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Short communication

DEVELOPMENT OF A NEW WHEAT MICROARRAY FROM A DURUM WHEAT TOTIPOTENT cDNA LIBRARY USED FOR A POWDERY MILDEW RESISTANCE STUDY

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Abstract: Totipotent cDNA libraries representative of all the potentially expressed sequences in a genome would be of great benefit to gene expression studies. Here, we report on an innovative method for creating such a library for durum wheat (*Triticum turgidum* L. var. *durum*) and its application for gene discovery. The use of suitable quantities of 5-azacytidine during the germination phase induced the demethylation of total DNA, and the resulting seedlings potentially express all of the genes present in the genome. A new wheat microarray consisting of 4925 unigenes was developed from the totipotent cDNA library and used to screen for genes that may contribute to differences in the disease resistance of two near-isogenic lines, the durum wheat cultivar Latino and the line 5BIL-42, which are respectively susceptible and resistant to powdery mildew. Fluorescently labeled cDNA was prepared from the RNA of seedlings of the two near-isogenic wheat lines after infection with a single powdery mildew isolate under controlled conditions in the greenhouse. Hybridization to the microarray identified six genes that were differently expressed in the two lines. Four of the sequences could be assigned putative functions based on their similarity to known genes in public databases. Physical

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Abbreviations used: 5-AzaC – 5-azacytidine; BAC – bacterial artificial chromosome; EST – expressed sequence tags; GST – glutathione s-transferase; HSP90 – heat shock protein; NIL – near-isogenic line

mapping of the six genes localized them to two regions of the genome: the centromeric region of chromosome 5B, where the *Pm36* resistance gene was previously localized, and chromosome 6B.

Key words: 5-Azacytidine, DNA methylation, Powdery mildew, Microarray, Durum wheat, Near-isogenic line, Candidate gene, Quantitative real-time PCR, Physical mapping, *Pm36* gene, Expressed sequence tag

INTRODUCTION

Isolating and cloning functional genes and studying their modes of action are essential in the quest to improve crops. Assigning functions to a newly isolated gene can be done using various approaches that exploit gene or deduced gene product similarity. The choice of approach depends on the level of genetic relatedness between the species under investigation.

In recent years, the pace of gene discovery in cultivated wheat species has greatly accelerated thanks to the development of molecular tools, including molecular marker-based maps, sequence libraries and expressed sequence tags (ESTs), and microarrays. Most recently, chromosome arm-based BAC libraries covering the whole genome of wheat were constructed for the model variety Chinese Spring (http://olomouc.ueb.cas.cz/dnalibraries/cereals). Gene isolation can be accomplished by chromosome walking within BAC libraries, which is the most direct but also the most difficult route. This difficulty is particularly evident for wheat, with its large genome size and abundance of repetitive DNA [1]. Despite the advances in genome sequencing technology, deciphering the logic of transcriptional control and gene regulation in plant systems is still an important and difficult challenge.

Discovery of QTLs and major genes using forward genetics is now routine in wheat, and there is a growing catalogue of mapped genes for both simple and complex traits. However, there is still a lack of knowledge of the mechanisms by which the mapped genes affect traits. Such functional genomics investigations often require the ability to simultaneously profile the differential expression of large numbers of genes.

cDNA microarrays are powerful tools that can be used to profile plant gene expression in different tissues or organs under various stimuli, such as compatible and incompatible pathogen infections. Most of the plant microarray experiments described in the literature focus on slides with bulked plant material. Specific genes expressed at low levels or only expressed during particular developmental stages and/or under certain environmental conditions can be missing from such analyses.

Recently, a new technology for the production of a totipotent cDNA library of durum wheat was developed to enable exploitation of the entire gene pool [2]. This innovative method was based on the use of suitable quantities of 5-azacytidine (5-AzaC) in the plant germination phase to induce demethylation of genomic DNA. 5-AzaC was first synthesized by Piskala and Sorm in 1964

and originally tested as a nucleoside anti-metabolite with a clinical specificity for acute myelogenous leukemia [3]. Early reports indicated that 5-AzaC was an inducer of chromosome breakage and a mutagenic agent [4-8]. Because it can be activated to its nucleoside triphosphate form and incorporated into both DNA and RNA, 5-AzaC treatment of cells leads to inhibition of DNA, RNA and protein synthesis [reviewed in 9]. Incorporation of 5-AzaC into tRNA was shown to inhibit tRNA methyltransferases [10] and to interfere with tRNA methylation and processing, leading to defective acceptor function of transfer RNA [11]. The finding that 5-AzaC is incorporated into DNA, where it can inhibit DNA methylation, led to its widespread use for demonstrating the correlation between loss of methylation in specific gene regions and activation of the associated genes [5-8]. This method obviates the need to construct multiple libraries from different tissues and developmental stages. It can capture the expression of genes for biotic and abiotic stress tolerance or resistance in plants without exposing them to the inducing stress.

The details of this technology (international patent no. WO2005003344 [12]) are described here for the first time. The totipotent cDNA library of durum wheat was used to construct a new microarray derived from an EST collection of about 11,000 sequences, of which 4925 were TUGs (tentative unique genes). The details of the sequence development, characterization and analysis were reported by Cifarelli *et al.* [2] and Gadaleta *et al.* [13].

The objectives of this study were the characterization of a durum wheat totipotent cDNA library and its validation through the assessment of differential gene expression correlated with powdery mildew resistance. Powdery mildew, which is caused by *Blumeria graminis* (DC) Speer f. sp. *tritici* Em. Marchal (syn. *Erysiphe graminis* f. sp. *tritici*), is one of the most significant wheat diseases in many regions of the world. The use of resistant cultivars has proven to be an effective and environmentally safe strategy for controlling wheat pathogens and eliminating the use of fungicides. However, due to frequent changes in the pathogen population, several wheat resistance genes have shown the tendency to become ineffective within a short period after their deployment. Therefore, it is necessary to search for new sources of resistance and to use available genes in combinations that will provide effective and lasting resistance [14].

Resistance introduced in a cultivar remains effective as long as the pathogen does not become virulent on the cultivar. Environmental changes that favor virulence undoubtedly play a role in reducing the effectiveness of resistance. Increased pathogenicity is probably the commonest cause of failure of resistance. In most cases, resistance and avirulence inherit in a dominant manner, while susceptibility and virulence inherit in a recessive manner. This implies that resistance will not remain effective if the pathogen acquires the corresponding virulence by losing the avirulence alleles that elicit resistance either by deletion or by genetic change. The gene-for-gene system occurs most clearly in pathosystems where a biotrophic, highly specialized pathogen is involved, such as in cereals, which are affected by various rusts, smuts and

bunts, and by powdery mildew. So far, forty-three genes for resistance to powdery mildew (*Pm1–Pm43*) have been identified in wheat and assigned to specific chromosomes or chromosome arms [see catalogue of genes in 15]. One such gene is *Pm36*, which has been mapped to chromosome 5B [14].
In this study, we use the totipotent cDNA microarray to analyze transcripts from infected and uninfected seedlings of near-isogenic durum wheat lines (NILs) that differ in the presence or absence of *Pm36*, making them respectively resistant or susceptible to powdery mildew. The resistant NIL 5BIL-42 was made by first crossing a *Pm36* donor line with the susceptible cultivar Latino to produce a heterozygous F1, and then repeatedly back-crossing the offspring to the recurrent parent Latino, retaining the *Pm36* gene in each successive generation. The resultant 5BIL-42 is genetically identical to Latino except for one or a few loci [14]. The use of these NILs in microarray experiments is described here. These allowed us to identify a small number of genes with expression levels that differ between the resistant and susceptible genotypes during infection, including a candidate sequence for the *Pm36* gene.

MATERIALS AND METHODS

Total plant DNA demethylation and cDNA library development and sequencing

Triticum turgidum cv. Ofanto seeds germinated in the dark in the temperature range 22-26ºC and in the presence of four different concentrations of 5-AzaC (0.1, 0.3, 0.5 and 1.0 mM). Sixteen seeds were used for each treatment. The experiments were replicated three times. Five micrograms of polyA mRNA were employed for cDNA synthesis and library construction, as reported in Cifarelli *et al* [2].
DNA was prepared from 15,000 recombinant clones with a Biomek 2000 robotic workstation using the Wizard SV96 Kit (Promega). The BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used with 0.5 µg plasmid DNA as a template and T3 primer (5'-ATTAACCCTCACTAAAG-3') to sequence the recombinant colonies.

Microarray construction

In order to evaluate the suitability of the totipotent library for large-scale sequencing projects, 11,000 clones were sequenced, analyzed and assembled into contigs [2]. The obtained sequences were subjected to both BLASTX and BLASTN searches. Analysis of the sequences revealed mRNA species expected to be present in all plant tissues, independent of the developmental stage and stress conditions [2, 12].
Out of 11,000 sequences, 4925 were not redundant (meaning that they were singletons, having only one TUG); these were double spotted on glass slides (MicroCribi, CRIBI, University of Padova, Italy). As a positive control for labeling, hybridization efficiency, and spotting, we used a housekeeping gene [16] mixture consisting of β-actin, RP18S, GAPDH and TUBB4 sequences. The *HSP65* gene of *Mycobacterium tuberculosis* was used as a negative hybridization control spotted on the array.

Plant material and powdery mildew infection

Two near-isogenic lines (NIL) of durum wheat, Latino and 5BIL-42, which are respectively susceptible and resistant to powdery mildew, were used for the microarray experiments. The two NILs are genetically identical except for a small stretch of DNA on chromosome arm 5BL containing the *Pm36* gene, which is introgressed in Latino from the wild emmer accession MG29896 by a backcross program, as described in [13]. The cultivar Pandas was grown under the same conditions as a susceptible control. The two NILs were infected under controlled conditions. Three replicas for each line were used and the infected plants were grown under same conditions as the control.

Six plants of each genotype were grown in a growth chamber at a temperature of 22ºC with a photoperiod of 18 h light, 6 h darkness. Plantlets were transferred to a greenhouse after 12 days (just after the first leaf expanded).

Three plants of each genotype were infected with the isolate O2 of *Blumeria graminis.* Isolate O2, which was obtained from experimental nurseries located in central Italy, was chosen because of its virulence to plants expressing many known mildew resistance genes, such as *Pm1*, *Pm2*, *Pm3c*, *Pm4a*, *Pm4b*, *Pm5*, *Pm6*, and *Mli*. It is avirulent to plants expressing *Pm3a*, *Pm3b* and *Pm3d.*

Inoculations were performed by brushing conidia from neighboring sporulating susceptible seedlings of *T. aestivum* cv. Pandas, and spread in a settling tower onto the plant materials, aiming for densities of 250-400 spores/cm^2. The incubation conditions were 22ºC, 80% relative humidity and continuous light. The success of the infection was controlled using plantlets of Pandas as a positive control. Three other plants of each genotype were grown under the same conditions without inoculation. Infection types (IT) were scored 12 days after inoculation, when the susceptible control was heavily infected. Leaves from infected and control plants were cut 24, 48 and 72 h after infection, immediately transferred to liquid nitrogen, and stored at -80ºC.

RNA isolation, labeling, and hybridization

Total RNA from leaf samples collected 24, 48 and 72 h after infection was extracted using the RNeasy Plant Mini Kit (QIAGEN) and checked on 1.5% denaturing agarose gel. The total amount of RNA and its purity were determined using a Nano-Drop ND1000 spectrophotometer (Thermo Scientific). All of the RNA samples were brought to the same concentration (1 μg) for subsequent treatment with DNase I Recombinant (Roche Applied Science) to remove genomic DNA, and then were reverse-transcribed into double-stranded cDNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). The cDNA was labeled using two fluorophores (Cy3, Cy5) and then hybridized simultaneously to each glass slide. The entire experiment was repeated to obtain independent biological replicates of infected and non-infected leaves, from which RNA was isolated and labeled with the other fluorophore. The dye-swap replicates were made to reduce the technical variation introduced during the handling of the samples. This consisted of two separate experiments: in the first

replicate, the sample was Cy3-labeled and the reference sample was Cy5-labeled, while in the second replicate the dyes were swapped. Eight labeling reactions were hybridized in pairs to four microarray slides.

Microarray analysis of durum wheat isogenic lines

Images were scanned with a ScanArray Express scanner (Perkin Elmer) at two wavelengths to obtain fluorescence intensities for both dyes with a resolution of 10 μm and PMT of 80%, and visually inspected to flag and exclude abnormal spots with irregular shapes or backgrounds. The signal on each slide was analyzed with the software of the scanner and normalized using within-print-tip-group lowness normalization with default parameters. Analyses were carried out on log2-transformed intensities and ratios. Only clones showing differential expression in all the replicas using the fold change method were selected. Clones considered to be differentially expressed had |log2-ratios| > 2, i.e., their intensity values differed by at least 4-fold.

Quantitative real-time PCR and sequence confirmation of clones

The identities of the clones showing differential expression were confirmed by re-sequencing the DNA in the spots using the method described above. The expression levels of selected expressed sequences were validated using real-time PCR by comparing their levels in the cDNA prepared from RNA from infected and uninfected samples from the two lines. The levels in infected and uninfected samples from the same line were also compared. The primers used to amplify the selected expressed sequences are listed in Table 1. All of the samples were

Table 1. Primers used for the real-time PCR analysis.

Gene	Primer	Primer sequence (5'-3')
AJ609811	For	ACTCAGCTCCAGGTACAGTTTATCC
	Rev	GTTTTCTTTAGCGTGTTTAGTTCTTTTATG
AJ610871	For	TCTCTCCTCCCAATCGAAGCT
	Rev	TGTCGTAACGCCATCGTGAT
AJ611689	For	ACCCGTCTCCACCCAGATAAC
	Rev	TCACTTTTCTCTGCCGTTCGT
AJ614358	For	TTCAGATTGCGAACATAGTTTGC
	Rev	CCTACCCTCATGTAAACCAATTACG
AJ716441	For	CACCGGCAAGTCCAGCTT
	Rev	TCGAGATCATACACGTCGAAATG
FM208374	For	TGGAGAGCGGGTTCAACCT
	Rev	TTTTGCACCGACCGGTAGA
18S*	For	GAAACGGCTACCACATCCAAG
	Rev	CCCCGTGTTAGGATTGGGT

*18S was used as reference gene [48]

analyzed in triplicate and compared with the housekeeping gene encoding 18S. Real-time PCR was conducted using SYBR Green Supermix (Invitrogen) according to the manufacturer's instructions for the iCycler (Bio-Rad).

Physical mapping of the differentially expressed sequences

Nulli-tetrasomic (NTs) and di-telosomic (DTs) lines of Chinese Spring [17-19] and the set of durum wheat Langdon D genome disomic-substitution lines [20] were used to assign the sequences that are differentially expressed in the two isogenic lines to durum wheat chromosomes. The physical locations of the sequences on chromosome bins was further mapped using a set of 58 common wheat deletion lines dividing the A and B genome chromosomes into 94 bins (provided by Professor B.S. Gill, USDA-ARS, Kansas State University) [21]. The lines selected were relatively easy to multiply and contained heterozygous and interstitial deletions [22].

RESULTS AND DISCUSSION

Development of a totipotent cDNA library

The details of the method (international patent no. WO2005003344 [12]) are described here for the first time. The method allows the isolation of cDNA from RNA that is normally expressed under various metabolic conditions and present in various plant tissues. Four different concentrations of 5-AzaC (0.1, 0.3, 0.5 and 1.0 mM) were used to treat a total of 64 seeds (16 seeds for each treatment) of *Triticum turgidum* cv. Ofanto. Approximately 21 days after germination, total DNA and RNA were extracted from the seedlings. Details on the time-course analysis were already reported in Cifarelli *et al.* [2]. To verify that germination and seedling growth in 5-AzaC resulted in demethylation, genomic DNA from the treated seedlings was digested with restriction enzymes sensitive to methylation, i.e., enzymes that preferentially cut hypomethylated DNA (*Cfo*I, *Hpa*2, *Msp*1). The extent of genomic DNA digestion with these enzymes increased with increasing concentrations of 5-AzaC in the treatment (Fig. 1). The lowest concentration of 5-AzaC that resulted in complete digestion by the methylation-sensitive enzymes, indicative of complete demethylation, was 0.3 mM.

In order to confirm that demethylation resulted in global clone expression, RNA from the seedlings was assayed by reverse-transcriptase PCR (RT-PCR) for the presence of transcripts from wheat genes normally expressed in growth stages other than seedlings. The expressed sequences used in these analyses are listed below, along with their accession numbers and tissue specificities**.**

- *Aespre-Ant* (BE500795), only expressed in precocious inflorescence phases;
- *Aesend* (BE401963), expressed in the immature endosperm;
- *Aesgliad* (U08287), encoding an α-gliadin storage protein, expressed in mature seeds;
- *Thiom* (AJ005840), expressing thioredoxin M in all tissues;
- *Thioh* (AJ001903), encoding thioredoxin H in all tissues.

Each expressed sequence tag was amplified using RT-PCR on total RNA and the experiments were replicated three times.

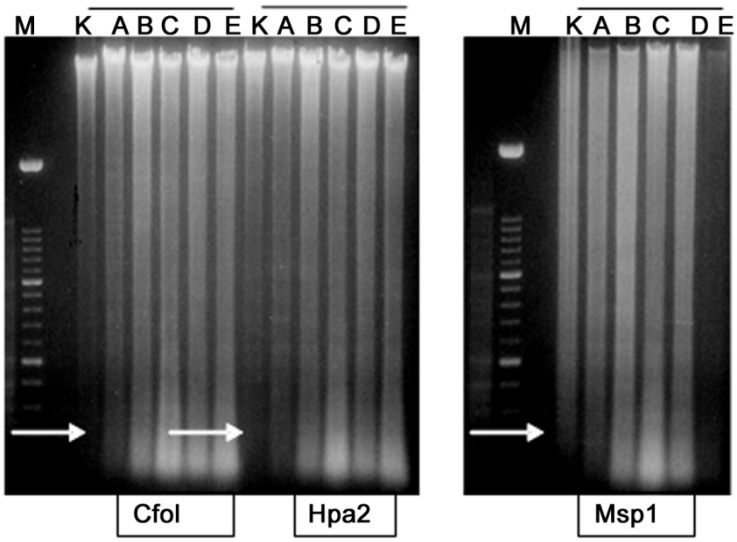

Fig. 1. Digestion of genomic DNA from the durum wheat cultivar Ofanto with methylation-sensitive restriction enzymes (*Cfo*I, *Hpa*2, *Msp*I). M = Marker, K = DNA from untreated plantlets. Lanes A-E contain DNA extracted from plantlets treated with various 5-AzaC concentrations (A = 0 mM, B = 0.1 mM, C = 0.3 mM, D = 0.5 mM, E = 1.0 mM). The arrows indicate the total RNA extracted.

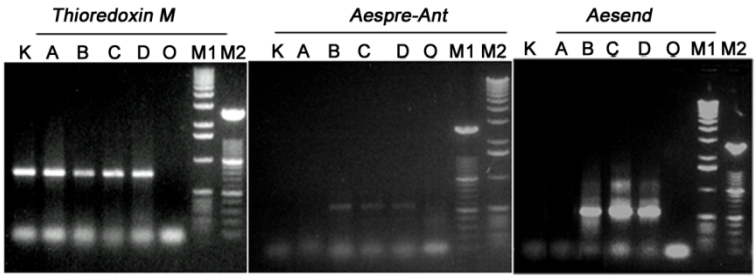

Fig. 2. RT-PCR amplification for two tissue-specific genes (*Aespre-Ant*, *Aesend*). The RT-PCR was conducted on total RNA extracted from durum wheat plantlets that were treated with 5-AzaC (A = 0.1 mM, B = 0.3 mM, C = 0.5 mM, D = 1.0 mM) or untreated (K). The ubiquitously expressed *Thioredoxin M* (*Thiom*) gene was used as a positive control. O = PCR reaction mix without nucleic acid template; M1 = 1 Kb Marker (Gibco) and M2 = Marker XIV (Roche). Arrows point to the expected amplification products for each primer pair.

Fig. 2 shows RT-PCR amplification products for three primer pairs. Transcripts from *Thiom* were detected in all of the samples (treated and untreated plants), while transcripts from *Aespre-Ant* and *Aesend* were only amplified from seedlings treated with various 5-AzaC concentrations. No PCR products were obtained for

these tissue-specific genes in the untreated samples. The amplified fragments had the expected sizes (Fig. 2) and their identities with the targeted genes were confirmed by DNA sequencing. In order to demonstrate that gene expression in Fig.2 follows demethylation of the respective gene regions, a quantitative RT-PCR was also conducted for each gene and each treatment. Correct quantification of the PCR product allowed us to correctly analyze gene expression.

Based on these results, a totipotent cDNA library was constructed as detailed in the Material and Methods section. In order to identify mutations induced by treatment with 5-AzaC, sequencing of a set of genes with known sequences was conducted for the treated samples. No differences were revealed by blast analysis between the sequences reported in databases and the sequences obtained for the same genes in samples treated with 5-AzaC. The library is expected to have a large proportion of the durum genome encoded mRNA, irrespective of the developmental stage or tissue in which they are usually found [2, 12].

Identification of differentially expressed sequences for powdery mildew resistance in two durum wheat near-isogenic lines (NIL)

A wheat microarray from the totipotent cDNA library consisting of 4925 non-redundant sequences was spotted on slides and used for the identification of putative sequences differentially expressed in two durum wheat near-isogenic lines (NILs) that differ in their resistance to powdery mildew. In our current microarray experiments, we only selected those sequences showing a difference of expression in all four replicates (two per slide) using the method of fold change. T-test analysis was conducted to select differentially expressed clones.

Six TUGs (*AJ609811*, *AJ610871*, *AJ611689*, *AJ614358*, *AJ716441*, *FM208374*) were expressed at higher levels in the resistant NIL 5BIL-42 after infection with powdery mildew than in infected Latino (Table 2).

Table 2. Expressed sequences identified in the microarray analysis as being differentially expressed in two near-isogenic lines of durum wheat that differ in their powdery mildew resistance.

Gene accession number	Best BLASTN hit	E value	Best BLASTX hit	E value	Putative function	Chromo-some locali-zation
AJ610871	Unknown	-	XP_003566365.1 *(Brachypodium distachyon)*	$5e^{-14}$	Uncharacterized protein	5BL
AJ611689	AK332251 (*Triticum aestivum*)	0.0	BAJ95474 (*Hordeum vulgare*)	0.0	Predicted protein	5BL
AJ716441	AK356753 (*Hordeum vulgare*)	0.0	BAJ92214 (*Hordeum vulgare*)	$1e^{-135}$	Hypothetical protein	5BL
FM208374	AK335765 *(Triticum aestivum)*	$4e^{-142}$	XP_003563275 *(Brachypodium distachyon)*	$1e^{-13}$	HSP90	5BL
AJ609811	AK374159 (*Hordeum vulgare*)	$3e^{-94}$	Unknown	-	Predicted protein	6B
AJ614358	AF002211 (*Triticum aestivum*)	0.0	O04437.1 (*Triticum aestivum*)		Glutathione s-transferase	6B

The physical map locations of these six sequences were assigned using PCR and gene-specific primers to amplify fragments from genomic DNA of various wheat nulli-tetrasomic and deletion lines (Table 2). As an example, the mapping data for accession AG611689 are shown in Fig. 3 and Fig. 4. This sequence could not be amplified from the Chinese Spring line lacking chromosome 5B (CS_N5BT5A), but was amplified from DNA of several lines missing short segments of the short and long arms of chromosome 5B. Using this method, the six sequences showing higher expression during infection of the powdery mildew-resistant NIL compared to its susceptible parent were assigned to only two locations of the B genome (Table 2). Accessions AJ611689, AJ610871, AJ716441 and FM208374 were localized on chromosome 5BL, where the *Pm36* resistance gene was previously localized [13], while accessions AJ609811 and AJ614358 were mapped to chromosome 6B.

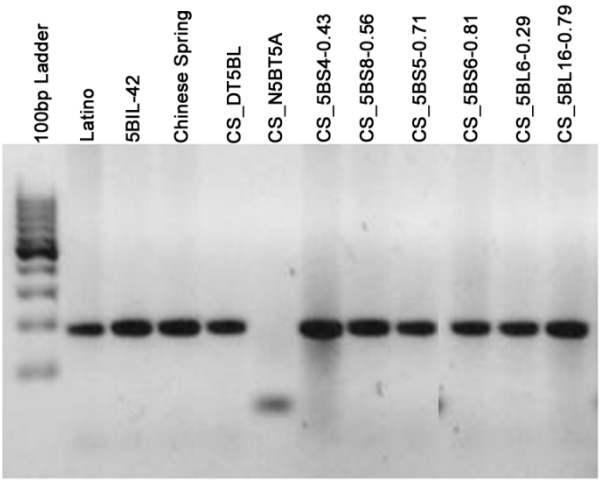

Fig. 3. Electrophoretic pattern of marker AJ611689 amplified in the lines Latino, 5BIL-42, Chinese Spring, di-telosomic line CS_DT5BL and nulli-tetrasomic line CS_N5BT5A, and the bin deletion lines CS_5BS4-0.43, CS_5BS8-0.56, CS_5BS5-0.71, CS_5BS6-0.81, CS_5BL6-0.29 and CS_5BL16-0.79.

Transcripts from the six TUGs were analyzed using real-time RT-PCR to quantify their expression levels in the two lines with and without infection, and to confirm the post-infection differences (24, 48 and 72 h) detected using microarray hybridization. The expression levels were compared between the two lines (5BIL-42 and Latino) after infection, between the two lines without infection, and for each line between infected and uninfected samples (Table 3). Real-time PCR confirmed that all six TUGs had higher transcript levels in the resistant NIL compared to the susceptible parent during the infection process, and that the response occurred in the first 24 to 48 h. For three of the TUGs, the difference in transcript levels between the NILs reversed by 72 h.

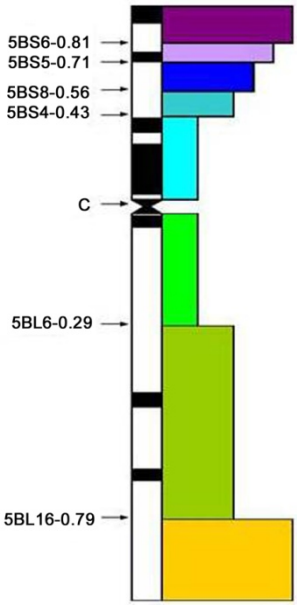

Fig. 4. Cytogenetic deletion map of 5B wheat chromosomes. This is a schematic representation of chromosome partitioning into bins, based on deletion breakpoints. The arrows indicate the name of deletion lines and the FL (fraction length) values of the breakpoints.

Table 3. Real-time PCR results using relative quantification normalized against a reference gene (18S). The normalized expression ratio x was calculated as 2-ΔΔCt, so x > 1 characterizes up-regulated sequences (UP), x = 1 means unvaried sequences, and x < 1 indicates down-regulated sequences (DOWN) in each pair-wise comparison. NA = not amplified.

Accession	Infected 5BIL42 *vs* infected Latino			Infected 5BIL42 *vs* uninfected 5BIL42 control			Infected Latino *vs* uninfected Latino control		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
AJ610871-5B (unknown)	UP (49.63)	UP (1.9)	UP (12.69)	UP (1.58)	UP (4.92)	UP (5.52)	DOWN (0.72)	DOWN (0.37)	DOWN (0.46)
AJ611689-5B (predicted protein)	UP (37.6)	UP (3.17)	UP (5.92)	DOWN (0.3)	UP (29.17)	UP (10.31)	UP (21.6)	UP (24.25)	UP (150.47)
AJ716441-5B (hypothetical protein)	UP (4.48)	UP (1.58)	DOWN (0.42)	DOWN (0.14)	DOWN (0.19)	UP (5.72)	DOWN (0.6)	DOWN (0.58)	UP (1.23)
FM208374-5B (HSP90)	UP (12.12)	UP (2.89)	DOWN (0.33)	UP (330)	UP (58.35)	UP (17.54)	UP (2.19)	UP (1.44)	UP (4.18)
AJ609811-6B (predicted protein)	UP (2.5)	UP (2.5)	UP (6.34)	DOWN (0.16)	DOWN (0.03)	N.A.	DOWN (0.001)	DOWN (0.04)	DOWN (0.05)
AJ614358-6B (glutathione s-transferase)	UP (5.52)	UP (3.1)	DOWN (0.4)	UP (57.01)	UP (16.37)	UP (9.1)	UP (2.75)	UP (1.41)	UP (11.05)

Functional annotation of differentially expressed sequences

The plant responses to infection or infection attempts by pathogens are complex and involve the over-expression of a wide range of genes and gene products [23]. The latter participate in the formation of chemical barriers, such as antifungal PR proteins (pathogen-related proteins), phytoalexins, and structural barriers, such as lignified walls [23]. The well-defined stages of powdery mildew disease development provide multiple opportunities to investigate the regulation of host genes in response to *Pm*-specified incompatible and compatible wheat-*Pm* interactions [24-27]. Information on transcript abundance can be used to describe a cellular state and predict the functional involvement of genes in the interactions between plants and pathogens [28-36].

The putative functions of the six sequences that differentially responded to powdery mildew infection in the two durum wheat NILs (Latino and 5BIL-42) were assigned based on homology searches against the GenBank non-redundant database using the BLASTN algorithm with the default parameters. The highest similarity score was considered as the best match for putative function of the corresponding TUG. Out of six TUGs identified, only two could be assigned putative functions based on nucleotide homology (Table 1). The EST AJ614358 has a high similarity to a gene encoding glutathione s-transferase (GST), and the EST FM208374 had a high similarity to a gene encoding a 90-kDa heat shock protein (HSP90). Both sequences were isolated from *Triticum aestivum*.

GSTs are a major group of detoxification enzymes. All eukaryotic species possess multiple cytosolic and membrane-bound GST isoenzymes, each of which displays distinct catalytic and non-catalytic binding properties. The cytosolic enzymes are encoded by at least five distantly related gene families (designated class alpha, mu, pi, sigma, and theta *GST*), whereas the membrane-bound enzymes, microsomal GST and leukotriene C4 synthetase are encoded by single genes, each of which arose separately from the gene for soluble GST. Evidence suggests that the level of expression of *GST* is a crucial factor in determining the cell sensitivity to a broad spectrum of toxic chemicals [37]. The *GST* genes are up-regulated in response to oxidative stress and are inexplicably over-expressed in many tumors, leading to problems during chemotherapy [38]. In plant species, GSTs are involved in protecting plants against both biotic and abiotic stresses. In this study, a putative *GST* gene AJ614358 was clearly up-regulated in both Latino and the resistant NIL 5BIL-42 after powdery mildew infection, indicating that at least one *GST* gene plays a role in the interaction with this fungus. However, this *GST* is not the *Pm36* resistance gene, because these two genes map to different chromosomes (Table 1). Transcripts from this gene were higher in the resistant NIL than in its susceptible parent during the first 48 h of infection, but lower 72 h post-infection (Table 2).

The involvement of heat shock proteins (HSP) in resistance responses has also been well documented. These molecular chaperones function by helping in the folding of nascent polypeptide chains, the refolding of denatured proteins, and the prevention of irreversible protein aggregation and insolubilization [39]. They

increase the rate of folding and thus increase the resistance of cells under stress conditions. The synthesis of such proteins was reported to increase after various forms of abiotic and biotic stress [40-41]. HSP90s are molecular chaperones that play important roles in plant growth and responses to environmental stimuli. For example, recessive loss-of-function phenotypes caused by mutations in an ATP-binding site have been described for the cytosolic heat shock protein HSP90.2 in *Arabidopsis* [42]. Three different amino acid substitutions in the ATP-binding site of HSP90.2 caused a destabilization of the RPM1 protein, and thus a loss of *RPM1*-mediated disease resistance, while a null mutation in *HSP90.2* had no effect on *RPM1*-mediated resistance [42]. However, little is known about the genes encoding Hsp90s in common wheat.

In a recent genetic and functional analysis of the genes specifying cytosolic Hsp90s in wheat species, Wang *et al.* identified three groups of homoeologous genes *TaHsp90.1*, *TaHsp90.2* and *TaHsp90.3*, encoding three types of cytosolic HSP90, and assigned these to group 2, 7 and 5 chromosomes, respectively [43]. Decreasing the expression of *TaHsp90.1* genes through virus-induced gene silencing (VIGS) caused pronounced inhibition of wheat seedling growth, whereas the suppression of *TaHsp90.2* or *TaHsp90.3* genes via VIGS compromised the hypersensitive resistance response of the wheat variety Suwon 11 to stripe rust fungus. It is interesting that the levels of transcripts encoding a 70-kDa heat shock protein (HSP70) are significantly increased in wheat organs challenged by *F. graminearum* infection [44].

The possible functions of the other four sequences that had higher levels of expression in the powdery mildew-resistant NIL than in its susceptible parent are less easily identified. The most similar nucleotide sequence for accession AJ716441 was to barley (*Hordeum vulgare)* TUG AK356753, which encodes an unknown protein. However, BLASTX analysis revealed a similarity (identity: 48%; coverage: 91%; E value: 1e-51) between the barley AK356753-encoded protein and a mitochondrial chaperone BCS1 from maize (*Zea mays*; NP_001147824.1). Wheat accession AJ611689 had close nucleotide similarity to the barley TUG AK364271, which encodes a predicted protein that BLASTX showed has significant similarity (max. score 93.3%; total score 96.3%; coverage 16%) with accession ACG2478, a 15-kDa dehydration-like protein from maize. Interestingly, a novel transcription factor has been identified recently: ERD15 (early responsive to dehydration 15), which connects endoplasmic reticulum stress with osmotic stress-induced cell death [45]. The endoplasmic reticulum (ER) is a key organelle that serves as the gateway for newly synthesized proteins into the secretory pathway. Following synthesis, secretory proteins are exported from the ER to various cellular compartments where they fulfill their inherent biological roles. Under normal conditions, the processing capacity of the ER is dynamically balanced with the protein synthesis rate. Disruption of the equilibrium between the secretory activity of the cell and the processing and folding capacities of the ER promotes a condition that is known as ER stress. In general, perturbation of ER homeostasis by ER stress

leads to the accumulation of unfolded proteins in the lumen of the organelle, which could also trigger the need for increased levels of chaperones such as HSP90 and BCS1 [46, 47].

Of the four gene sequences identified in the hybridization analyses that map to chromosome 5BL, the expression profile of accession *AJ610871* makes it the best candidate for the monogenic resistance gene *Pm36*. This TUG is the only one that has elevated transcripts in the infected 5BIL-42 line compared to its uninfected state and to the infected state of its susceptible parent Latino. By contrast, transcripts of the other three sequences localized on 5BL chromosome were increased (*AJ611689*, *FM208374*) or decreased (*AJ716441*) 48 h after infection in both Latino and 5BIL-42 compared to uninfected seedlings. No sequence matches for *AJ610871* were found in the Genbank database.

The technology described herein has the international patent No. WO2005003344 [12] and can be successfully applied to any species. The method allows the generation of cDNA that is representative of various metabolic states that are normally present in the different plant tissues and developmental stages from RNA in young tissue, thus bypassing the need to construct multiple libraries. It is important to emphasize that this system simplifies the screening of cDNA libraries for rare mRNAs and can lead to the discovery of new genes.

High-quality cDNA library construction is essential for comprehensive cDNA analysis that is critical for complementing genomic sequence information. Mammalian cDNA analysis, for example, is often indispensable in reliably predicting the protein-coding sequence from the genome sequence. The use of a TUG approach had also been found to be a very efficient and successful way of identifying genes in plants.

This method allows the generation of a set of cloned cDNA fragments containing an approximately equal representation of all of the mRNA sequences from any starting material, regardless of the relative abundance of the mRNA in the material generating the libraries. The use of demethylation maximizes the representativeness of TUG collections originating from a single plant tissue.

The TUG collections made by this method can be employed in cDNA arrays that are useful for differential gene expression studies, such as the one presented here, where this technology has been efficiently employed in the study of the powdery mildew resistance of wheat. Hybridization to the totipotent durum wheat BioChip substantially reduced the experimental time needed and increased the success of identifying genes that are putatively involved in the response to powdery mildew infection, such as GST, HSP90 and a dehydration-induced protein. The identified sequences map to only two regions of the genome, suggesting that some of the genes with specific functions or involved in specific processes are clustered.

While the involvement of these proteins that are components of the main systems of response to biotic and abiotic stress in plants is not unexpected, the more interesting result appears to be the over-expression of three genes with

currently unknown functions in the early stages of infection. One of these maps to the same chromosome region as the *Pm36* gene and has the expression profile expected for a dominant resistance gene. This result is a starting point for new experiments to discover the functions of these proteins and how they fit into the complex defense system of plants, especially in the responses of durum wheat to *B. graminis* attack.

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