably resides in the genes that are solely expressed by LDN(Dic-3A)10. In addition, a binding site for ABRE-OSRAB21, related to ABA-induced genes (Kang et al. 2002), was detected in the promoter regions of genes associated with TDFs#6 (Group A2) and #58 (Group A1) and another TDF from Group B2, TDF#77. Thus, the coexistence of similar TFBSs in the promoter regions suggests that metabolic pathways could be shared by more than one kind of stress.

Five TDFs showed homology to *F. graminearum* genes. These genes are apparently expressed in the fungal propagation stage since they were not observed in samples of inoculum in liquid medium. Moreover, two of these TDFs, including sequences that encode DnaJ and Rho GTPase domains, were observed only in the samples from the susceptible genotype LDN. This is suggestive of a higher or earlier pathogenic activity in LDN as compared to LDN(Dic-3A)10, or, alternatively, of different fungal genes being expressed both genotypes, the latter less probable since Type II resistance to FHB (resistance to spread of pathogen within heads). Whatever the case, such differences might be reflecting differences in susceptibility between genotypes. Even when no genes from the mycotoxin synthesis pathways were identified, TDF#9 (Group B1; susceptible genotype) encoded a gene domain RhoGAP. Although *F. graminearum* virulence mechanisms remain to be fully characterized, Rho GTPases such as FgCdc42 have been implicated in *F. graminearum* hyphal development, conidium formation and deoxynivalenol production and thus to the pathogenic capacity (Zhang et al. 2013a,b). Our findings suggest that the expression of fungal genes can be dependent on the resistance level of the host, in contrast to previous results (Steiner et al. 2009).

Overall, the present work allowed the identification of genes differentially expressed in spikes of resistant and susceptible durum wheat genotypes upon *F. graminearum* inoculation. Plant–pathogen interaction induces specific and general responses, revealed by the modifications in gene expression. Several transcripts were expressed in both materials, suggestive of a common defense response that, in spite of being gene-specific, does not explain difference in susceptibility. Changes in gene expression in genotype LDN are not effective to impede the pathogen colonization, probably since the lack of the introgressed genes impedes the full expression of the resistance pathway. The broad spectrum of induced genes and their distribution across the whole genome is suggesting that differences in pathogen susceptibility among the studied genotypes are governed by the genes located into de introgressed fragments but other genes, probably located throughout the genome, and contributes to the defense pathways. The transcripts expressed solely in LDN(Dic-3A)10 may be related to the introgression of the QTL *Qfhs.ndsu-3AS*. Our findings, including the identification of the likelihood of common transcription regulation mechanisms among certain TDFs, suggest that LDN and LDN(Dic-3A)10 interactions with *F. graminearum* are governed by gene-for-gene relationships (Li et al. 2015) and evidenced several changes in transcripts expression attributable to the presence of the QTL *Qfhs.ndsu-3AS*.

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