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ORIGINAL PAPER

Transcriptional responses of Italian ryegrass during interaction with *Xanthomonas translucens* pv. *graminis* reveal novel candidate genes for bacterial wilt resistance

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Abstract *Xanthomonas translucens* pv. *graminis* (*Xtg*) causes bacterial wilt, a severe disease of forage grasses such as Italian ryegrass (*Lolium multiflorum* Lam.). In order to gain a more detailed understanding of the genetic control of resistance mechanisms and to provide prerequisites for marker assisted selection, the partial transcriptomes of two Italian ryegrass genotypes, one resistant and one susceptible to bacterial wilt were compared at four time points after *Xtg* infection. A cDNA microarray developed from a perennial ryegrass (*Lolium perenne*) expressed sequence tag set consisting of 9,990 unique genes was used for transcriptome analysis in Italian ryegrass. An average of 4,487 (45%) of the perennial ryegrass sequences spotted on the cDNA microarray were detected by cross-hybridisation to Italian ryegrass. Transcriptome analyses of the resistant versus the susceptible genotype revealed substantial gene expression differences (>1,200) indicating that great gene expression differences between different Italian ryegrass genotypes exist which potentially contribute to the observed phenotypic divergence in *Xtg* resistance between the two genotypes. In the resistant genotype, several genes differentially expressed after *Xtg* inoculation were identified which revealed similarities to transcriptional changes triggered by pathogen-associated molecular patterns in other plant–pathogen interactions. These genes represent candidate genes of particular interest for the development of tools for marker assisted resistance breeding.

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Introduction

Bacterial wilt is a major disease of various forage grasses including Italian ryegrass (*Lolium multiflorum* Lam.), causing substantial losses in forage crop production and infestation of important breeding material. The disease is caused by the bacterial pathogen *Xanthomonas translucens* pv. *graminis* (*Xtg*; Egli et al. 1975), Vauterin et al. (1995), which occludes the xylem vessels, and causes wilting symptoms and necrosis of the leaves. Susceptible plants may die within only a few days after infection. Breeding for resistant cultivars is a major objective and has lead to development of cultivars with increased resistance to bacterial wilt. Nevertheless, despite intensive breeding efforts, no complete resistance has been achieved to date, and highly susceptible individuals still occur in advanced breeding populations (Michel 2001). This may be due to the out-breeding reproductive mode of this species and the population-based breeding schemes often used to improve ryegrasses. In such complex interactions, a detailed understanding of the genetic control of resistance mechanisms is crucial for further improvements in *Xtg* resistance breeding.

Transcriptome and quantitative trait loci (QTL) analyses represent powerful tools for elucidating host defence responses and for the identification of genetic markers

linked to disease resistance. QTL mapping in a *L. multiflorum* mapping population identified one major QTL on linkage group (LG) 4 associated with bacterial wilt resistance explaining up to 84% of the total phenotypic variance (Studer et al. 2006). This major QTL indicated the presence of major qualitative resistance. However, phenotypic and molecular genetic evaluation of race-specific interactions between different *Xtg* isolates and *L. multiflorum* genotypes revealed no indication of major qualitative *Xtg* resistance (Wichmann et al. 2010). Thus, although major QTL may be found in particular germplasm, additional unknown genes or QTL control quantitative *Xtg* resistance in *L. multiflorum*. The analysis of transcripts differentially expressed as a response to infection may allow further elucidation of the genetic control of host resistance. Preliminary transcriptome analyses of a resistant *L. multiflorum* genotype inoculated with *Xtg* using cDNA-amplified fragment length polymorphism (AFLP) identified a number of differentially expressed transcript-derived fragments (TDF) at different time points after inoculation (Rechsteiner et al. 2006) and therefore demonstrated the suitability of the approach. However, the small number and the short lengths of the TDF analysed limited the utility and generality of the results for further, candidate gene-based investigations of the genetic control of *Xtg* resistance. In addition, the cDNA–AFLP analysis focused only on one single *L. multiflorum* genotype not taking into account the high genetic diversity of individual genotypes present in populations and cultivars due to the out-breeding reproduction system of the species. Therefore, a more comprehensive transcriptome analysis using more than one genotype and a larger number of transcripts is needed to gain a more detailed insight into genes and pathways involved in defence responses against *Xtg*.

Microarray-based transcriptome analyses allow for the simultaneous detection of modulated expression of several thousand transcripts and are therefore suitable for large scale analyses. In crops such as rice, cassava, citrus or tomato, microarray analyses of interactions with *Xanthomonas* spp. have revealed differential expression of specific genes encoding cell-wall modifying proteins, protein kinases involved in signalling pathways and genes triggered by pathogen-associated molecular patterns (PAMPs; Cernadas et al. 2008; Gibly et al. 2004; Li et al. 2006). For example, a number of genes are co-regulated during the interactions of rice with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and with *Magnaporthe grisea* (the fungal pathogen causing rice blast) indicating shared defence pathways (Li et al. 2006). In addition, a number of candidate genes involved in signal transduction co-locating with a major QTL for broad-spectrum *Xoo* resistance and a QTL for submergence tolerance on chromosome 5 have been identified in rice, indicating that submergence tolerance and broad-spectrum *Xoo* resistance share a common signalling system (Kottapalli et al. 2007). In citrus, many genes associated with PAMP recognition were commonly modulated after *Xanthomonas axonopodis* pv. *aurantifolii* and *X. axonopodis* pv. *citrii* infection (Cernadas et al. 2008). Although *L. multiflorum* as well as *Xtg* share phylogenetic similarities with some of the host and pathogen species described above, the *L. multiflorum* × *Xtg* interaction is unique in many respects. The pathogen has a relatively broad host range (Egli and Schmidt 1982), host resistance is characterised by partial rather than complete resistance and race-specific interactions have not, so far, been observed (Wichmann et al. 2010). In addition, *L. multiflorum* populations and cultivars are characterised by a high genetic diversity, while *Xtg* isolates have been shown to share high genetic similarity (Kölliker et al. 2006). A detailed transcriptome analysis of this host × pathogen interaction is therefore crucial for a more comprehensive understanding of resistance mechanisms and for developing tools for marker assisted resistance breeding. However, to date, there is no representative microarray available for *L. multiflorum* and sequence information available is limited. In such cases, cross-species hybridisation (CSH) using microarrays with sequences from related species represents a valuable tool for transcriptome analyses (Bar-Or et al. 2007). Due to the close phylogenetic relationship of *L. multiflorum* and *Lolium perenne* (Catalan et al. 2004), the 9,365 unique expressed sequence tag (EST) sequences developed at Det Jordbrugvidenskabelige Fakultet (DJF), Aarhus University (Asp et al. 2007) represent a promising resource for transcriptome analysis in *L. multiflorum*.

The first aim of this study was to develop a cDNA microarray using a unique EST sequence set of *L. perenne* and to evaluate this cDNA microarray for transcriptome analyses in *L. multiflorum*. The second aim was to compare the transcriptomes of an inoculated and control-treated *L. multiflorum* genotype with a high level of resistance to bacterial wilt in order to identify candidate resistance genes for marker assisted selection. The third aim was to compare the transcriptomes of a resistant and a susceptible *L. multiflorum* genotype in order to elucidate genotypic differences contributing to varying levels of resistance to bacterial wilt.

Materials and methods

Construction of a *Lolium perenne* cDNA microarray

The set of unique genes for construction of the cDNA microarray was identified by clustering and assembly of 25,744 high-quality *L. perenne* EST sequences. The 25,744 ESTs were assembled into 3,195 tentative consensus

sequences and 6,170 singletons, thus representing 9,365 unique sequences (Asp et al. 2007). In addition, a total of 625 expressed sequences of *Lolium* either retrieved from public databases (http://ncbi.nlm.nih.gov), or representing candidate genes for vernalization (Andersen et al. 2006), for *Xtg* resistance identified by cDNA–AFLP (Rechsteiner et al. 2006), laccases (Schejbel et al. 2008), and resistance gene analogues (RGA; Ikeda 2005) were included, resulting in a total of 9,990 unique genes. Plasmid DNA of the unique genes was prepared from all clones by MWG Biotech AG (Ebersberg, Germany). The cDNA inserts were amplified by PCR using standard M13 forward and reverse primers, *Taq* DNA polymerase (0.8 U; Fermentas, Vilnius, Lithuania) in reaction volumes of 100 µl containing reaction buffer [10 mM Tris, pH 8.8; 50 mM KCl; 0.08% Nonidet P40; 0.5 mM $MgCl_2$; 0.3 mM (each) dNTP]. The PCR reactions were performed in a MJ Research PTC-225 Thermal Cycler (MJ Research, Waltham, MA, USA) and the cycling conditions were 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 61°C, 3 min at 72°C, and a final extension step of 10 min at 72°C. PCR products were precipitated at −20°C for 12 h with 3 (v/v) 96% ethanol and 1/10 (v/v) 3 M sodium acetate, pH 5.2, and subsequently centrifuged at 4,000 rpm for 1 h in an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany). The DNA pellet was air-dried and resuspended in 20 µl of water. The quality of the PCR products was visually inspected by agarose gel electrophoresis. A total of 10 µl (200–500 ng) of each sample was vacuum-dried and resuspended in 5 µl of 50% dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The samples were spotted onto Nexterion Slide A (Schott Nexterion, Jena, Germany) using a QArrayMini spotter (Genetix, New Milton, UK). Following spotting, the microarrays were UV crosslinked at 250 mJ in a UV Stratalinker 1800 (Stratagene, Santa Clara, CA, USA) and kept dry in the dark at room temperature until hybridisation.

Bacterial isolates and plant material

The *L. multiflorum* genotypes *Lm*B-01 (partially resistant to *Xtg*) and *Lm*K-01 (highly susceptible to *Xtg*) previously characterised for *Xtg* resistance (Wichmann et al. 2010) were used for transcriptome analyses. Genotype *Lm*B-01 originated from a Syn1 progeny of a polycross with nine elite genotypes from Agroscope Reckenholz-Tänikon, and genotype *Lm*K-01 is an individual selected from the commercially available cultivar Adret (Verneuil Recherche, Verneuil-Etang, France). Both *L. multiflorum* genotypes were clonally propagated and *Xtg* inoculation and control treatment were performed using the leaf clipping method as described in Kölliker et al. (2006). Plants were arranged in a split-plot design with the sampling time point as block factor and 4 biological replicates per treatment and genotype, resulting in 16 clones per genotype. The *X. translucens* pv. *graminis* isolate *Xtg*29 (Kölliker et al. 2006), which was stored at −70°C in GYC [glucose 2% (w/v), yeast extract 1% (w/v), $CaCO_3$ 2% (w/v)] broth containing 15% (v/v) glycerol, was used for inoculation. For the control treatment, plants were cut with sterile scissors without inoculum. Plant leaves and sheaths were cut at 2 cm above soil and the total harvest was used for total RNA extraction at four time points post-inoculation or control treatment: 8, 48, 192 and 288 h post-inoculation (hpi) or hours post-control treatment (hpc). Early disease symptoms such as wilting of the tips of the leaves were only observed on the plants of the susceptible genotype sampled at the last time point after infection at 288 hpi (data not shown). Dye swaps were included into the experimental design with two biological replicates assigned to each labelling dye (Fig. 1). Direct comparisons between the control-treated susceptible and the inoculated susceptible plants were not included into the experimental design since the main interest was laid on the comparison of non-inoculated versus inoculated plants of the resistant genotype and the comparison of the two genotypes under the two different treatments. However, effects of the indirect comparisons may still be estimated. All tissue samples were immediately frozen in liquid nitrogen and stored at −70°C prior to total RNA extraction.

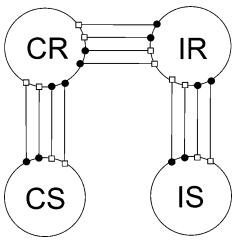

Fig. 1 Experimental design of microarray analyses performed for each sampling time point. *Circles* represent labelled mRNA samples with the following genotypes and treatments: C, control treatment; I, inoculated with *Xanthomonas translucens* pv. *graminis*; R, resistant *Lolium multiflorum* genotype; S, susceptible *L. multiflorum* genotype. *Lines* indicate mRNA samples hybridised to the same microarray. *Black circles* indicate samples labelled with Cy3 and *white squares* indicate samples labelled with Cy5

RNA extraction and reverse transcription

The plant leaves were ground in liquid nitrogen and 300 mg of plant material per plant sample was used for total RNA isolation using the FastRNA Pro Green Kit® (MP Biomedicals, Irvine, CA, USA) and FastPrep® Instrument (MP Biomedicals). The FastPrep® setting 6.0 was used for 40 s. Thereafter, RNA extraction and reverse

transcription were performed as described by Gregersen et al. (2005). The amino-allyl cDNA concentration was measured using the Synergy2 plate reader (BioTek, Winooski, VT, USA) measuring the absorbance at 260 nm. After cDNA quantification, the samples were dried in a Vacufuge (Eppendorf) and resuspended in 10 µl of 0.1 M sodium bicarbonate pH 9.0. Labelling of second strand cDNA was performed with the CyDye (Cy3/Cy5) Post-Labelling Reactive Dye Pack (Amersham Pharmacia, UK) in the dark for 2 h at 35°C. After labelling, the samples were purified using the Qiaquick PCR purification kit and PB buffer (Qiagen, Hilden, Germany). Quantification of labelled cDNA and incorporated CyDye was carried out with a spectrophotometer measuring the absorbance at 260 and 550 nm for Cy3, and 260 and 650 nm for Cy5, respectively.

Hybridisation, washing and scanning of spotted microarrays

The target cDNA was prepared from approximately 25 pmol of each the Cy3- and Cy5-labelled cDNA sample, dried with the Vacufuge (Eppendorf) and resuspended in 5 µl of sterile water and 45 µl of Nexterion® Hyb buffer (Schott Nexterion). Before hybridisation, the spotted microarrays were blocked in 5 g of succinic anhydride, 280 ml of 1-methyl-2-pyrrolidinone and 12.5 ml of sodium borate (1 M, pH 8) for 15 min. After blocking, the microarrays were immersed into 0.1% (w/v) SDS and water for 20 s followed by a denaturation of 3 min in boiling water and spin-drying in at 1,500 rpm for 8 min.

Before hybridisation, the target cDNA was denatured at 95°C for 3 min and cooled for 30 s on ice. The target cDNA was pipetted on the middle of the spotted area of the microarray and covered carefully with a LifterSlip (Eerie Scientific Company, Portsmouth, NH, USA). The microarray was placed in a hybridisation chamber containing 1× SSC and incubated at 65°C for 16 h. The LifterSlip was removed by gentle agitation in 1× SSC and 0.2% SDS. Then the microarrays were washed once in pre-warmed 1× SSC and 0.2% SDS for 10 min, twice in pre-warmed 0.2× SSC and 0.1% SDS and twice at room temperature in 0.1× SSC for 1 min. After washing, the microarrays were again spin-dried and scanned. Scanning was performed with a GenePix® Personal 4100A (Axon Instruments, Union, CA, USA) microarray scanner and the PMT gains (exposure settings) were optimized individually for each microarray.

Microarray data analyses and statistics

Quantification of hybridisation signals was performed using the GenePix® Pro 6.0 software (Axon Instruments) aligning the spot grids for each spot automatically with manual adjustments. The R software 2.8 and the LIMMA package (Smyth 2005) were used to normalise the microarray data. Normalisations within arrays were performed in a signal-dependent manner using the LOWESS (locally weighted linear regression) method to remove intensity-dependent variation in dye bias by applying a smoothing adjustment that removes such variation (Yang and Speed 2002). This was followed by between array normalisations of the four replicates per comparison using the quantile method as proposed by Bolstad et al. (2003). Diagnostic plots were created using "maQualityPlots" function of the arrayQuality package (Paquet and Yang 2008). Data producing unsatisfactory diagnostic plots were discarded. Generation of lists of differentially expressed genes was performed by means of the moderated *t* statistics (Lonnstedt and Speed 2002) using a *P* value threshold of $P < 0.01$. In addition, a $\log_2$ fold change (FC) threshold of 0.8 was used for the comparison between genotypes, in order to reduce the number of differentially expressed genes. Since subtraction of background signals increase spot variation (Qin and Kerr 2004), foreground signal alone was used for normalisation and analysis of differential gene expression. DNA sequences and predicted protein sequences were analysed and annotated using GenBank and the blastx database (http://ncbi.nlm.nih.gov/BLAST/) considering annotations with *E* values <1E−06. Assignment of the genes to functional categories was performed following the description of the genes in public databases according to functional categories in the GO database (Ashburner et al. 2000).

Quantitative real-time PCR

Differential expression of genes observed with the microarray assay was verified by quantitative real-time PCR (qPCR) using an iCycler (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR® Green Supermix (Bio-Rad). The plant material from the four replicates of each treatment and time point sampled for the microarray experiment was pooled for total RNA extraction as described above. Before cDNA synthesis, total RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen) including DNase treatment (Qiagen). cDNA synthesis was performed in a 60 µl volume from 3 µg of total RNA using an oligodT_{25}-primer (Microsynth, Balgach, Switzerland) and Superscript II (Invitrogen, Carlsbad, CA, USA) according to the manufacturers recommendations. The primer pairs for a number of selected genes potentially involved in *Xtg* resistance or with a high FC value were designed with the Primer3 tool (Rozen and Skaletsky 2000) to amplify fragments of approximately 150–250 bp and to be of similar GC content and melting temperature (Table 1). The specificity of primer pairs was verified by melting curve analysis. The

Table 1 Sequence IDs, forward and reverse primer sequences and annotations of the genes confirmed by quantitative real-time PCR

Sequence ID[a]	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Comparison	Annotation[b]
r_010d_c02	AGCAAACCTCGACAAGCCTA	AGCACCGTGAGGATCTCTGT	CR ↔ IR	Germin-like protein (GLP6)
r_010d_c04	TTCAGGTCCCGTTCTACTGG	CACCGCTCTGTTGTCTGTTG	CR ↔ IR	Low silicon protein (Lsi1)
rg6_008b_d08	TCATCGCCCTCATCCTTATC	GGGCCAGAGCACACTAAGAG	CR ↔ IR	Synaptobrevin-like protein
gsa_007d_f02	GGTTTTCTTCCCATTTGGACC	CGAGGTAAAGCTCAACAGACG	CR ↔ IR	TMBIM4
ve_007b_e10	CATGGTTGGTTGGTTCTGTG	TGCCTCAAGAACAGCAACAG	CR ↔ IR	Structural protein MFS18
gsa_007c_h09	GGGACAAGGAGAAGATGCTG	CAAACAGGCCACGGTTATTT	CR ↔ IR	Ankyrin
sb_001a_f10	GTGTATGTATGGATGTGTGTGTG	TGGTCTCATTCATCGCAAGA	CR ↔ IR	Unknown
rg3_011d_b12	GACATACGTGGTGCAGGATG	GGTAGCTACAGCCTCCTCGT	CR ↔ IR	Unknown
rg3_011a_h07	CGATACCTTTCCCGACATTG	GCCAAGGGATAAATCGAGGA	CR ↔ IR	Unknown
r_003d_g12	TGCCAGAGCTTCGTGAATAA	CGTAGCTTCCCAAGACATGC	CR ↔ CS	Unknown
rg1_014c_b10	CATGTGCCAGCTCTGACCTA	CCAAGGTATTCGATGCCACT	CR ↔ CS	Leucine rich repeat
Lac_11	CACCAAGAGCATCGTGACAG	CCGGTGATGGTGAAGTTGTA	CR ↔ CS	Laccase 11
rg1_011a_g05	CGCTCTGGACCCTAACAGTC	GCATTCATCAAAGTCGAGCA	CR ↔ CS	Wheat-induced resistance (WIR 1)
rg1_015d_f09	ATCCCTCAAGGCTTCCAGAT	AGTTTCCTCACGGCAATCAC	CR ↔ CS	Serine/threonine protein kinase
sb_003c_e08	GGCAGTACGGGAGGATTACA	TCTGTACTGTTCGGCTGTGG	CR ↔ CS	Peroxin 14
sb_007b_a08	TGCGTGGATTACTACGACCA	GGGAAGGTATTCAGCAGCAG	CR ↔ CS	Peroxidase 53
eIF-4a	GGTCGTGTGTTTGACATGCT	CCTTGAAACCACGAGAAAGC	All	Eukaryotic Initiation factor 4a
eEF-1α	GGCTGATTGTGCTGTGCTTA	CTCACTCCAAGGGTGAAAGC	All	Eukaryotic elongation factor 1α

The comparison in which the genes were detected to be differentially expressed is indicated: C, control treatment; I, inoculated with *Xanthomonas translucens* pv. *graminis*; R, resistant *Lolium multiflorum* genotype; S, susceptible *L. multiflorum* genotype

[a] Unique identifier. Contains information about the cDNA library (Asp et al. 2007) that the sequence originated from

[b] Derived from GenBank and the blastx database (http://ncbi.nlm.nih.gov/BLAST/)

eukaryotic initiation factor 4a (eIF-4a) and the eukaryotic elongation factor 1α (eEF-1α) were used as internal reference genes, since they were shown to be most suitable for mRNA quantification due to stable expression in different tissues and under different conditions in *L. perenne* (Martin et al. 2008). PCR amplifications were performed using primers described by Martin et al. (2008) in 25 µl volumes using 2 µl of 1:10-fold diluted cDNA. Thermal-cycling conditions were as follows: initial denaturation step at 95°C for 3 min, 45 cycles of 30 s at 95°C, 30 s at 61.4°C, 30 s at 72°C, final extension at 72°C for 5 min. Expression ratios of three technical replicates were averaged for each sample. Quantification of the relative changes in gene expression was performed using the Pfaffl method and the REST software (Pfaffl et al. 2002).

Results

Overview of global gene expression

An average of 4,487 ± 196 ESTs (45%) of the 9,990 *L. perenne* EST sequences spotted on the cDNA microarray were detected in both channels with a signal/background ratio >1.5 by target cDNA from *L. multiflorum* across all hybridisations included in the analysis. In addition, the average background intensity value across all hybridisations for both channels was at 294.4 ± 19.8.

Transcriptional changes following *Xtg* inoculation in the resistant genotype

Comparisons of control-treated and *Xtg* inoculated plants were performed with the partially resistant *L. multiflorum* genotype *Lm*B-01. The transcriptome analysis of the resistant genotype revealed in total 158 genes differentially expressed after *Xtg* inoculation (Fig. 2, Supplementary Table 1). Twenty up-regulated genes were observed at 48 hpi, 52 genes were differentially expressed 192 hpi (42 up- and 10 down-regulated), and 124 genes were differentially expressed 288 hpi (76 up- and 48 down-regulated). No genes revealed significant differential expression 8 hpi. Of the 158 differentially expressed genes in total, 33 genes (21%) were differentially expressed at more than one time point after inoculation (Fig. 2, Supplementary Table 1). For example, 5 genes were up-regulated at the three sampling time points 48, 192 and 288 hpi, 2 were up-regulated at 48 and 192 hpi, 6 were up-regulated both at 48 and 288 hpi and 20 were up-regulated at 192 and 288 hpi.

Of the 158 genes differentially expressed in the resistant genotype *Lm*B-01 after *Xtg* inoculation, 56% showed no sequence homology to genes deposited in public databases

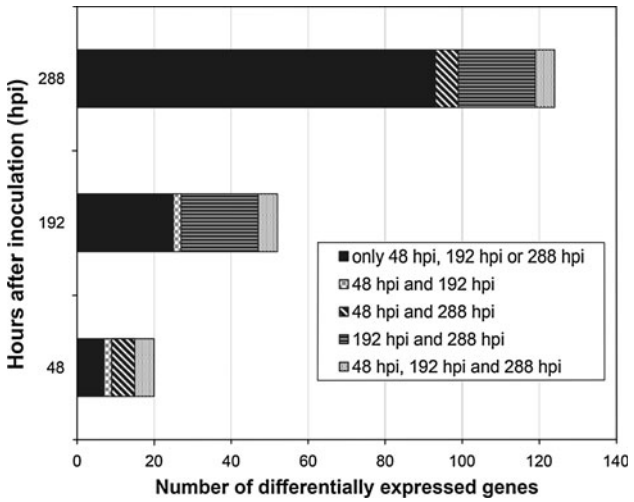

Fig. 2 Number of genes differentially expressed in the partially resistant *Lolium multiflorum* genotype *Lm*B-01 after *Xanthomonas translucens* pv. *graminis* (*Xtg*) inoculation at three time points post-inoculation (48, 192 and 288 hpi). *Identical filling patterns* indicate genes differentially expressed at multiple time points

or were homologous to plant genes with unknown functions and hypothetical proteins (*E* value <1E−06; Supplementary Table 1). Differentially expressed genes that revealed significant homology to genes deposited in public databases were assigned to functional categories according to the gene ontology (GO) database (Ashburner et al. 2000).

Defence and stress-related genes

Defence-related genes that were up-regulated after *Xtg* inoculation included a gene encoding the germin-like protein 6 (GLP6; r_010d_c02), the Verticillium wilt disease resistance protein (Ve2; rg3_008d_a09), the precursor of the pathogenesis-related protein 5 (csAtPR5; r_008d_h09), the non-specific lipid-transfer protein (LTP) 2 (sb_004a_g05), and the transmembrane BAX-inhibitor motif containing protein 4 (TMBIM4; gsa_007d_f02). Down-regulated defence-related genes included genes encoding the laccase 11 (LAC11) and the pathogenesis-related protein 4 (PR4; rg5_007a_c11). The stress-related gene encoding the Low silicon protein 1 (Lsi1; r_010d_c04) was also up-regulated after *Xtg* inoculation.

Genes involved in signal transduction

Genes involved in signal transduction that were up-regulated after *Xtg* inoculation included genes encoding a victorin-binding protein (ve_005b_b02), an ankyrin (gsa_007c_h09), the Pto kinase interactor 1 (p_001c_b08), a leucine rich repeat protein (rg2_004_f05), an annexin (r_004d_e11) and the brassinosteroid-insensitive 1 (r_014a_c01) protein, and a signal peptidase containing a 18K chain (rg6_008c_g08). A gene encoding a signal recognition receptor (sb_005a_a05) was down-regulated after *Xtg* inoculation.

Other genes

Two genes encoding glutathione transferases (r_003c_g07 and rg1_008a_h04) responsible for detoxification and a gene encoding a synaptobrevin-like vesicle-associated membrane protein (rg6_008b_d08) were up-regulated after *Xtg* inoculation.

Transcriptional differences between the resistant and the susceptible genotype

Comparisons of expression profiles of the resistant genotype and the susceptible genotype across all sampling time points revealed a total of 1,203 differentially expressed genes (Fig. 3, Supplementary Tables 2 and 3). Thereof, 624 genes were up-regulated in the resistant genotype and 579 genes were up-regulated in the susceptible genotype. Of the 624 genes up-regulated in the resistant genotype, 38 (6.1%) genes were only detected after control treatment, 525 (84.1%) were only detected after *Xtg* inoculation, and 61 (9.8%) were detected both after control treatment and after *Xtg* inoculation (Fig. 3). Of the genes up-regulated in the resistant genotype, 426 (68%) showed no sequence homology to genes deposited in public databases or were

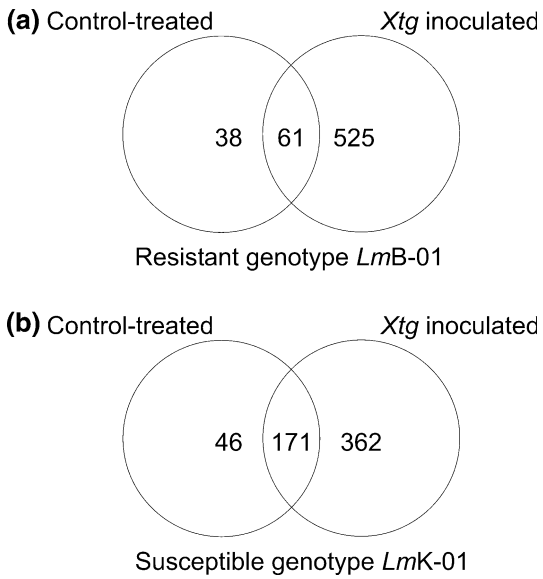

Fig. 3 Venn diagram of the genes from all time points up-regulated in the resistant *Lolium multiflorum* genotype *Lm*B-01 when compared to the susceptible genotype *Lm*K-01 (**a**) and in the susceptible genotype *Lm*K-01 when compared to the resistant genotype *Lm*B-01 (**b**) after control treatment and inoculation with *Xanthomonas translucens* pv. *graminis* (*Xtg*)

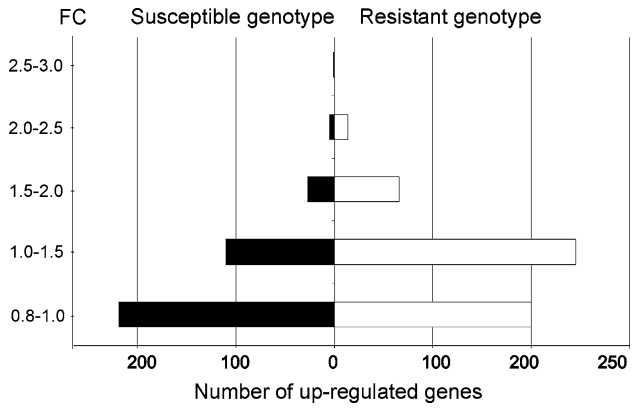

Fig. 4 Histogram of the fold change (*FC*) of the numbers of genes from all sampling time points up-regulated in the susceptible (*black*; $n = 362$) and the resistant (*white*; $n = 525$) genotype after *Xanthomonas translucens* pv. *graminis* infection

homologous to plant genes with unknown functions and hypothetical proteins. Of the 579 genes up-regulated in the susceptible genotype, 46 (7.9%) genes were only detected after control treatment, 362 (62.5%) genes were only detected after *Xtg* inoculation, and 171 (29.5%) genes were detected after control treatment and after *Xtg* inoculation. The number of genes with average FC between 0.8 and 1 after *Xtg* infection was comparable between the resistant and the susceptible genotype (Fig. 4). On the other hand, the numbers of genes with FC values between 1 and 1.5, and 1.5 and 2, were twofold higher in the resistant genotype when compared to the susceptible genotype. At the four sampling time points (8, 48, 192 and 288 hpc), 25, 34, 34 and 29 genes were up-regulated in the resistant genotype and 105, 119, 86 and 53 genes in the susceptible genotype, respectively (Fig. 5). Through comparison of the transcriptomes of the two genotypes after *Xtg* inoculation, 112, 125, 452 and 34 genes were up-regulated in the susceptible and 18, 483, 318 and 51 in the resistant genotype 8, 48, 192 and 288 hpi.

Of the 579 genes up-regulated in the susceptible genotype, 370 (64%) genes showed no similarities to other plant genes or were homologous to genes with unknown functions and hypothetical proteins (*E* value <1.00E−06). Functional categorization was performed as described above and genes with ontologies related to defence and stress are described in more detail.

Defence- and stress-related genes up-regulated in the resistant genotype

Defence-related genes up-regulated in the resistant genotype included genes encoding a BAX-inhibitor 1 (rg6_008a_f08), a 23 kDa jasmonate-induced protein (rg3_005a_h01), a homeobox-like resistance protein (rg3_012a_e02), LAC11, resistance gene analogue 7 (RGA7; rg1_006b_g04), the wheat-induced resistance protein 1 (WIR1; rg1_011a_g05), avrRpt2-induced protein 2 (AIG2; rg1_013a_d11). Stress-related genes included genes encoding two heat shock proteins (gsa_001b_f05 and r_009c_h12), the universal stress protein (rg3_009a_b11), the chaperonins (rg3_009d_e09 and ve_004a_e03) and the salt tolerance protein 5 (rg3_011a_d10).

Defence- and stress-related genes up-regulated in the susceptible genotype

Defence-related genes up-regulated in the susceptible genotype included genes encoding the powdery mildew resistance protein MLA6 (r_007d_d10), two different LTP (gsa_007d_e04; sb_001b_b03) and the *Erwinia*-induced protein 2 (r_007d_d06). The genes encoding stress-related proteins included genes encoding a ferredoxin (rg6_014b_f03), a wound-induced protease inhibitor (r_004a_g09), a HVA22-like protein (r_006b_g06), the low molecular mass heat shock protein Oshsp17.3 (r_007d_d03), an early responsive to dehydration protein (r_009a_c10), the 17.8 kDa class II heat shock protein (r_010c_b08), the cytosolic chaperonin delta-subunit (r_012b_b08) and the chloroplast heat shock protein 70 (ve_006a_c02).

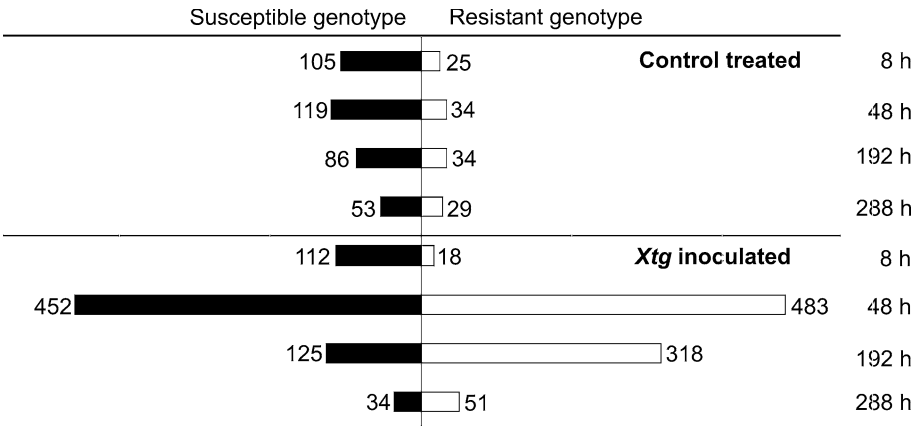

Fig. 5 Number of up-regulated genes in the susceptible *Lolium multiflorum* genotype *Lm*K-01 (*black*) and in the partially resistant genotype *Lm*B-01 (*white*) 8, 48, 192 and 288 h after control treatment and inoculation with *Xanthomonas translucens* pv. *graminis* (*Xtg*). A number of genes were simultaneously up-regulated at more than one time point or both after control treatment and after *Xtg* inoculation

Confirmation of differential expression

Nine of the genes differentially expressed in the resistant genotype after *Xtg* inoculation were analysed using qPCR. Up-regulation was confirmed for the six genes with known functions (Fig. 6) as well as for genes with the sequence IDs rg3_011a_h07, rg3_011d_b12 and sb_001a_f10, which did not reveal any sequence homology to sequences deposited in public databases. In addition, real-time quantitative PCR confirmed that the genes with the IDs: r_003d_g12, rg1_014c_b10, Lac_11, rg1_011a_g05, rg1_015d_f09, sb_003c_e08 and sb_007b_a08 were up-regulated in the resistant genotype when compared to the susceptible genotype.

Discussion

Transcriptome analyses using a cDNA microarray developed from 9,990 unique EST sequences from perennial ryegrass (*L. perenne*) revealed 158 genes differentially expressed in a resistant Italian ryegrass (*L. multiflorum*) genotype after inoculation with *X. translucens* pv. *graminis* (*Xtg*) and 1,203 genes differentially expressed between a resistant and a susceptible *L. multiflorum* genotype. CSH in which the target cDNA and the cDNA spotted on the microarray are from different species have been shown to produce reliable results providing the phylogenetic distance between the two species in not too extreme (Gilad et al. 2006). In this study, both the target species, and the species that was used for the development of the cDNA microarray, belong to the genus *Lolium* and are very closely related, as fully fertile F1 hybrids may be formed between the two species (Catalan et al. 2004). Consistent signals were detected for an average of 45% of the spotted EST sequences. This is higher than the 25–35% reported for hybridisation of *Arabidopsis halleri* cDNA to a microarray designed for *Arabidopsis thaliana* (Becher et al. 2004; Weber et al. 2004) but comparable to the 45–52% that were achieved when hybridising pepper (*Capsicum annuum*) and eggplant (*Solanum melongena*) cDNA to a microarray designed for tomato (*Solanum lycopersicum*; Moore et al. 2005). In view of the close phylogenetic relationship of *L. multiflorum* and *L. perenne*, the observed level of cross-species hybridisation of 45% is rather low. However, the EST sequences used for microarray development were derived from cDNA libraries from a broad range of different tissues including roots, seed and leaves and a similar level of hybridisation (50%) was observed in preliminary experiments using RNA from *L. perenne* leaves (data not shown). The finding that no differentially expressed genes were detected 8 hpi when comparing inoculation with *Xtg* and the control treatment is in agreement with the observations of Rechsteiner et al. (2006), in which only three differentially expressed TDF were observed at 12 hpi and no differentially expressed TDFs were detected before this time point using cDNA–AFLP. Although the rate of bacterial invasion may depend on the host–pathogen system (Wang and Sletten 1995), recognition of bacterial effector proteins and the induction of the hypersensitive response (HR) usually occur within 24 hpi (Scheideler et al. 2002). Thus, transcriptional changes leading to a HR were either absent or below the detection threshold of microarray and cDNA–AFLP analysis. It could also be that the host transcriptional response was manipulated by bacterial effectors initially suppressing host defences as it has been shown to occur during other *Xanthomonas* × host plant interactions (Kay and Bonas 2009) or that there was no recognition of effector proteins secreted by *Xtg*.

Reproducibility of the results obtained with the cDNA microarray was demonstrated by verification of transcriptional changes using qPCR (Fig. 6). The cDNA microarray and the qPCR results were in good agreement with respect to trends of regulation. However, for some of the genes, identical patterns could not be reproduced as for example for rg6_008d_b08, which was significantly up-regulated at 48 and 288 hpi according to the microarray analyses, but showed the highest FC at 192 hpi according to qPCR analysis (Fig. 6, Supplementary Table S1). This could be due to the fact that qPCR is a very sensitive method for differential gene expression discovery of even very small amounts of transcripts (reviewed in Valasek and Repa 2005) or due to a relatively stringent P value threshold ($P < 0.01$) chosen in this study for the microarray experiments which failed to identify genuinely differentially expressed (false negatives) genes.

The comparisons between the resistant and the susceptible *L. multiflorum* genotype were based on non-isogenic, highly diverse genotypes. Differences in transcriptome profiles may therefore reflect general genetic differences and are not limited to differences related to resistance characteristics. Consequently, transcriptome analyses revealed 1,203 genes differentially expressed between the two genotypes, representing a wide range of different functions (Table 2). Although the use of genetically largely identical individuals derived from near isogenic lines would allow to reduce general expression differences, such individuals are difficult to obtain and often suffer from severe inbreeding depression (Posselt 2010). The comparison of responses between a larger number of independently sampled genotypes from different phenotypic classes could present a valuable means to mitigate effects caused by disparate genetic backgrounds, but may be restricted by limited resources available. The approach based on only two genotypes used in this study may serve

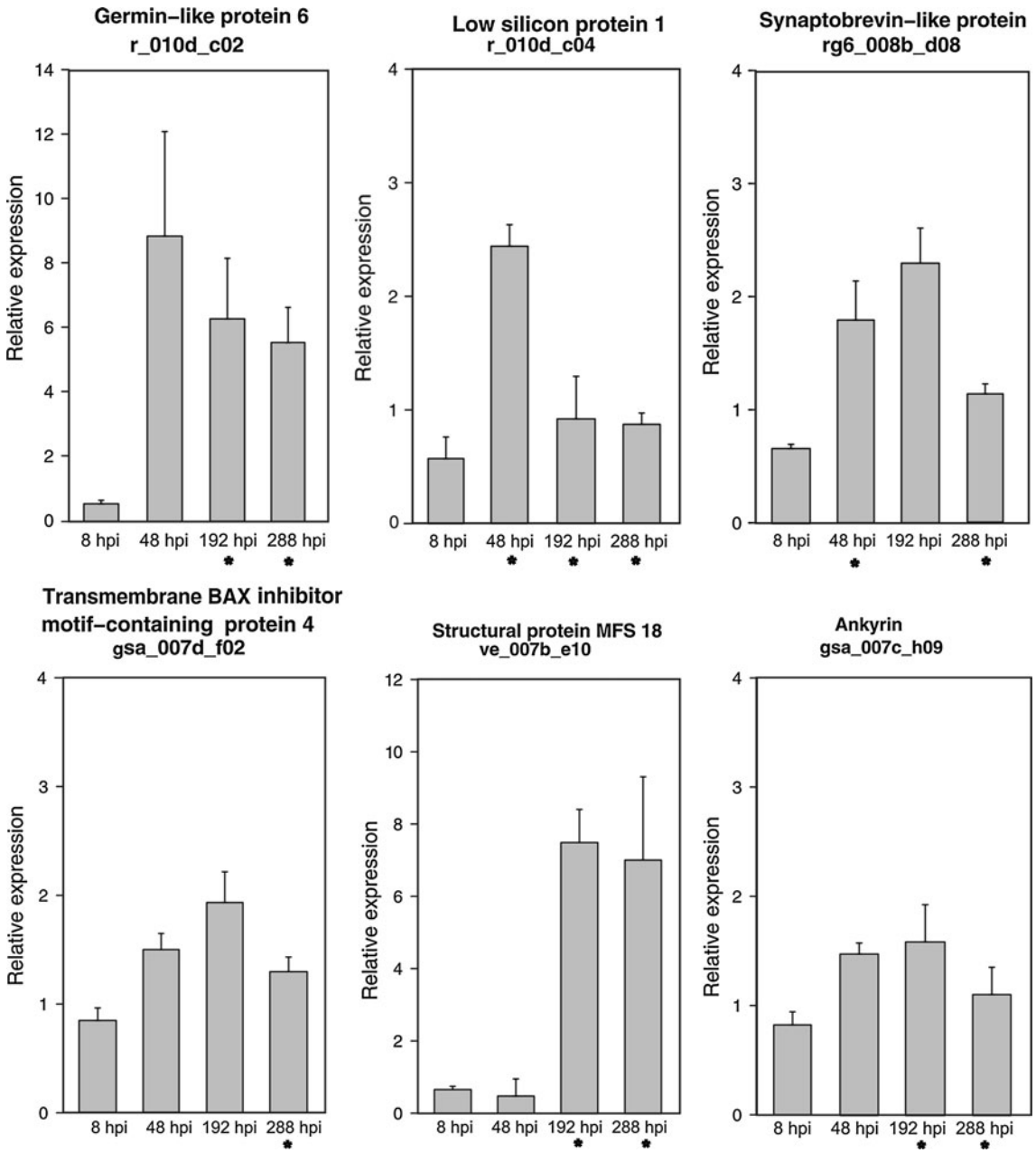

Fig. 6 Quantitative real-time PCR analysis of genes differentially expressed in the partially resistant genotype after inoculation with *Xanthomonas translucens* pv. *graminis* compared to the control treatment. *Protein names above* represent the annotation derived from GenBank and the blastx database (http://ncbi.nlm.nih.gov/BLAST/) and the *sequence ID* represents the unique identifier with information about the cDNA library (Asp et al. 2007) that the sequence was taken from. *Error bars* indicated standard errors and *asterisks* indicate that the gene was significantly up-regulated according to the microarray analysis

as a proof of concept for further investigations. Interestingly, a remarkably high number of genes were differentially expressed between the resistant and the susceptible genotype after *Xtg* infection when compared to the number of genes differentially expressed after control treatment (Fig. 5). Further, in the susceptible genotype (29.5%) were up-regulated both after control treatment and *Xtg* inoculation, and 62.5% of the genes were detected to be up-regulated only after *Xtg* inoculation (Fig. 3). A much lower number (9.8%) was observed in the resistant genotype both after control treatment and *Xtg* inoculation and a higher percentage (84.1%) was observed only after *Xtg* inoculation. This indicates that gene expression in the resistant genotype is more variable in response to *Xtg* inoculation compared to the susceptible genotype. Further, the greatest gene expression differences between the two genotypes were observed at 48 hpi and the number of up-regulated genes in the susceptible genotype decreased between 48

Table 2 Functional categorization of genes up-regulated in the partially resistant and the susceptible *Lolium multiflorum* genotypes after control treatment (C) and inoculation with *Xanthomonas translucens* pv. *graminis* (I) according to the GO database (Ashburner et al. 2000)

Functional category	Resistant genotype			Susceptible genotype		
	C	I	C and I	C	I	C and I
Amino acid and carboxylic acid metabolism	2	7	2	0	5	6
Autophagy	0	2	0	0	0	0
Carbohydrate metabolism	1	17	1	0	9	4
Cell-wall modification	0	2	0	0	1	1
Defence-related	2	9	2	0	3	2
Detoxification	0	2	0	0	2	3
Hormone pathway	0	4	0	0	2	4
Lipid metabolism	0	8	1	1	5	1
Oxidative burst	1	3	3	0	2	1
Phosphorylation/dephosphorylation	1	11	0	0	10	2
Secondary metabolism	0	1	1	0	0	2
Signal transduction	2	7	0	1	7	2
Stress-related	0	6	0	1	7	1
Transcription factor	1	14	0	2	9	3
Transport	0	5	2	1	6	1
Ubiquitination	0	5	1	0	3	4
Uncategorized	7	60	7	10	61	29
Unknown function	21	362	41	30	230	105
Total	38	525	61	46	362	171

and 192 hpi (Fig. 5). The number of genes up-regulated in the resistant genotype after *Xtg* inoculation remained high (318) and did not decrease until 288 hpi, indicating that important genotype-specific processes potentially involved in defence responses occur between 48 and 192 hpi. Additionally, more defence-related genes were up-regulated in the resistant genotype when compared to the susceptible genotype (Table 2). Up-regulated genes in the resistant genotype after *Xtg* inoculation may contribute to the phenotypic divergence in *Xtg* resistance between the resistant and the susceptible genotype. Genetic mapping of these up-regulated genes in the resistant genotype after *Xtg* inoculation compared to the susceptible genotype and subsequent expression QTL (eQTL) analyses is capable of assisting further elucidation of the genetic control of this variation as it has been shown for barley leaf rust in different barley genotypes or for stress response in different rice cultivars (Chen et al. 2010; Inoue et al. 2004).

Transcriptome analyses of the resistant genotype after *Xtg* inoculation revealed a number of differentially expressed genes previously reported to be involved in resistance and stress tolerance pathways. These genes represent candidate genes of particular interest for the development of tools for marker assisted resistance breeding. For example, the gene with the greatest FC according to the microarray analyses (r_010d_c04) encodes Lsi1, which belongs to a Nodulin26-like major intrinsic protein sub-family of aquaporins (Supplementary Table 1). Transcription of a Nodulin26-like major intrinsic protein has also been found to be up-regulated in citrus after infection with *X. axonopodis* pv. *axonopodis* (Cernadas et al. 2008). Further, Lsi1 is known to be involved in silicon uptake in rice and barley, which is thought to be important for resistance against biotic and abiotic stress (reviewed in Ma and Yamaji 2006). Silicon treatment has also been shown to reduce the chlorotic area of wheat leaves infected with the bacterial leaf streak causing pathogen *X. translucens* pv. *undulosa* (Silva et al. 2010).

Another gene showed high sequence similarity to members of the family of germin-like proteins (GLP; r_010d_c02) which are known to be involved in broad-spectrum basal defence against various pathogens and are also induced upon abiotic stress (Manosalva et al. 2009). Some germin-like proteins exhibit oxalate oxidase activity (Bernier and Berna 2001). r_010d_c02 also revealed high sequence similarity to an oxalate oxidase mRNA from wheat, indicating that this GLP up-regulated by *Xtg* inoculation may also exhibit oxalate oxidase activity. In rice, a putative QTL for bacterial blight resistance caused by *X. oryzae* pv. *oryzae* (*Xoo*) mapped to chromosome 3 and was closely associated with the candidate gene oxalate oxidase (Ramalingam et al. 2003). Chromosome 3 of rice displays conserved synteny with LG 4 of *L. multiflorum* (Devos 2005) where a major QTL for bacterial wilt

resistance was identified (Studer et al. 2006). Further, an oxalate oxidase gene in *L. perenne* (*Lp*OXO) was mapped to a similar location as the major QTL for bacterial wilt resistance on LG 4 of *L. multiflorum* (Dracatos et al. 2009; Studer et al. 2006).

Synaptobrevin-like transcripts such as rg6_008d_b08 which was up-regulated in the resistant genotype after *Xtg* inoculation have also been shown to be up-regulated in tomato and citrus infected with *Xanthomonas* spp. (Balaji et al. 2007; Cernadas et al. 2008). Synaptobrevin-like proteins are vesicle-associated membrane proteins involved in vesicle trafficking. The non-specific resistance genes *HvMLO* and *HvROR2* regulate accumulation of large vesicle-like structures in barley during powdery mildew attack (Collins et al. 2003). Large vesicle-like particles contain small cell-wall appositions, in which small vesicles accumulate between the plasma membrane and the cell wall (An et al. 2006). Thus, polar vesicle trafficking has been shown to be involved in the formation of new cell-wall appositions which is important for basal defence (An et al. 2006) presumably also supporting the mediation of partial resistance and defence to *Xtg*. Interestingly, rg6_008d_b08 also mapped to LG 4 in the VrnA mapping population (Studer et al. 2010) indicating that this gene may be associated with the major QTL for bacterial wilt resistance observed on LG 4 (Studer et al. 2006).

Expression of the flowering gene MFS18 (ve_007b_e10) was strongly induced in *L. multiflorum* leaves of the resistant genotype at 192 and 288 hpi with *Xtg*. MFS18 is a structural protein with sequence similarity to a gene expressed in male flowers (MFS18) of maize (Wright et al. 1993). It has been previously documented that flowering time is strongly correlated with disease resistance, such that the expression of flowering genes is induced with progressing disease development (Collins et al. 1999).

A gene encoding an ankyrin (ANK) repeat protein (gsa_007c_h09) was up-regulated after *Xtg* inoculation in *L. multiflorum* in the resistant genotype. The major role of plant ANK repeat proteins has mainly been related to signalling in defence and development mechanisms in *Arabidopsis* (Cao et al. 1997). In pepper, the ANK domain C_3H_1 zink finger was not only up-regulated in response to infection with *X. axonopodis* pv. *glycines* but also as response to abiotic stresses such as cold and salt stress (Seong et al. 2007). Therefore, the up-regulation of this gene encoding an ANK repeat protein may be associated with the specific signalling pathway triggered by *Xtg* infection.

In conclusion, the cDNA microarray developed using EST sequences from *L. perenne* provides an efficient means to identify differentially expressed genes in *L. multiflorum* genotypes during pathogen infection. *L. multiflorum* defence responses detected by transcriptome analysis display many similarities to those of other species such as rice, cassava and citrus after inoculation with *Xanthomonas* spp. Interestingly, the genes differentially expressed in the resistant *L. multiflorum* genotype after *Xtg* inoculation are remarkably similar to transcriptional changes triggered by PAMPs in other plant–pathogen interactions. Mapping of these candidate genes on the genetic linkage map of *L. multiflorum* developed by Studer et al. (2006) and subsequent QTL analyses may allow for the verification of genes co-locating with the major QTL on LG 4. Although the exact mechanism of putative *Xtg* resistance mediation by increased silicon transport needs to be further elucidated, the gene encoding Lsi1 represents a promising candidate gene for marker assisted selection. The differentially expressed genes identified in this study represent a crucial element in understanding *Xtg* resistance in *L. multiflorum* and in the future may significantly facilitate the development of molecular markers as tools for resistance breeding. In addition, it was demonstrated that two genotypes with a contrasting level of *Xtg* resistance reveal substantial transcriptional difference especially at the 48 and 192 hpi time points. The genes expressed at higher levels in the resistant genotype may be particularly useful to perform eQTL analyses in order to further understand the networks and pathways involved in *Xtg* resistance to identify genotypes with high levels of *Xtg* resistance.

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