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Barley elicits a similar early basal defence response during host and non-host interactions with *Polymyxa* root parasites

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Abstract Plant viruses transmitted by the obligate root-infecting plasmodiophorid parasites *Polymyxa graminis* and *Polymyxa betae* cause devastating yield losses to cereal and sugar beet crops worldwide. Barley is a non-host for *P. betae* but is a host for *P. graminis*. Using the Barley1 GeneChip® microarray we have investigated the transcriptional re-programming of barley roots during the earliest non-host and host interactions with zoospores of these protist species. At high confidence levels we detected 20 and 13 genes with increased transcriptional activity in response to *P. betae* and *P. graminis*, respectively, compared to unchallenged barley roots. Functional classification of the induced genes showed that a majority of the genes from both responses were associated with a classic defence response. Validation by quantitative RT-PCR analysis indicated that all of the genes examined were induced to comparable levels in both non-host and host responses. Our results also demonstrated that the barley defence-associated genes, *RAR1*, *ROR1* or *ROR2* were not essential for limiting the establishment of *P. betae* infection in barley. These data suggest that in barley roots the *Polymyxa* species induce a similar basal defence response whether the interaction is with a non-host or host. Thus, the early response to protist plant parasites appears to be part of the general 'frontline' defence against invading microbes.

Keywords Barley · Microarray · PAMP-triggered immunity · Plasmodiophorid · *Polymyxa* · Resistance

Abbreviations

ELISA	enzyme-linked immunosorbent assay
FDR	false discovery rate
PAMP	pathogen-associated molecular pattern
qRT-PCR	quantitative reverse transcription polymerase chain reaction

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Introduction

Plant disease is the exception rather than the rule within plant–pathogen interactions, as most pathogens are unable to infect most plant species (Thordal-Christensen 2003). This innate form of immunity, termed non-host resistance, can involve both pre-formed physical or chemical barriers and inducible

defence responses (Heath 2000). These different defence mechanisms are layered and the defence pathways involved can be individually dispensed without adverse effect on non-host resistance, which remains fully functional (Ham et al. 2007). The molecular basis of non-host resistance is still largely unknown but is thought to involve pathogen-associated molecular pattern (PAMP) perception, the basal response, and *R*-gene mediated recognition leading to pathogen exclusion (Jones and Dangl 2006). PAMP receptors recognise universal characteristics of microbes and are the first line of defence, whilst resistance genes tend to recognise pathogen effector molecules used by the invading pathogen to circumvent the early PAMP-triggered immunity. Together, the early PAMP-triggered immunity and later, effector-triggered immunity contribute to the multi-layered innate immunity of plants (Jones and Dangl 2006). Successful pathogens have evolved mechanisms to suppress the basal PAMP-mediated defences and evade the later resistance gene-mediated responses. Transcriptome profiling has provided much insight into the molecular processes that occur between plants and pathogens, including incompatible and compatible host interactions as well as non-host responses to fungi, bacteria and oomycetes (Caldo et al. 2006; de Torres et al. 2003; Huitema et al. 2003; Thilmony et al. 2006; Zimmerli et al. 2004).

Polymyxa species are obligate root-infecting protist parasites belonging to the order Plasmodiophorales. There are two species of *Polymyxa*, *Polymyxa graminis* and *Polymyxa betae*, which are morphologically similar but can be distinguished by their host range and rDNA sequences (Ward and Adams 1998). *Polymyxa graminis* mostly infects the Gramineae whereas *P. betae* colonises members of the Chenopodiaceae, including sugar beet (*Beta vulgaris*; Barr 1979; Kanyuka et al. 2003; Rush 2003). The *Polymyxa* parasite is able to survive for long periods as thick-walled resting spores which upon germination release a single zoospore. The zoospores are biflagellate, motile and initially probe host plant roots until a suitable point for attachment is identified (Keskin 1964). Once attached the zoospores encyst on the host cell wall and develop a tubular structure, the rhor, and a dagger-like body, the stachel, over a period of approximately 2 h. Once these structures have developed an adhesive outgrowth forms, through which the stachel and the contents of the zoospore are rapidly injected (within 1 min or less) through the host cell wall and plasma membrane into the host cytoplasm (Fuchs 1966; Keskin 1964). Previous studies have indicated that attachment of *Polymyxa* zoospores to the roots of their host plant species can occur within 1 h of inoculation (Barr et al. 1995) and that penetration and injection of the zoospore contents can occur within 2–3 h post-inoculation (Adams and Swaby 1988). However, *Polymyxa* zoospore probing, attachment and penetration events are not synchronised and can occur concurrently, such that zoospores at different stages of infection can be seen adjacent to one another on a plant root (Barr et al. 1995; Fuchs 1966; Keskin 1964).

Although *Polymyxa*-infection causes negligible crop losses, unlike the related plasmodiophorid *Plasmodiophora brassicae*, both *Polymyxa* species are vectors to a range of at least 15 economically important soil-borne plant viruses including *Beet necrotic yellow vein virus*, *Barley yellow mosaic virus*, and *Soil-borne wheat mosaic virus* (Kanyuka et al. 2003; Rush 2003). There is no known host resistance to either *Polymyxa* species in elite sugar beet or cereal crop cultivars, but resistance to *P. betae* has been documented in the wild *Beta* species *B. procumbens* and *B. patellaris* (Barr et al. 1995). Previous studies have shown that *P. graminis* elicits an active transcriptional response in the non-host sugar beet during the early stages of zoospore challenge, indicating an induced response rather than a solely preformed physical barrier (McGrann et al. 2007). In this study we have used a transcriptomics approach to elucidate the molecular responses of barley (*Hordeum vulgare*) roots during the early non-host and host interactions with zoospores of *P. betae* and *P. graminis* and show that both species elicit a similar basal response. Furthermore, we demonstrate that the barley defence-related genes *RAR1*, *ROR1* and *ROR2* are not essential to prevent the establishment of *P. betae* infection in its non-host barley.

Materials and methods

Polymyxa species cultures and inoculation

Polymyxa betae was propagated to the zoosporangial stage in sugar beet (*B. vulgaris* cv. Roberta) roots,

grown in a 50:50 mixture of sterilised sand and untreated *P. betae*-infested soil (Bullrush isolate, Broom's Barn Research Centre) for 21 days as previously described (Kingsnorth *et al*. 2003). *Polymyxa graminis* isolate 1 was cultured in barley (*H. vulgare* cv. Regina) roots in sand culture for 28 days at 22°C as described (Adams and Swaby 1988). Zoospores of both *Polymyxa* species were released into zoospore extraction buffer as previously described (Adams and Swaby 1988) and the zoospore concentration was determined using a haemocytometer. The suspensions were adjusted to 1×10^6 zoospores per millilitre in zoospore extraction buffer. Barley seeds were imbibed overnight in distilled water and allowed to germinate and grow for 3 days at room temperature on damp 2EW sand (W.E. Hewitt and Son Ltd., Petersfield Growing Mediums, Leicester, UK) prior to inoculation. The roots were immersed in a zoospore suspension of either *P. betae* or *P. graminis*. Unchallenged control material was prepared as above, except that seedlings were immersed in zoospore-free extraction buffer.

To identify genes differentially expressed during the earliest responses of barley to *Polymyxa* species, ten seedlings were sampled from both challenged and unchallenged treatments at 15, 30, 45 min, 1, 2, 3, 4, 5, 6 and 7 h. On each occasion the roots were removed with a sterile scalpel, frozen in liquid nitrogen and stored at −80°C until required. Root samples collected from each time point were combined to give a pooled sample of the time-course. Three biological replicates were used for each experiment.

RNA isolation and microarray analysis

Total RNA was extracted from the pooled barley-*Polymyxa* zoospore challenged and unchallenged roots using Trizol (Invitrogen, Paisley, UK) followed by DNase I (Ambion, TX, USA) treatment according to the manufacturer's protocol. RNA samples for microarray hybridisation were further purified using RNeasy Mini Spin column purification (Qiagen, Hilden, Germany).

Microarray hybridisation was conducted through contract research services provided by Geneservice Ltd. (www.geneservice.co.uk). The integrity of the RNA samples was confirmed using the BioAnalyzer 2100 (Agilent Technologies, Stockport, UK) before it was converted to cRNA with Message Amp II (Ambion). The triplicate barley-*Polymyxa* non-host or host-challenged root material and their respective unchallenged controls were hybridised to Affymetrix Barley1 GeneChip® microarrays using standard Affymetrix protocols (www.affymetrix.com). The data were analysed in-house using the Genespring (Silicon Genetics, Redwood, CA, USA) software package and by Geneservices Ltd. using the LIMMA (Smyth 2004) software package to produce a list of differentially transcribed genes for subsequent analysis.

Quantitative RT-PCR expression analysis

Pooled RNA from the host and non-host barley-*Polymyxa* interactions and their respective unchallenged controls were used for validation of the microarray data by quantitative RT-PCR (qRT-PCR). To further reduce DNA contamination following DNase I treatment, the total RNA for qRT-PCR was precipitated with LiCl. cDNA for qRT-PCR was synthesized from 2 μg total RNA using AffinityScript QPCR cDNA synthesis kit with Oligo(dT) primer (Stratagene, La Jolla, CA, USA) at 50°C. The cDNA was amplified using gene-specific primers (Table 1) and the Brilliant SYBR Green QPCR master mix (Stratagene). PCR amplification and real-time analysis of product formation was carried out using the MxPro-Mx3000P v3.20 QPCR System (Stratagene). The cycling conditions included an initial activation step at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 1 min at 60°C and 45 s at 72°C. Melt-curve analysis was performed at the end of each reaction to monitor primer–dimer formation and the amplification of gene-specific products. The average threshold cycle (C_T) value for each gene was calculated from duplicate samples for each triplicate experiment. A standard dilution series of barley root cDNA was used to validate the efficiency of each primer set prior to expression analysis.

Three housekeeping genes were selected for normalisation of qRT-PCR data. The choice of normaliser genes was based on previously published reports, indicating their high degree of stability under differing environmental conditions and pathogen stresses (Burton et al. 2004; Faccioli et al. 2007). These included the barley elongation factor 1-α, glyceraldehyde 3-phosphate dehydrogenase, and cyclophilin (see Table 1 for primer sequences). Using the geNorm programme v3.5 (http://medgen.ugent.be/

Table 1 Primers used for quantitative RT-PCR analyses

Affymetrix probe ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product (bp)
AF069331_s_at	ATGATCATCACTAGTTCATGTGC	AAAATACAGCAACACGGACT	163
Contig10624_at	CAACTTTGGTATGCGCTTTTCCC	CCGGTTCGTCAACTTATGTATGGCA	179
Contig12584_s_at	CCTCTACTCGTGCGGCTACA	AGCTCTCGGTACTCCTGGCA	126
Contig1334_at	CGGCGAGGAGAAGATCTTGGGGTT	GCCACATGTTCAGCTTCAGCATG	173
Contig1567_x_at[a, b]	CATGTGTCCGTTTCTGTAATGATGG	AAAATGACAATACGACCTTG	103
Contig1579_s_at[a]	CCATTGGATGCAGGAATTCTGTCTG	CCATCATTACAGAAACGGGCACA	115
Contig1580_x_at[a, b]	CATGTGTCCGTTTCTGTAATGATGG	AAAATGACAATACGACCTTG	103
Contig2214_s_at	AGCACGAAGCTGCAGGCGTA	TCTCGTCCACCCACAGCTTCAC	160
Contig2672_at	CGTTCGTGGCGTCGTACAAG	TCTTCTGCACCCACTGGAGG	141
Contig2787_s_at	ACCTACTGCTGCCGTGGCCAGTTT	ATCTGGTAGTTGGTTCCGGCAGGG	149
Contig2975_s_at	GTTTATGAGCGCGGGTGTGTGTGTG	AATCAGGCTCGCTCGCACGAAC	125
Contig3776_s_at	TGCATGTGCATGTGTAGTCG	CGTACGGGATACAATGATCG	126
Contig3783_at/ Contig3783_s_at	TGCTGAAGCTCAAGCTCGGCGT	ATTCCACGTACATATATCCTTTTAC	455
Contig4111_at	GGAGACGACTATTACTTCCGGGCGT	GCAGCCTGCTATTCTTCCCACTG	115
Contig4433_s_at	GCGCTCAAGGCCAACATCCT	GGGTTCTAAGAGGGGCACTGGAA	116
Contig4435_at	TGCGTGTACGTATCTGTGCG	GCGGTACGTGCAGGAGATCA	130
Contig4887_s_at	GTATGCGCGCAACGTAATGTGCTAT	GATACGGTGCCAAAGTAATT	107
Contig4953_at	GAAGAACAACTAAACTCGCAAGCCG	GGAGTCTCCGAGAAAGGGAATACCA	147
Contig5299_at	TGCATGGTGGATTCGTTGTTCGGT	TGCAGCCTGAACAAACGCAGTT	160
Contig6276_s_at	GCAATTGGAAGGCCATGAGACCC	ATACCTTGCATCGCCTCTCCCAGG	138
Contig6688_s_at	GGATGTCCGCGAGCCTTTCGTACT	TCAATGCGCTCATAGGTGTGTGC	112
Contig8185_at	GAGCCGCTGCTGCACAAGTTCT	TGGTCTCGATGATGGCCTGC	103
Contig845_s_at	ATCGAACGCGTGCTTCCATC	CAACGTACAGCAAACTCCCGT	250
Contig9925_at	CACCACTGAGCCGAGCATGA	AGAACACAGGGCTAGCTGCG	100
EBem05_SQ002_D05_s_at	CTACTCCTGGGGCTACTGCTTC	TGTGGGAGATCTGGATGGGC	128
EBma01_SQ002_F07_s_at	GCGTCCTACCCAGTCAAGACCTCA	GTCGAAACCGCATTACAGGTGTG	216
EBma03_SQ003_J21_s_at	CATACGTACGATATTGAGCAATAA	CATTAATTGCCTGAACAGGTAGGTC	206
EBma05_SQ003_C16_s_at	ATAGAAAAGTGCGGGAGTCG	GCTCCCATGGCTGATTTG	100
Normalisation genes			
Elongation factor 1α[c]	ATGATTCCCACCAAGCCCAT	ACACCAACAGCCACAGTTTGC	101
GAPDH[d]	CCTTCCGTGTTCCCACTGTTG	ATGCCCTTGAGGTTTCCCTC	124
Cyclophilin[e]	TTGAGGACGAGATAAGGCCAG	GCGACTGACAAGGTGCAAGAG	120

[a] Thionin-like multigene family primers are likely to cross-anneal.

[b] The same primer set represents both probe sets.

[c] HvGI TC146566

[d] GenBank accession number M36650

[e] GenBank accession number CV056520

~jvdesomp/genorm/; Vandesompele et al. 2002) these housekeeping genes demonstrated a high degree of stability in our treatments and were used to generate a normalisation factor (NF) for each cDNA sample. The normalised expression value of each gene of interest in *Polymyxa*-challenged roots compared to its unchallenged control was calculated following application of the NF to the ΔC_T. Analysis of variance (ANOVA) was used to compare the expression levels of each gene between the non-host and host responses (GenStat® (2007) Tenth Edition, © Lawes Agricultural Trust (Rothamsted Research) VSN International Ltd., UK).

Screening barley *rar1, ror1* and *ror2* mutants for susceptibility to *P. betae*

Seeds of the barley genotypes *rar1-1*, *rar1-2*, cv. Sultan 5 (*RAR1* wild-type (WT)), and *ror1*, *ror2*, cv. Ingrid (*mlo5*-backcross, *ROR1* WT, *ROR2* WT) were germinated on damp Schleicher and Schuell paper flutes for 3 days at room temperature and then grown for 21 days at 22°C in a *P. betae* infested soil–sand mix. Barley cv. Regina and sugar beet cv. Roberta were used as negative and positive controls for *P. betae* infection, respectively. Seedlings of each geno-

type screened were also grown in sand as uninoculated controls. The roots were washed free from any soil and/or sand under tap water for sample collection. The levels of *P. betae* in the roots were quantified using the ELISA method of Kingsnorth et al. (2003) to detect *P. betae* glutathione S-transferase. Samples showing an OD_{405nm} greater than three times the mean OD_{405nm} of their respective uninoculated control were considered infected.

Results

Transcription profiling of the barley-*Polymyxa* non-host and host responses

To identify barley gene transcripts differentially activated or repressed during the early stages of non-host (*P. betae*) and host (*P. graminis*) interactions we used the Affymetrix Barley1 GeneChip® for a microarray hybridisation approach. Barley roots were sampled over a 7 h period following exposure to zoospores of the different *Polymyxa* species and pooled prior to nucleic acid extraction.

Two different approaches were used to analyse the raw microarray data. Using Genespring, data values <0.01 were transformed to 0.01, then normalised to the 50th percentile per chip and to the median per gene. The data were filtered on expression to remove data values <10, and filtered on fold-change to identify differentially transcribed genes with a minimum fold change of 1.5 (Log_2 0.58) in challenged roots compared to the unchallenged control. The second approach for microarray analysis used Robust Multiarray Average measure (RMA; Irizarry et al. 2003) to normalise the data which were subsequently analysed with the LIMMA package (Smyth 2004) to identify differentially transcribed genes. Genes were selected if they showed a minimum fold change of 1.5 (Log_2 0.58) and a false discovery rate (FDR) of <0.05 (Benjamini and Hochberg 1995). Only genes that matched the selection criteria in both analysis methods were considered differentially transcribed.

A total of 20 induced genes were identified in the non-host response and 13 induced genes in the host response, of which four genes were common to both interactions (marked with superscript b in Tables 2 and 3). There were no down-regulated genes identified in either interaction. Annotations of all up-regulated genes were based on those provided for each of the probe sets present on the Barley1 GeneChip® by Affymetrix. The relative values for transcript induction in these genes were generally higher in the analysis produced by GeneSpring compared to that from LIMMA analysis (Tables 2 and 3).

Within this set of induced genes was a large proportion of genes encoding well characterised pathogenesis-related (PR) proteins such as PR1a, PR5, lipid transfer proteins, thionins, cysteine proteases, glutathione S-transferase and chitinase (Tables 2 and 3). We also identified genes encoding stress-related low temperature-induced proteins as well as some genes involved in cell wall modifications, metabolism, transport, or with no similarity to existing sequences in the databases (Fig. 1). Twenty-two of the 29 genes identified by microarray transcription profiling were predicted to enter the secretory pathway based on TargetP analysis (http://www.cbs.dtu.dk/services/TargetP/; data not shown), consistent with the active mobilisation of defence compounds to the sites of potential pathogen challenge.

Validation of the microarray data by qRT-PCR

To validate the microarray results we performed qRT-PCR analysis for all of the genes that had an FDR <5% in either the barley-*Polymyxa* non-host or host interaction using gene specific primers (Table 1). Three probe sets (Contig1567_x_at, Contig1579_s_at and Contig1580_x_at) represent highly homologous thionin-like gene sequences and cross-hybridisation is likely to be a key consideration for the microarray data. Indeed, we were unable to design qRT-PCR primers that would specifically amplify individual members with confidence; therefore the primers used could amplify more than one member of the thionin-like gene family (Table 1). Probe sets Contig3783_at and Contig3783_s_at are designed to the same barley exemplar (Barley1_03783) and therefore only one set of primers was used to confirm the microarray data for these probe sets.

The qRT-PCR data for each of the genes tested confirmed the significant up-regulation of those genes in both host and non-host responses to *Polymyxa* species (Fig. 2) and correlated well with the microarray data (Tables 2 and 3). Notably, despite high stringency analysis of the microarray data showing

Table 2 Genes identified by microarray analysis as transcriptionally activated during non-host response of barley roots against *P. betae* zoospores

Affymetrix probe ID	Annotation	Expression relative to unchallenged control[a]	
		GeneSpring	LIMMA
AF069331_s_at	Low temperature-induced protein	1.6	1.1
Contig10624_at	Hypothetical protein	2.8	1.9
Contig1334_at	Low temperature-induced protein	4.3	3.5
Contig1567_x_at	Thionin precursor	5.7	4.5
Contig1579_s_at[b]	Thionin	7.4	6.1
Contig1580_x_at[b]	Thionin precursor	1.5	2.1
Contig2214_s_at	Pathogenesis-related protein 1	2.1	1.8
Contig2787_s_at	Pathogenesis-related protein 5	3.4	3.3
Contig2975_s_at	Glutathione S-transferase 1	1.5	1.2
Contig3776_s_at[b]	Putative lipid transfer protein	3.8	3.1
Contig3783_at[b]	Putative lipid transfer protein	3.9	3.0
Contig4111_at	Nuclease I	1.1	1.0
Contig4433_s_at	Extensin-like protein	1.9	1.1
Contig4887_s_at	Cysteine protease	2.4	1.8
Contig4953_at	High affinity nitrate transporter (NAR2.1)	1.5	1.1
Contig5299_at	Ferredoxin precursor	1.0	0.8
Contig6688_s_at	Cytosolic 6-phosphogluconate dehydrogenase	1.4	1.1
Contig8185_at	Ferredoxin-nitrite reductase	1.9	0.9
EBma01_SQ002_F07_s_at	Cysteine endopeptidase	2.0	1.5
EBma05_SQ003_C16_s_at	Nitrate reductase apoenzyme	2.0	1.4

Values calculated using both the GeneSpring and LIMMA software packages are presented.

[a] Expression data shown as Log_2 transformed value.

[b] Probe sets also identified as induced in Barley-*P. graminis* host interaction microarray data.

Table 3 Genes identified by microarray analysis as transcriptionally activated during host response of barley roots against *P. graminis* zoospores

Affymetrix probe ID	Annotation	Expression relative to unchallenged control[a]	
		GeneSpring	LIMMA
Contig12584_s_at	No significant similarity	1.8	0.9
Contig1579_s_at[b]	Thionin	6.6	5.9
Contig1580_x_at[b]	Thionin precursor	3.3	2.0
Contig2672_at	Xyloglucan endo-transglycosylase	2.2	1.2
Contig3776_s_at[b]	Putative lipid transfer protein	4.1	3.3
Contig3783_at[b, c]	Putative lipid transfer protein	3.7	2.9
Contig3783_s_at[c]	Putative lipid transfer protein	4.0	2.6
Contig4435_at	Extensin-like protein	1.2	0.8
Contig6276_s_at	Abscisic acid-induced protein ABA7	3.4	2.3
Contig845_s_at	Nonspecific lipid-transfer protein precursor	2.9	1.8
Contig9925_at	Putative glycine-rich protein	3.3	2.0
EBem05_SQ002_D05_s_at	Class I chitinase	3.2	1.9
EBma03_SQ003_J21_s_at	Lipid transfer protein	2.9	2.0

Values calculated using both the GeneSpring and LIMMA software packages are presented.

[a] Expression data shown as Log_2 transformed value.

[b] Probe sets also identified as induced in Barley-*P. betae* non-host microarray data.

[c] Probe sets match different regions of the same barley exemplar.

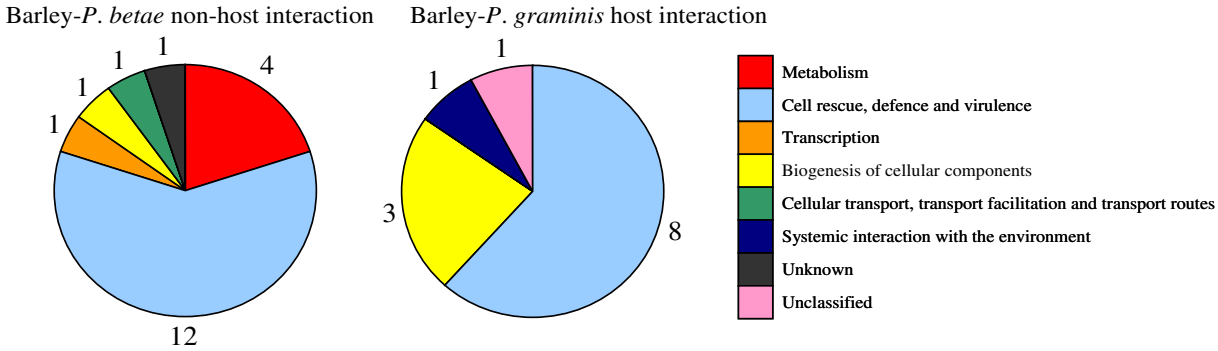

Fig. 1 Functional classification of the barley-*Polymyxa* non-host (*P. betae*) and host (*P. graminis*) microarray data showing that the majority of the genes identified in both responses are related to cell rescue, defence and virulence. *Numbers* around chart indicate genes present in each category

that only four genes were induced in common between the two interactions, qRT-PCR validation showed that all the genes were in fact induced to similar levels in both interactions (ANOVA, all genes $P>0.05$). This observation highlights the significance of the FDR when considering robustness of the data and that relaxing the FDR to allow for more variation between biological replicates would provide a larger

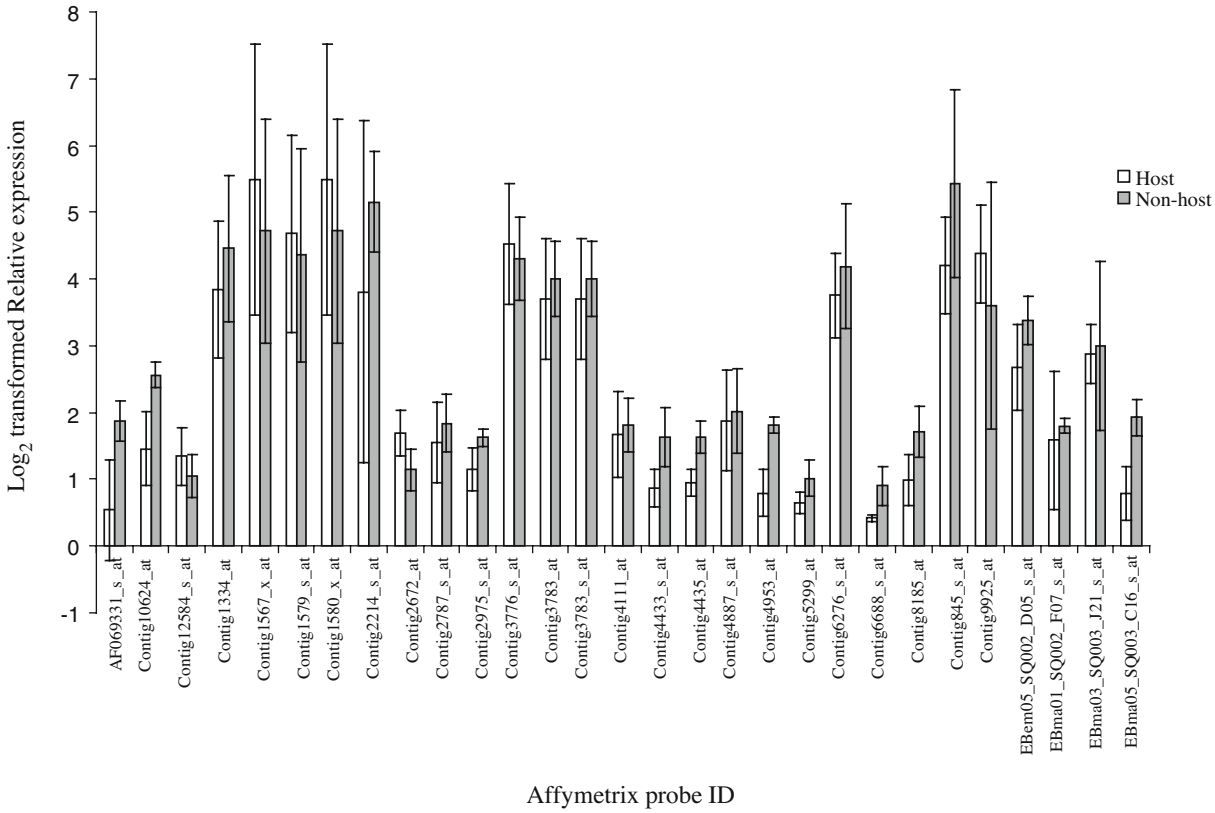

Fig. 2 Validation of the microarray data using qRT-PCR for genes identified as induced in response to challenge with zoospores of *P. betae* or *P. graminis*. Shown are Log_2 transformed relative expression values for each gene in the non-host (*P. betae—grey bars*) or host (*P. graminis—white bars*) response to *Polymyxa* species. The mean values of three independent experiments with standard errors are shown. There were no significant differences in levels of gene induction between the non-host and host interactions for any of the genes identified by microarray analysis (ANOVA, all genes $P>0.05$)

set of differentially expressed genes, yet potentially increase the number of aberrant genes identified. In this case, relaxation of the FDR to the 0.20 level increased the number induced genes that were common to both the host and non-host microarray experiments to 18 (data not shown).

RAR1, ROR1 and *ROR2* are not essential for prevention of *P. betae* establishment in barley

Mutant barley genotypes have been characterised that possess lesions in the defence-related genes *RAR1*, *ROR1* and *ROR2*. *RAR1* is required for the full function of particular resistance genes involved in race specific resistance to powdery mildew (*Blumeria graminis*) and has been implicated as a key regulator of early resistance gene–triggered defences in both monocotyledonous and dicotyledonous plant species (Muskett et al. 2002; Shirasu et al. 1999). *ROR1* and *ROR2* are necessary for non-race specific powdery mildew resistance in barley specified by the *mlo* resistance gene (Freialdenhoven et al. 1996). Resistance mediated by *mlo* is broad spectrum, appears highly durable and it has been suggested that *mlo*-mediated- and non-host resistance may share a common mechanism of action (Humphry et al. 2006).

Our microarray data suggest that *Polymyxa* species elicit a basal defence response during the early interactions with barley roots. *RAR1*, *ROR1* and *ROR2* have all been implicated in basal and non-host resistance responses (Freialdenhoven et al. 2005; Holt et al. 2005; Peterhansel et al. 1997). The Barley1 GeneChip® contains a representative of *RAR1* (Barley1_08942; Close et al. 2004) and *ROR2* (Barley1_22370). Based on our data that neither of these genes was significantly differentially transcribed in response to either *Polymyxa* species (data not shown), but because of the relationship these three genes have with basal and non-host resistance, we tested whether mutations in any one of these genes would be sufficient to enable *P. betae* to infect and develop within barley, a non-host to this parasite. An ELISA-based method was used to screen for *P. betae* in all of the barley genotypes exposed to infection, including the *rar1-1*, *rar1-2*, *ror1* and *ror2* mutants. After a 3-week exposure period none of the mutant genotypes contained levels of *P. betae* normally associated with established host infection, as was detected in sugar beet by ELISA (Fig. 3). These data indicate that *RAR1*, *ROR1* or *ROR2* barley mutants are not compromised in their ability to mount resistance against *P. betae* establishment.

Discussion

Polymyxa betae and *P. graminis* are vectors of a wide range of important plant viruses worldwide (Kanyuka et al. 2003; Rush 2003). Despite this, very little is known about the molecular interactions between *Polymyxa* species and their host and non-host plant species (McGrann et al. 2007). Using transcriptome analysis of the barley-*Polymyxa* species non-host and host interactions we have for the first time identified genes that are transcriptionally activated in each interaction. qRT-PCR analysis validated the microarray analysis and also indicated that the identified genes were induced to comparable levels in barley roots in response to both *Polymyxa* species. This result indicates that *P. betae* and *P. graminis* elicit a similar basal defence response in their interactions

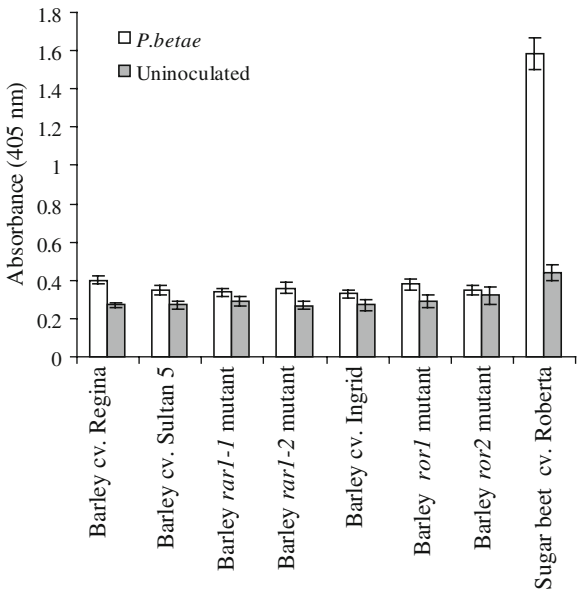

Fig. 3 Barley *RAR1*, *ROR1* and *ROR2* are not essential to prevent the development of *P. betae* in the non-host plant barley. ELISA was used to detect *P. betae* glutathione *s*-transferase levels in barley roots from plants grown in *P. betae*-infested soil/sand (white bar) or in *P. betae*-free sand (uninoculated controls—*grey bar*). *Bars* indicate standard error. Results of the mean ELISA values from a minimum of 35 inoculated and 20 uninoculated plants for each genotype, from at least three independent experiments are shown

with barley roots. de Torres *et al*. (2003) reported a similar finding during the initial 2 h of exposure to different strains of *Pseudomonas syringae* pv. *tomato* that elicited host (susceptible), incompatible (resistant) and non-host responses in *Arabidopsis thaliana*. The initial transcriptional re-programming observed in these experiments was common to all three challenges and resulted from PAMP recognition rather than specific strain effectors (de Torres et al. 2003).

Barley roots challenged by zoospores of either *P. betae* or *P. graminis* responded by increasing the transcription of a range of PR genes such as *PR1a*, *PR5*, chitinase, glutathione S-transferase, lipid transfer proteins and thionins. Regulation of these genes in other plant species is typically observed during PAMP-induced transcriptional re-programming in host, incompatible and non-host responses to plant-pathogens (de Torres et al. 2003; Huitema et al. 2003; Thilmony et al. 2006). There was also transcript induction of genes involved in cell wall modifications including xyloglucan endo-transglycosylase (XTH) and glycine-rich and extensin-like genes which have been reported as PAMP-inducible (Thilmony et al. 2006). Of particular interest was the up-regulation of the barley XTH gene in both non-host and host responses. A similar XTH gene has been isolated from a sugar beet root cDNA library specifically selected by differential screening to identify highly expressed transcripts in sugar beet roots heavily infected with *P. betae* (Dimmer et al. 2004; Mutasa-Göttgens et al. 2000). This suggests that both the monocotyledonous and dicotyledonous host plants of *Polymyxa* species respond to these parasites by repairing damaged cell walls.

The evidence presented here suggests that the early non-host and host responses of barley to the two *Polymyxa* species are very similar; however, this result may be influenced by limitations of the current barley gene set. Whilst the Barley1 GeneChip® contains around 22,000 probe sets representing non-redundant barley exemplar sequences (Close et al. 2004), a more conservative estimate of the actual number of unique genes represented on the Barley1 GeneChip® is closer to 14,000, the equivalent of approximately 30% of the barley genome (Close 2005). With this in mind, genes that condition barley for being a non-host for *P. betae* but a host for *P. graminis* may not be represented on the GeneChip®. de Torres et al. (2003) identified a large number of transcripts responsive to different *P. syringae* pv. *tomato* strains that were not present on the *A. thaliana* Affymetrix GeneChip®. Techniques such as cDNA-AFLP or representational difference analysis still have an important role in transcription profiling to identify unknown transcripts (de Torres et al. 2003; McGrann et al. 2007).

Non-host resistance is believed to be the product of multiple barriers to disease establishment (physical and/or chemical, preformed and/or inducible) which a successful pathogen must overcome (Thordal-Christensen 2003). Analysis of plant mutant genotypes has helped to decipher the pathways involved in plant defence responses as well as to demonstrate which of these pathways is essential for resistance against different plant pathogens. Our data indicate that the barley defence-related genes *RAR1*, *ROR1* and *ROR2* are not essential to prevent the establishment of *P. betae* infection in barley roots. However, *RAR1*-, *ROR1*- and *ROR2*-mediated defences may still contribute to non-host resistance in our experimental system. The pathways disrupted by these mutations appear, however, to be individually expendable for non-host resistance to function in barley against *P. betae*, as has been observed for other well characterised plant defence pathways in non-host systems (Ham et al. 2007).

The possibility also exists that in barley-*Polymyxa* interactions, differentiation between establishing disease in a host or rejection in a non-host may occur later in the interactions than the early events studied here. We specifically chose to examine the transcriptional responses up to 7 h after challenge. This time period was selected to include the probing, attachment and penetration stages of the *Polymyxa* life-cycle which occur in an overlapping series (Adams and Swaby 1988; Barr et al. 1995) and has been used previously to identify differentially transcribed genes in sugar beet-*Polymyxa* species interactions (McGrann et al. 2007). Furthermore, it has been previously demonstrated that transmission of virus particles by *Polymyxa* can occur within this time window (Adams and Swaby 1988). Samples were taken as early as 15 min post-inoculation to cover initial probing and attachment events through to 7 h when only empty zoospores have been observed at the root surface in host interactions (Barr *et al*. 1995). These samples were then pooled prior to microarray analysis to include transcripts representative of all

stages in the infection process whilst preventing the experiments from being prohibitively expensive. Ultimately a kinetic approach to dissect the processes of probing, attachment, and penetration would be desirable; however, the asynchronous nature and extremely rapid penetration step would render this extremely challenging.

Our transcriptome profiling did not identify any genes involved in signal transduction pathways, or any genes that were repressed. It is possible that such signals and gene repression may have occurred early and transiently such that, using the pooled sampling approach, the transcripts could not be resolved by our analysis. Similarly, the pooling approach is likely to contribute to an inherent degree of biological variation in our system, for which we have accounted by using the FDR as a measure of confidence.

Although our work has provided insight into the downstream genes associated with *Polymyxa* interactions in barley, the perception of *Polymyxa* elicitors remains undefined. We conclude that zoospores of *P. betae* and *P. graminis* appear to elicit similar transcriptional changes in barley roots during the first 7 h of non-host and host interactions, comparable to the basal defence response induced by PAMPs.

It remains possible that modifications in the transcriptional re-programming which prevents (non-host) or allows (host) *Polymyxa* biotrophy may occur at a later stage than we investigated, and could possibly involve effector-triggered immunity. Alternatively, the host specificity determinants may be encoded not in the plant but within the *Polymyxa* genomes. Circumstantial evidence to support this exists from the finding that glutathione S-transferase, a well known defence protein in plants (Jwa et al. 2006) and a gene which is induced in *Mycosphaerella graminicola* when the fungus was under oxidative stress *in planta* (Keon et al. 2007), is highly expressed in *P. betae* during plant infection (Mutasa-Göttgens et al. 2000). Clearly, an important key to improved understanding of the *Polymyxa* plant parasite and virus vector is the availability of reliable and comprehensive genome sequence data which are still lacking. Genome sequencing together with improved knowledge of the infection biology of *Polymyxa* species are important goals for future research. This should assist in the development of strategies for crop improvement to ensure continued sustainability in the warmer climates predicted for the future, and which also favour the growth and spread of *Polymyxa* and the soil-borne viruses it carries.

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