Gene expression of plant defence pathways using *Lr1* transgenic lines and the Affymetrix wheat chip

Cloutier S[1], Wang Z[1], Banks TW[1], Jordan MC[1], McCallum BD[1]

[1] Agriculture and Agri-Food Canada, Cereal Research Centre, 195 Dafoe Road, Winnipeg, Manitoba, Canada R3T 2M9

ABSTRACT

Leaf rust resistance gene *Lr1* was cloned and demonstrated by transformation of the susceptible cultivar Fielder. Transgenic line T_0-938 had a single hemizygote *Lr1* insertion and segregated in subsequent generations. Unlike the majority of R-genes, *Lr1* has a co-dominant gene action, i.e., the heterozygotes exhibit an intermediate level of resistance. A gene expression experiment using T_0-938 derived lines was performed to study the resistance, intermediate and susceptible pathways triggered in the presence of *Lr1* in its homozygous or heterozygous states and in the absence of *Lr1*. The 27-Affymetrix chip experiment comprised 3 independent biological replicates, 3 genotypes (T_4-938-homozygous for *Lr1* (R), T_4-938-sister line without *Lr1* (S) and F_1-938-heterozygous for *Lr1* (I)), and 3 time points (before inoculation, 6h and 24h after inoculation). These lines genetically differed almost exclusively by their *Lr1* gene dosage and provided near perfect isogenic lines for profiling the expression pathways triggered during the compatible and incompatible *Lr1* wheat leaf rust pathosystem. Gene expression was measured by comparison to the expression levels before inoculation. A total of 584 of the 61,127 probe sets were differentially regulated in at least one of the 6 possible genotype × time point comparisons. Only 4 and 10 of these differentially regulated genes showed the same trends in all three genotypes at 6 and 24 hours after inoculation, respectively. The majority of genes differentially expressed in the three genotypes and at the two time points differed, indicating that pathways to resistance and susceptibility were largely not shared. Interestingly, the strict number of differentially regulated genes was higher in the intermediate and compatible reactions as it was in the incompatible reaction. Expression profiling analysis across genotypes and time points will be presented.

INTRODUCTION

Resistance or susceptibility symptoms are generally visible on wheat leaves 10-14 days after infection by leaf rust. In the *Lr1-Avr1* pathosystem, the incompatible interaction is observed as a fleck while large pustules with necrosis are easily visible in the compatible interaction. Resistance or susceptibility commitments, however, took place well before the appearance of symptoms, i.e. immediately upon infection and in the hours that followed. In order to understand the defence pathways activated upon leaf rust infection, it is necessary to determine early onset gene expression modulations.

The wheat leaf rust pathosystem fits the gene-for-gene model (Flor, 1956). Depending on the *Lr* gene, resistance can be dominant, recessive or intermediate (Kolmer and Dyck, 1994). We have cloned leaf rust resistance gene *Lr1* from bread wheat variety Glenlea (Cloutier et al. 2007). A member of the large Psr567 NBS-LRR gene family, *Lr1*, in its heterozygous state, provides an intermediate level of resistance.

Complementation of *Lr1* was achieved by the production of 17 independent transgenic lines in the Fielder background. Line T_0-938 was characterized by a single hemizygous Lr1 insertion and segregated for the transgene in a 1:2:1 ratio of resistant, intermediate and susceptible. These perfect near isogenic lines (NILs) are ideal for expression profiling experiments because they differ almost exclusively by the presence of the gene investigated, namely *Lr1*. In comparison, NILs produced through 6 or even 8 backcrosses will differ by an estimated 200 to 1000 genes or more depending on the size of the introgression(s), thereby creating large noise in expression profiling experiments.

The objectives of the present research included the identification of the pathways activated in transgenic sister lines with *Lr1*/*Lr1*, *Lr1*/*lr1* and *lr1*/*lr1* genotypes, upon infection with a leaf rust race carrying *Avr1*, and measured at 6 and 24 hours after infection. Expression profiling comparative analysis was performed to unravel the molecular mechanisms of resistance and susceptibility.

MATERIALS AND METHODS

Fielder-*Lr1* transgenic line T_0-938 was selfed to the T_4 generation and its progenies were tested every generation to determine the segregation of *Lr1*. Homozygous *Lr1* T_2-938-34-1 was crossed to its revertant sister line T_2-938-36-1 to produce *Lr1*/*lr1* heterozygous F_1 seeds. Five T_4-938-34-1, five T_4-938-36-1 and 5 F_1 plants were grown for each of the three biological replicates performed one week apart. Plants were grown in a growth chamber with a 16/8 hour photoperiod and a constant 20°C temperature. At the 4-5 leaf stage, just prior to inoculation, one random leaf from each plant was sampled in liquid nitrogen to serve as time zero samples. The plantlets were then inoculated with *Puccinia triticina* spores race CCDS which carries avirulence gene *Avr1* and is virulent on Fielder. Inoculated plants were placed in a misting chamber for 24 hours. One random leaf was sampled from each plant 6 hours after inoculation and again 24 hours after

inoculation i.e., just prior to returning the plants to the growth chamber where they were kept until apparition of symptoms. Rating was performed 11 and 14 days after inoculation.

RNA was extracted from each leaf independently and verified for integrity. Presence or absence of the transgene as expected for each of the three genotypes was performed by RT-PCR (Cloutier et al. 2007). RNA samples from the same treatment (0, 6h, 24h), genotype (T_4-938-34-1, T_4-938-36-1, F_1) and replicate were pooled. mRNA was extracted using the Ambion Poly-A Purist kit according to manufacturer's instructions. cRNA was produced using the complete One-cycle target labelling and control reagent kit from Affymetrix according to manufacturer's instructions. Purified cRNA from each of the 27 samples was hybridized to the Affymetrix wheat gene chip. CEL files were processed with the R – Bioconductor package "affy" (Gautier et al. 2004) using the following settings: background correction using robust multi-chip analysis, loess normalization and summarized using medianpolish algorithm. Student's t-tests were performed to detect significant differences in expression between time points. Benjamini-Hochberg correction was applied to the p-values to control false discovery rate. Probe sets were declared differentially expressed at an adjusted P-value <0.05. Differentially expressed probe sets were annotated and classified by biological functions. Identification of gene network was performed using Pathway Studio 5.0 (Aradne).

RESULTS AND DISCUSSION

Expression levels at 6 and 24 hours were compared to their corresponding 0 hour genotype within replicate. A total of 13 to 196 genes were differentially expressed depending on the comparison (Table 1). The incompatible interaction had the fewest differentially expressed genes while the interaction of the heterozygote F_1 had the most. Regardless of the interaction, there were more genes up-regulated than down regulated (Table 1). Only 4, 27 and 17 probe sets were differentially expressed at both 6 and 24 hours after infection in the incompatible, intermediate and compatible interactions, respectively. The majority of the differentially regulated genes were therefore transient i.e. differentially regulated 6 hours but not 24 hours after infection or vice-versa.

Nucleotide sequences of the contigs/ESTs used to design the differentially regulated probe sets was used to attempt to annotate the genes by their molecular functions. Nearly a third of the probe sets could not be annotated and are classified as unknown molecular function (Table 1). The wheat Affymetrix chip was designed as a "discovery" chip, and as a consequence of this design, more than half of the probe sets were from less reliable input sequences, thereby impeding our annotation abilities. This high proportion of unknowns also reflects the annotation gap of monocots in general.

The incompatible and the intermediate reactions shared 5 and 23 probe sets at 6 and 24 hours respectively. The majority of these probe sets have cell wall, defence and transport functions. Similarly, 38 and 28 probe sets were shared between the intermediate and the compatible interactions at 6 and 24 hours respectively, including 6 probe sets common between the genotypes at both time points. The intermediate reaction seemed to combine defence pathways of both incompatible and compatible reactions.

Table 1. Differentially regulated genes, classified by gene function, of the resistant (R), susceptible (s) and intermediate (I) reactions between isogenic transgenic wheat lines and *Puccinia triticina* at 6 and 24 hours after infection, compared to their expression prior to infection (0h).

Function	R0-6h	R0-24h	I0-6h	I0-24h	S0-6h	S0-24h
Biogenesis				1		
Biosynthesis		2	7	16	9	2
Cell wall		4		5		3
Cell division			1			
Defence	2	5	6	11	12	8
Develop. proc.			3		5	2
DNA binding	1	1	5	1	8	
Energy	1	1	2	4	3	1
Kinase activity				5	1	1
Hydrolase act.	1	5	1		1	
Metabolism		3	11	15	2	2
Mutase activity						1
Nucleot. bind.					1	
Other binding		1	2	1	5	
Peroxisome					1	
Protein binding		1	2	6	2	2
Protein synth.	1			1	2	1
Protein fate			1	1		
Resp. to stress	1	2	3	8	4	3
Ribosome				1		
RNA binding			3	7		
Signal transd.	1	4	11	11	9	6
Transcription		1	9	13		3
Transfer. act.				1	6	
Translation				2	6	1
Transport	1	3	7	27	8	9
Transporter			2	6	3	
Ubiquitin cycle						1
Unknown	4	11	45	53	56	20
Up-regulated	**10**	**29**	**92**	**113**	**114**	**39**
Down-regulat.	**3**	**15**	**29**	**83**	**30**	**27**
Total	**13**	**44**	**121**	**196**	**144**	**66**

Only 4 probe sets were differentially regulated at 6 hours in all three interactions. They were DNAJ, HLH DNA

binding and LEA proteins. At 24 hours after infection, a total of 10 probe sets were found to be common across all reaction types. These included 3 XET, 3 hypothetical, 1 aspartokinase and 3 unknown proteins. None were common across all three reaction types at both time points. DNAJ heat shock proteins are involved in a number of cellular functions including cell division and protein folding. XETs are xyloglucan endotransglycosylases involved in cell and tissue growth and are regulated by xyloglucan and hormones such as auxin and giberellic acid. One of the hypothetical genes had some similarity to a *BBI* gene encoding a Bownam-Birk proteinase inhibitor; however this was annotated with a lower confidence level. Pathway analyses have started to reveal links between these genes and their functional organization into networks. Incompatible, intermediate and compatible interactions will trigger the gene regulation of some common genes/gene networks as illustrated in Figure 1, but the majority of them were found to be unique to a reaction type with the intermediate reaction sharing pathways with both compatible and incompatible reactions.

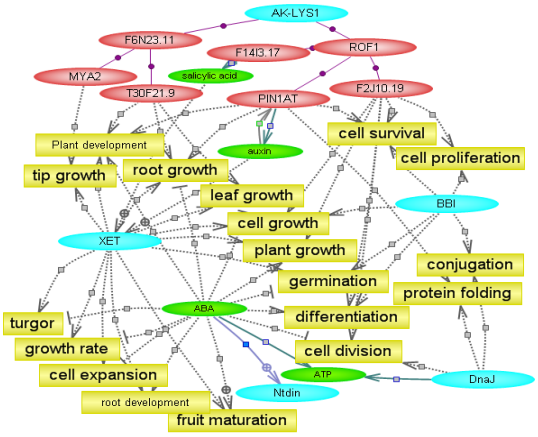

Figure 1. Pathways involving genes differentially regulated in incompatible, intermediate and compatible wheat leaf rust interactions. Genes in blue were differentially regulated in all three interactions at either 6 or 24 hours after infection.

CONCLUSION

Near isogenic transgenic lines with leaf rust resistance gene *Lr1* in its homozygous or heterozygous state and revertant line were ideal for gene expression studies of the wheat leaf rust pathosystem because they were of uniform genetic background, thereby isolating the gene expression changes to the system under study. In this pathosystem gene expression modulations are both rapid and transient. The compatible interaction was characterized by a larger number of gene expression changes than the incompatible interaction. The intermediate reaction provided by the heterozygous *Lr1/lr1* genotype was the most complex sharing pathways with both incompatible and compatible interactions.

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