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Cloning and mapping of genes involved in wheat-leaf rust interaction through gene-expression analysis using chromosome-deleted near-isogenic wheat lines

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Abstract Molecular markers on wheat chromosome 6BL were isolated using mRNA differential display. Two wheat isolines inoculated with *Puccinia recondita* were analysed: Sinvalocho MA line carrying the *Lr3* gene for leaf rust resistance on distal chromosome 6BL, and a rust-susceptible derivative of the Sinvalocho MA line with a deletion at the distal end of chromosome 6BL. Comparison of mRNA fingerprinting profiles, obtained from control and rust-inoculated plants, let to the isolation of 34 differentially displayed cDNAs. All these genes, except TaRr16, were up-regulated in the rust-inoculated resistant line. TaRr16 has constitutive expression in the rust-resistant line while no expression was detected in the rust-susceptible line. A number of those cDNAs revealed homology to genes previously identified in other plant-pathogen interactions. Two out of the 34 cDNAs, mapped in the distal part of chromosome 6BL and TaRr16, was genetically linked to the *Lr3* gene. DNA sequence differences and differential expression between non-allelic copies of TaRr16, are also reported.

Keywords mRNA fingerprinting · Wheat chromosome deletion lines · Rust resistance · cDNA mapping · Plant-pathogen interaction

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Introduction

Mapping and cloning of wheat rust resistance genes would permit marker-assisted selection and facilitate the study of the molecular mechanisms involved in rust-disease resistance. Among wheat rusts, leaf rust, caused by *Puccinia recondita*, is one of the most important rust diseases of wheat all over the world. Although many wheat leaf rust-resistance genes have been described in genetic terms, the biochemical and molecular basis of this disease remains poorly understood. To-date, a single putative rust-resistance gene of bread-wheat has been cloned and partially characterised at the molecular level (Feuillet et al. 1997). A number of wheat genes involved in the wheat-pathogen response have been identified, however, no differences between resistance and susceptibility were detected. Early induction of gene expression correlates with pathogen resistance, while susceptibility is characterised by delayed induction of the same genes (Schweizer et al. 1989; Dudler et al. 1991; Bull et al. 1992). Due to the large genome of hexaploid wheat (about 16,000 Mbp), it is difficult to apply positional cloning or tagging methodologies in order to isolate genes (Tanksley et al. 1995; Somerville and Somerville 1996). Alternative strategies for cloning genes involved in the expression of a particular trait are those based on the study of gene expression. Several authors have reported the successful use of mRNA Differential Display (RT-PCR-DD) for the isolation of differentially expressed genes in plant-pathogen interactions (Benito et al. 1996; Bertinetti and Ugalde 1996; Oh et al. 1999; Collinge and Boller 2001).

The wheat cultivar "Sinvalocho MA" (R-SV8) has been extensively used for many decades in Argentina as a leaf rust-resistance gene donor. R-SV8 carries a recessive allele of leaf rust-resistance gene *Lr3* mapping in the distal region of chromosome 6BL (6BL-dr). *Lr3* is the genetic determinant for resistance to race 66 of *P. recondita* (Suarez and Favret 1982; Sacco et al. 1995). A spontaneous mutant line susceptible to race 66 of *P. recondita*, named S-SV8, has been previously isolated

from R-SV8. It has been shown, by cytogenetic analysis, that a small deletion (15–20%) on 6BL-dr is associated with the loss of resistance to race 66 of *P. recondita* (Sacco et al. 1998).

The present paper reports an attempt by RT-PCR-DD to isolate the *Lr3* gene for leaf rust resistance, as well as genes differentially expressed between the R-SV8 and the S-SV8 following leaf rust inoculation. This approach led to the isolation of cDNA clones for genes located on 6BL-dr, as well as cDNA clones corresponding to genes differentially regulated in the resistant and susceptible lines.

Materials and methods

Plant material and rust inoculation

Standard Sinvalocho MA (R-SV8) wheat carrying the *Lr3* gene on distal chromosome 6BL and the spontaneous susceptible S-SV8 line isolated from Sinvalocho, lacking 15–20% of the distal region of chromosome 6BL, were used to perform RT-PCR-DD experiments. Seeds of R-SV8 and S-SV8 lines were germinated and grown in the greenhouse under the following conditions: 14 h light/10 h dark at 15–20 °C. Plants were mock- and rust-inoculated at the first leaf stage (approximately 10 days after germination). Inoculations were carried out using a suspension of urediospores (50 mg spores/L) of *P. recondita* race 66 in water, to which Tween-20 (0.01%) was added, and kept overnight in moist chambers. Mock-inoculated plants were sprayed using the same conditions, but with a solution of 0.01% Tween-20. Plants were grown under greenhouse conditions until RNA extraction was carried out, 48 h after inoculation. Some susceptible rust-inoculated plants were kept in the greenhouse as controls for confirmation of successful infection.

Mapping analysis was performed on a segregating population of 109 F_2 plants from a cross between the rust-susceptible Gama-6 line and the R-SV8 line carrying the *Lr3* gene. The Gamma-6 line was used for the genetic linkage analysis of cDNAs to the *Lr3* gene. This line was used because of the high number of RFLPs on the 6BL chromosome (Sacco et al. 1998).

To determine the phenotype for the rust reaction of F_2 plants, F_3 families of 15 seedlings from each F_2 parent were grown and inoculated as described above.

RNA and DNA isolation

Total RNA was extracted from leaves by using Trizol-Reagent (Gibco-BRL). PolyA-RNA was purified from total RNA using the PolyA-Track Purification System (Promega, Madison, WI) following the manufacturer's instructions. Genomic DNA was isolated as described by Sacco et al. (1998) except that fresh material was used instead of lyophilised leaves.

mRNA Differential Display, cloning and sequencing procedures

Total RNA extracted from leaves of R-SV8 and S-SV8, mock- and rust-inoculated, were treated with RNAse-free DNAse (Promega, Madison, WI) according to the manufacturer's instructions. Genomic DNA contamination was tested by PCR amplification with Actin primers against total RNA as a template. cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Gibco-BRL) on 5 μg of total DNA-free RNA, using a T_{12}MC (where "M" means A, G or C) oligonucleotide as the 3′ primer. PCR reactions were carried out as described previously (Liang and Pardee 1992) using T_{12}MC and OPA, OPB or OPZ random decamer primer series (Operon Technology) as the 5′ primers in a Biometra-UNO II thermocycler (Biometra). Amplified products were analysed by electrophoresis in 6% denaturant polyacrylamide gels. Differentially expressed bands from two independent sets of RNA were recovered from the gel and re-amplified by PCR (Liang and Pardee 1992). Re-amplified PCR products were cloned in pGEM-T Easy plasmid (Promega, Madison, WI) and transformed into *Escherichia. coli* DH5α. Plasmids were purified and sequenced by the dideoxy chain-termination method, using an ABI-377 automatic sequencer (Applied Biosystem).

PCR amplification of the TaRr16 clone on genomic DNA

Genomic DNA (100 ng) extracted from the R-SV8 and S-SV8 lines were used as templates for PCR amplification with 5′-GCATTCTTGACGTCTCTGGT-3′ and 5′-CATCTTGCATGCCGACCAAT-3′ upper and lower oligonucleotides, respectively.

Semiquantitative RT-PCR

Total cDNAs were obtained from DNA-free total RNA of R-SV8 or S-SV8 leaves 48 h after mock- and rust-inoculation. Genomic DNA contamination was tested by PCR amplification with actin primers. Reverse transcription was carried out with SuperScript II (Gibco-BRL) on 2 μg of DNA-free RNA with Oligo-dT_{18} oligonucleotide as a 3′ primer. PCR amplifications were stopped at a fixed number of cycles, in the exponential phase of amplification. The exponential phase of amplification was estimated by incorporation of P^{32}. PCR products were submitted to 6% polyacrylamide-gel electrophoresis, dried and quantified densitometrically over scanned autoradiographies (Kodak X-OMAT AR).

Semiquantitative RT-PCR reactions procedures were carried out as described previously (O'Rourke et al. 2000) using 5′-CATCTTGCATGCCGACCAAT-3′ and 5′-GCATTCTTGACGTCTCTGGT-3′ as primers for TaRr16. Total cDNA subjected to PCR was normalised according to the amount of wheat actin mRNA. The specific primers 5′-CTCATACGGTCAGCAATAC-3′ and 5′-ATGTGGATATCAGGAAGGA-3′ were used for actin amplification. Reaction products were submitted to electrophoresis in a 2% agarose gel, stained with ethidium-bromide and photographed under U.V. light.

DNA gel-blot analysis and probe labelling

Genomic DNA (30 μg) was digested using different restriction enzymes, electrophoresed in 1 × TAE 0.8% agarose gels and then transferred to nylon membranes (GIBCO BRL, Nylon-1) using 10 × SSC as a transfer solution. Probes were labeled by PCR incorporation of Digoxigenin-11-dUTP using plasmids as a template and M13 universal primers. The labelling reaction was carried out in a Progene thermocycler (Techne, Cambridge, UK). The DNA blots were pre-hybridised, hybridised, washed and detected following procedures described by Hoisington et al. (1994) using the DIG Labelling and Chemiluminescent Detection Kit from Boehringer Mannheim. For chemiluminescent detection, CDP Star Ready (Promega, Madison, WI) was used and filters were exposed to Kodak X-OMAT AR films for 1–2 h at room temperature.

RNA gel-blot analysis and probe labelling

Sample preparation, electrophoresis and transfer were performed as described by Fourney et al. (1988) except for the use of 5 μg of PolyA RNA. Dried membranes (Hybond-N, Amersham) were prehybridised for 2 h and hybridised overnight at 65 °C in a phosphate buffer solution pH 7.2 (0.5 M PO_4^{-3}, 7% SDS, 1% Bovine sero-albumin, 1 mM EDTA). After hybridisation, membranes were washed twice in 2 × SSC with 0.1% SDS for 10 min at room temperature and twice in 0.1 × SSC with 0.1% SDS for 10 min at 65 °C. Filters were exposed to Kodak X-OMAT AR films at –70 °C with an intensifier screen.

Antisense probes were P^{32}-labeled (α-P^{32}-dCTP) by the antisense primer-extension reaction from 10 ng of plasmid containing TaRr16 or wheat Actin cDNAs. The reaction was performed using *Taq* DNA polymerase, and 5′-CATCTTGCATGCCGACCAAT-3′ and 5′-CTCATACGGTCAGCAATAC-3′ oligonucleotides were used as TaRr16 and Actin antisense primers, respectively.

Results

Differentially expressed genes detected by mRNA Differential Display

Total leaf RNA from isolines R-SV8 and S-SV8, extracted 48 h after inoculation (h.a.i.) from mock and rust-inoculated seedlings, were independently tested with 60 different primer combinations. RT-PCR-DD fingerprinting profiles revealed the presence of about 100 cDNA products for each primer combination. If we assume non-redundancy in the displayed cDNA products, we may have screened a total of 6,000 mRNAs. Most of the observed differences corresponded to genes expressed in the rust-inoculated R-SV8 line (Fig. 1B, C, D and E). One gene showed constitutive expression in R-SV8 and was absent in the S-SV8 line (Fig. 1A). Although cDNA products with different intensity were observed among treatments, only those present or absent in a given treatment compared to the control (mock-inoculation) were further analysed. Sixteen of the 60 assayed primer combinations showed differentially expressed bands. Seventy five percent of the isolated bands contained more than one cDNA sequence. Five primer combinations gave more than one differential band running at different positions in the polyacrylamide gel. It is worth noting that three of these five primer combinations yielded the same unknown mRNAs that migrated at different positions of the gel. The size of these clones depended on the priming site of T_{12}MC on the same mRNA molecule, which strongly suggests a differential representation of these mRNAs.

Thirtyfour wheat cDNA clones were isolated. Most of them (19 out of 34) did not show any homology to protein sequences annotated in public databases. The fact that the mRNA Differential Display technique yields mainly the 3′-untranslated region of genes (3′-UTR), could explain this observation. Table 1 shows a list of the isolated cDNA clones with sequence homology to proteins annotated in public gene databases (cDNAs with homology probability values lower than 1 e^{-5} were not included in the list). In order to test the putative differential expression of the isolated cDNAs, the 34 clones were blotted on hybridisation membranes and massively screened by Reverse Northern analysis using as probes total P^{32}-labeled cDNAs from R-SV8 and S-SV8 challenged, or not challenged, with *P. recondita*. By this method, most analysed clones did not show differential expression and some of them did not show signal hybridisation at all, probably due to the low mRNA expression level (data not shown).

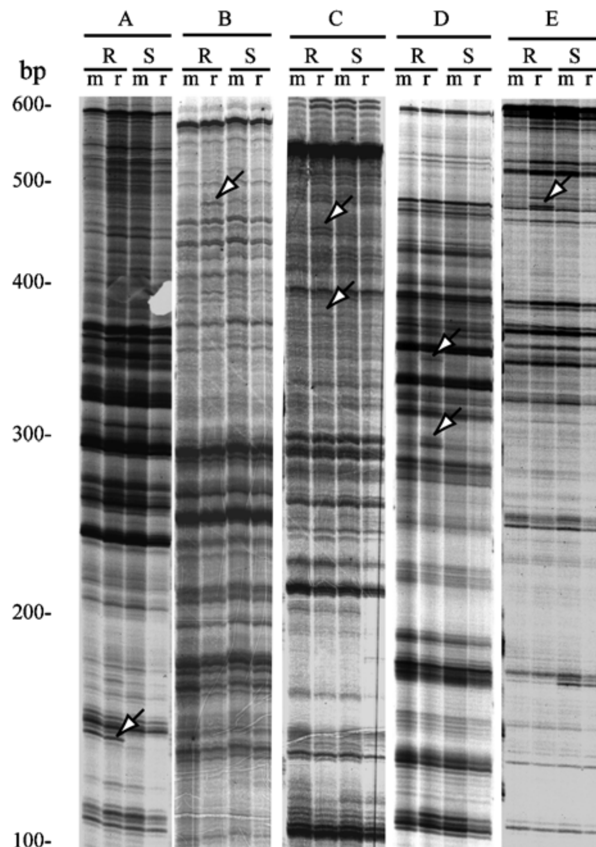

Fig. 1 Illustrative fingerprinting profiles obtained by mRNA Differential Display reactions. Primer T_{12} MC was used as the 3′primer in all reactions, combined with the following 5′primers: (**A**) OPA-10, (**B**) OPA-4, (**C**) OPA-6, (**D**) OPA-18 and (**E**) OPA-19. *White arrow heads* indicates differentially displayed bands. "*R*" and "*S*" means R-SV8 (rust-*R*esistant) and S-SV8 (rust-*S*usceptible) respectively. *m*: *m*ock-inoculated. *r*: *r*ust-inoculated. Molecular-weight markers are indicated at the left

Physical mapping of cDNA clones on the distal chromosome 6BL arm

Given the hexaploid nature of the wheat genome, redundancy in expressed genes may exist. This redundancy could be an obstacle to detect differences in mRNA expression for a specific gene. Even if a gene is differentially expressed between the two lines, cross-hybridisation of probes with related mRNAs could mask the signal obtained with a specific probe. Consequently, we decided to use all the 34 isolated cDNA clones as probes in Southern-blot experiments to test if they were present or absent in the genome of the rust-susceptible line. Southern blots were carried out using wheat genomic DNA, digested with two or three restriction endonucleases. Most of the cDNAs showed three hybridising bands, which suggests that they correspond to single-copy genes. The same RFLP pattern was observed between R-SV8 and S-SV8 with most of probes (data not shown).

Two clones (TaRr01 and TaRr16) were physically mapped on 6BL-dr, corresponding to the deleted distal

Table 1 Characterisation of the cDNAs isolated by RT-PCR-DD from rust-inoculated SV8 leaves

cDNA clone[a]	5′ primer	Insert size (bp) PolyA tail excluded	Homology[b]	Blastx probability (*P*) value
TaRr01	OPA-6	377	Serine/threonine protein kinase protein[d]	2 e-10
TaRr02	OPA-6	491	Hypothetical protein	1 e-15
TaRr03	OPA-6	491	Histone deacetylase	1 e-11
TaRr04[c]	OPA-18	228	Chloroplast translation elongation factor	1 e-13
TaRr05	OPA-20	436	Phosphoshikimate-1-carboxyvinil transferase	5 e-34
TaRr06	OPB-8	187	Reverse transcriptase	1 e-12
TaRr07	OPB-8	165	Phothosysthem II 4kD rection centre protein	1 e-12
TaRr08	OPB-9	386	Prolyl-hydroxylase	1 e-30
TaRr09	OPB-18	229	Cytochrome-like p450 monooxygenase	2 e-6
TaRr10[c]	OPB-18	410	Chloroplastic ATP-synthase beta-chain	7 e-28
TaRr11	OPZ-1	487	Proton-pump ATPase	2 e-19
TaRr12	OPZ-6	279	Hypothetical protein	1 e-7
TaRr13	OPZ-7	394	Ribosomal 60s L1 protein	1 e-11
TaRr14	OPZ-7	368	Chloroplast heat shock protein 70[d]	4 e-13
TaRr15	OPZ-7	371	Cell division cycle protein 48 homologue	2 e-18

[a] cDNA sequences were compared to the non-redundant gene sequence data base by the BLASTX and BLASTP sequence analysis program. GenBank accession number from TaRr01 to TaRr15 are BI502693 to BI502707

[b] Each cDNA clone showed homology to the same protein irrespective of the use of BLASTX or BLASTP analysis programs. All out of one translated cDNAs (clone TaRr09) showed higher homology values with BLASTP than BLASTX protein analysis

[c] cDNA amplified with the decamer primer at both 5′ and 3′ sides

[d] Conserved activity domains identified by BLASTP

part of the S-SV8 line. Three hybridising bands were observed for clone TaRr01 on the R-SV8 genome using the restriction enzymes *Eco*RI, *Bam*HI and *Hin*dIII (Fig. 2A). A band (11-kbp *Eco*RI, 8-kbp *Bam*HI and 2-kbp *Hin*dIII fragments) was absent in the genome of the S-SV8 line. Sequence analysis of this clone, named TaRr01, showed high homology to various serine/threonine-protein kinases (Table 1).

Figure 2B shows that the TaRr16 clone revealed the presence of two hybridising bands on R-SV8 and one band on the S-SV8 line. The absence of the 11-kb *Bam*HI-fragment or the 8-kb *Hin*dIII-fragment in the S-SV8 line, suggests that this cDNA is transcribed from a gene located on the 6BL-dr. This assumption was confirmed by the PCR reaction using genomic DNA as a template and specific primers. The absence of PCR products from S-SV8 genomic DNA at a high annealing temperature (60 °C), provides further evidence supporting this conclusion. By decreasing the annealing temperature 10 °C during the PCR reaction (50 °C), a weak PCR signal was detected using S-SV8 genomic DNA (Fig. 2C) or cDNA (Fig. 3B) as a template. These PCR products were cloned and further analysed (see below).

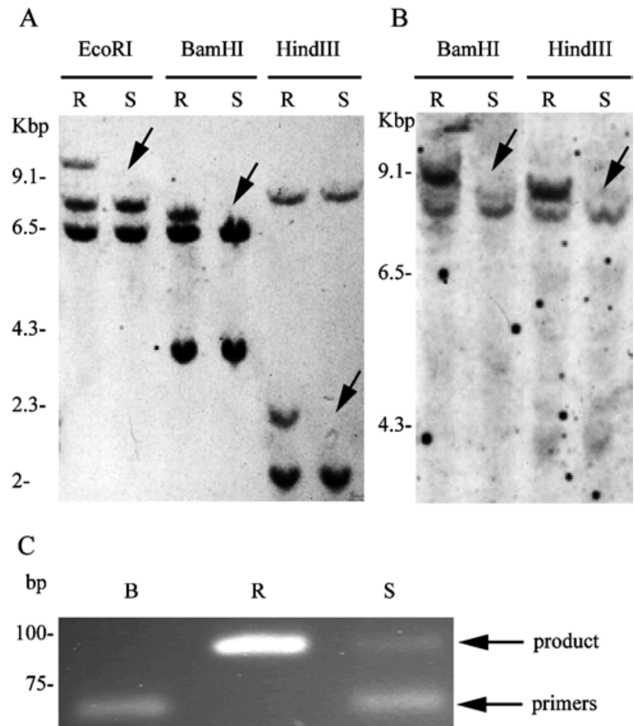

Fig. 2A–C Physical mapping of TaRr01 and TaRr16 cDNA clones. **A** RFLP pattern of the TaRr01 clone. Genomic DNA was digested with *Eco*RI, *Bam*HI or *Hin*dIII restriction endonucleases. **B** RFLP pattern of the TaRr16 clone. Genomic DNA was digested with *Bam*HI or *Hin*dIII restriction endonucleases. *Black arrows* indicate absence of the band in the S-SV8 line. **C** TaRr16 differential PCR amplification from genomic DNA. "*R*" and "*S*" means R-SV8 (rust-*R*esistant) and S-SV8 (rust-*S*usceptible) lines respectively. "*B*" indicates PCR negative control. Molecular-weight markers are indicated at the left

Expression analysis of TaRr01 and TaRr16 genes

As shown in Fig. 1C, a band that corresponds to the TaRr01 cDNA clone was RT-PCR-DD-amplified only in the R-SV8 line after pathogen infection. On the other hand, RT-PCR-DD revealed that the TaRr16 clone was constitutively expressed in R-SV8 and absent in the

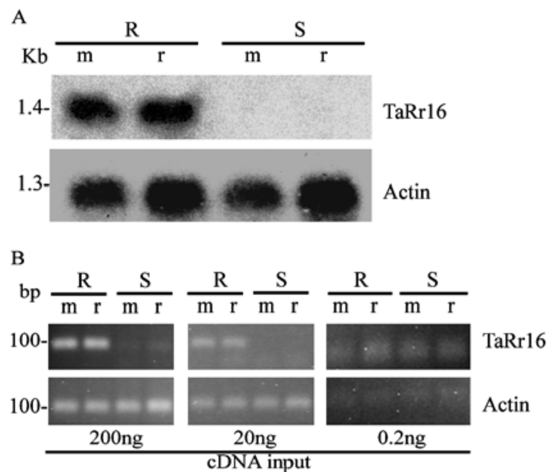

Fig. 3A, B Analysis of the expression of the TaRr16 clone in R-SV8 leaves. **A** Northern blot on leaf Poly-A RNA. **B** Semiquantitative RT-PCR on RNA obtained 48 h.a.i. PCR products were submitted to electrophoresis in 2% agarose gels. Total cDNA input in the PCR is indicated at the bottom of each panel. TaRr16 and Actin are shown in the upper and lower panels respectively. "*R*" and "*S*" means R-SV8 (rust-*R*esistant) and S-SV8 (rust-*S*usceptible) lines respectively. *m*: *m*ock-inoculated. *r*: *r*ust-inoculated. Molecular-weight markers are indicated at the left

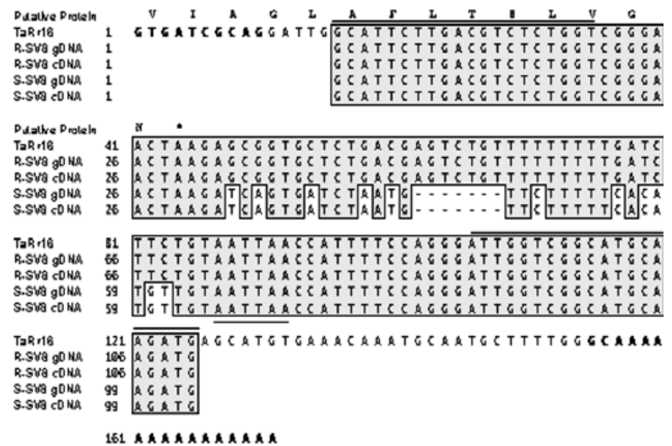

Fig. 4 Sequence comparison of TaRr16 genomic and cDNA clones of R-SV8 and S-SV8 lines. Primers used in the RT-PCR-DD reaction are shown in *bold type*. Specific primers designed for TaRr16 used in the PCR reaction over genomic DNA or cDNA are *overlined*. Homology between clone TaRr16 and TaRr16-homologous, is boxed. The Putative protein encoded by TaRr16 is shown at the top. An asterisk indicates the putative stop codon. Putative polyadenylation signal is italic

S-SV8 line, indicating that inoculation was not required for expression (Fig. 1A).

The low mRNA expression level of the TaRr01 gene made the analysis by Northern-blot hybridisation difficult. However, semiquantitative RT-PCR experiments revealed the presence of the corresponding mRNA, though no differential expression was detected among both lines and treatments (data not shown).

Northern-blot analysis revealed that expression of TaRr16 mRNA was detected only in R-SV8 plants. The single 1.4-kb signal observed in R-SV8 was not detected in leaves of S-SV8 plants. No difference in signal intensity was observed between R-SV8 mock- and rust-inoculated plants (Fig. 3A). The absence of the TaRr16 signal in the S-SV8 line suggested that the homologue gene copy (TaRr16-h), detected by Southern-blot hybridisation (Fig. 2B) in the S-SV8 line, had such a low level of mRNA expression that it was not possible to detect it under this experimental condition. Northern-blot results were in agreement with those obtained at 48 h.a.i. by semiquantitative RT-PCR (Fig. 3B). At low annealing temperature (50 °C), a low level of expression was detected for clone TaRr16 in the S-SV8 line (Fig. 3B). Semiquantitative RT-PCR showed that TaRr16 mRNA was 100-fold more represented in R-SV8 than its homologue (TaRr16-h) mRNA in S-SV8 plants.

Sequence analysis of TaRr01 and TaRr16 clones

In order to detect nucleotide differences among clones, PCR products from R-SV8 and S-SV8 isolines were cloned and sequenced. PCR reactions were carried out with specific primers using genomic DNA or cDNA as templates. Ten cDNA clones from each line and treatment were sequenced and compared with genomic DNA clones obtained from both isolines. No nucleotide differences among R-SV8- and S-SV8-genomic DNA and cDNA clones were detected for TaRr01 (data not shown).

The low annealing temperature (50 °C) required for PCR amplification of the TaRr16 gene from the S-SV8-genomic DNA or cDNA, suggested that PCR-products obtained from the two isolines might be different at the nucleotide level. Only one type of sequence was recovered from each line regardless of the template origin (genomic or cDNA). No nucleotide differences were detected between the products obtained from the cDNA or genomic DNA of each isoline, which indicates that introns were not present in the putative 3′-UTR of the TaRr16 gene. However, sequence differences were detected between clones obtained from the R-SV8 and S-SV8 lines: a 110-bp amplified product was obtained from R-SV8 leaf cDNA or genomic DNA, whereas a 103-bp product was obtained from S-SV8 cDNA or genomic DNA. Sequence analysis of the TaRr16 clone using Blastn and Blastx data search programs revealed no homology to previously reported genes in public databases. However, a putative ORF in the first 42 nucleotides was detected and a putative polyadenylation signal is present between position 87–92, suggesting that this region might be the 3′-UTR of the TaRr16 gene. As is shown in Fig. 4, the 3′UTR sequence of the gene expressed in the S-SV8 line compared to R-SV8 revealed the presence of a micro-deletion from position 64 to 70 and nucleotide changes at positions 48, 50, 56, 60, 62, 73, 79, 81, 82, 84 and 85.

All nucleotide differences between TaRr16 and its homolog (TaRr16-h) are located between the putative stop codon and the polyadenylation signal. TaRr16 and TaRr16-h are 86% identical at the nucleotide level. This sequence micro-heterogeneity suggests that the gene expressed in the S-SV8 line is different from that expressed in the R-SV8 line, at least in the 3′-UTR.

Genetic linkage analysis of the TaRr01, TaRr16 and *Lr3* genes

In order to asses the genetic linkage among the TaRr01, TaRr16 and *Lr3* genes, RFLP studies were carried out between R-SV8 carrying the *Lr3* gene and the rust-susceptible Gama-6 line. No polymorphisms for clone TaRr01 were detected using 18 restriction enzymes (*Hin*dIII, *Eco*RI, *Bam*HI, *Bgl*II, *Eco*RV, *Sal*I, *Sma*I, *Pst*I, *Pvu*II, *Sac*I, *Xho*I, *Xba*I, *Hinc*II, *Apa*I, *Nco*I, *Kpn*I, *Nar*I and *Sph*I).

By contrast, *Hin*dIII and *Bam*HI polymorphic fragments were detected using the TaRr16 clone as a probe. One of these polymorphisms (*Hin*dIII) was employed to determine the genetic linkage analysis between the TaRr16 and *Lr3* loci. The RFLP linkage study was performed by using a population of 109 F_2 plants from the cross Gama-6 × R-SV8. F_2 plants were progeny tested using the race 66 of *P.recondita* to establish the genotypes for the leaf rust reaction. A segregation of 29 susceptible homozygous, 56 susceptible heterozygous and 24 resistant homozygous types was found, reflecting a single 1:2:1 recessive gene segregation for the leaf rust reaction ($p < 0.9$). No recombination was observed between TaRr16 and the *Lr3* gene.

Discussion

Cloning of genes involved in the wheat-rust interaction would facilitate the study of molecular mechanisms underlying resistance to rust diseases. Here we report that, for near-isogenic wheat lines, all the differences in mRNA expression observed at 48 h.a.i. were either upregulated or were constitutively expressed genes of the rust-resistant line (Fig. 1). The differences detected by mRNA fingerprinting profiles show that rust-resistant and rust-susceptible isolines respond in a different way to the pathogen attack.

Among the 34 isolated cDNAs, only the differential expression of TaRr16 was confirmed by the hybridising or RT-PCR methods. Reverse Northern hybridisation revealed that most of the isolated genes have no differential expression among both lines and treatments, while others have not shown signal hybridisation at all. It is well-documented that RT-PCR-DD produces a high percentage of false positive clones. This may be the case for most of the 34 isolated cDNAs. However, since a hybridisation signal was not detected for a number of the isolated clones, we can not rule out the possibility that some of them could be differentially expressed between both isolines.

Interestingly, none of the pathogenesis-related genes (like PR1, PR2, PR3, PR4, PR5, WIR and the WheatWin genes) previously reported to be up-or down-regulated in other wheat-pathogen interactions were isolated in this study (Schweizer et al. 1989; Munch-Garthoff et al. 1997; Molina et al. 1999). This would not be surprising since most of the PR genes are expressed in mock- as well as pathogen-inoculated wheat plants (Pritsch et al. 2000). Furthermore, most of the up- or down-regulated genes during the wheat-pathogen interaction display a similar response regardless of whether the pathogen is being virulent or avirulent (Rebmann et al. 1991a, b). In the present work, quantitative differences were not considered since from the RT-PCR-DD gels we have isolated only those cDNA products present or absent in a given treatment compared to the control condition. None of the cDNA clones isolated in the present work have been previously reported in other wheat-pathogen interactions (Table 1). However, putative proteins encoded by a number of these cDNAs show high homology to proteins involved in other plant-pathogen interactions, such as the *Arabidopsi thaliana* Cytochrome p450-Monooxygenase that was implicated in the resistance to a fungus pathogen (Zhou et al. 1999); a H^+ Pump ATPase probably involved in the barley powdery mildew interaction (Zhou et al. 2000) and tomato pathogen-defence reactions (Schaller and Oecking 1999); a bean mRNA coding for Prolyl Hydroxylase that is induced by a phytopathogenic fungus elicitor (Bolwell et al. 1985); and the chloroplastic Heat-Shock Protein 70 that participates in the protection of H_2O_2-damaged photosystem II under stress conditions (Schroda et al. 1999) which could be part of the programmed cell death in response to pathogen attack (Mullineaux et al. 2000).

Interestingly, the TaRr01 clone displays a high homology to serine/threonine-protein kinases. A number of reports have demonstrated the participation of signal transducer proteins in plant-pathogen interaction and several pathogen-resistant genes encode serine/threonine-protein kinases (Hammond-Kosack et al. 1994; Zhou et al. 1997; Wang et al. 1998). Although differential expression and the genetic linkage analysis for the TaRr01 gene was not discerned, it is an interesting gene for future studies considering its sequence homology and its physical mapping near to the *Lr3* gene.

Two hybridising bands for the TaRr16 clone were observed by Southern blots. Sequence analysis of the genomic and cDNA clones indicates that two different copies of the TaRr16 gene are present in the R-SV8 line, but only one in the S-SV8 line. A TaRr16-homologue was amplified by PCR using TaRr16-specific primers only at a low annealing temperature from S-SV8 genomic DNA, which indicates that the two non-allelic copies are heterogeneous at the nucleotide level. It is worth noting that the TaRr16-homologue was PCR-amplified and cloned only from the S-SV8 line. We hypothesise that, from the R-SV8 background, the TaRr16-homologue was not

PCR amplified because of primer competition with the TaRr16 gene. We cannot rule out the possibility that two or more identical copies of the TaRr16 gene (or the TaRr16-homolog) may be present in the R-SV8 genome and we could not distinguish between them. In any case, the active copy(ies) of the TaRr16 gene(s) is(are) located near the *Lr3* locus, suggesting that each chromosome of group-6 of hexaploid wheat may express different genes (chromosome differentiation).

Most of the resistance genes, which may be sensing the presence of an avirulent factor of pathogens, are constitutively expressed. Interesting, the TaRr16 gene is expressed in a similar way. It was reported previously for the flax rust-resistance L6 gene that no induction of L6 expression was observed following infection of resistant plants with an avirulent pathogen (Ayliffe et al. 1999).

No recombination was detected between the TaRr16 and *Lr3* genes in 109 gametes, indicating that they are tightly linked in a telomeric region. This means that, if both loci are not the same, they are not far apart.

In the present paper we have observed a frequency of 1 over the 16 analysed clones mapped on 6BL-dr, which is similar to the efficiency obtained by using AFLP-based mRNA fingerprinting in the study of wheat flower-morphogenesis (Kojima et al. 2000).

R-SV8 and S-SV8 near-isogenic lines were previously analysed by RAPDs using more than 400 different primers, and no polymorphism was detected (Sacco F, unpublished results). This suggests that mRNA Differential Display may be a powerful technique to generate molecular markers on highly related genomes differing for specific traits.

Some genes identified in this study encode putative proteins, highly homologous to proteins involved in plant-pathogen interactions. All the genes up-regulated by rust inoculation were detected in the rust-resistant line. Furthermore, in some cases, the same cDNA clone was recovered from different positions of the RT-PCR-DD gels. These results suggest that some of the genes isolated here could play a critical role during the response of wheat plants to *P. recondita* attack. On the other hand, the use of deletion lines combined with mRNA differential display have demonstrated to be useful to generate molecular markers for an unknown chromosomal region, yielding a valuable tool for marker-assisted selection for wheat leaf rust resistance.

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