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Gene expression profiles unique to chile (*Capsicum annuum* L.) resistant to *Phytophthora* root rot

Richard D. Richins, Sandra Micheletto, Mary A. O'Connell *

Department of Plant and Environmental Sciences, New Mexico State University, Las Cruces, NM 88003, USA

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ABSTRACT

Transcriptome profiling in chile (*Capsicum annuum*) roots was used to determine those genes with expression profiles correlated with a resistance phenotype to the pathogen, *Phytophthora capsici*. Two microarrays were generated; a 10K element array printed with cDNAs from a library of transcripts expressed in Criollo de Morelos-334 variety of *C. annuum* 6 h post-challenge with *P. capsici* and a 2K element array printed with sequenced cDNA clones selected based on their preliminary patterns of expression. This second array was enriched for clones that were differentially expressed in susceptible (New Mexico 6-4) vs. resistant (CM334) varieties of *C. annuum*. Gene expression profiles were revaluated at 0, 4 and 24 h post-inoculation. Control treatments included samples collected at 0, 4, and 24 h post-mock-inoculation. In addition to the parental CM334, a resistant backcross line (01-1688) was also used to identify gene expression patterns associated with the resistance phenotype. Based on a principal component analysis, CM334 samples showed the most significant transcription induction at 4 and 24 h post-inoculation, while the predominant variability in the susceptible line was in genes repressed at 24 h. A set of 168 genes with significant changes in expression following *P. capsici* challenge was identified; of these, 22 were uniquely expressed only in the resistant lines (CM334 or 01-1688). This set of genes represent candidates for further study as markers for recurrent selection programs and as candidate genes for the mechanism of disease resistance.

1. Introduction

Phytophthora capsici is a soil born pathogen, which causes root rot, foliar blight and pod rot in nearly all cultivars of *Capsicum annuum* [1,2]. The pathogen is among the most economically destructive to chile production causing losses of tens of millions of dollars annually in chile growing regions throughout the world. Among the disease syndromes, the root rot is the most destructive form of the disease causing wilting and death in infected plants. The spread of the fungus is exacerbated by periods of water-saturated soil and warm temperatures – conditions that are common at times to most of the major chile production regions in the world [3,4]. Chemical control of the root rot is only minimally effective, hence efforts to identify and introduce resistance into economically important chile cultivars is a priority for chile breeders.

The best source of *P. capsici* resistance is observed in the Criollo de Morelos-334 (CM334) variety of *C. annuum* [3,5]. CM334 shows a very high degree of resistance to all of the disease syndromes caused by even the most virulent strains of *P. capsici* [6–9]. Attempts have been made to introduce the resistance phenotype from CM334 into commercially grown chile cultivars via traditional breeding. These attempts have not been entirely successful due in part to a lack of understanding of the resistance mechanisms employed by CM334. Resistance in CM334 to *P. capsici* is thought to be polygenic [10,11]; with at least some differences in the complement of genes responsible for conferring resistance to the foliar blight vs. those involved in resistance to the root rot [12]. QTL maps and molecular markers associated with the root rot resistance in CM334 have been determined supporting the polygenic nature of the resistance [10,11,13–15]. These markers and maps are useful for plant breeding purposes to introgress disease resistance, but to date, give no indication of the specific genes or mechanisms involved in the resistance phenotype.

Alterations in gene expression in response to pathogen challenge is commonly observed in plants, and there are numerous examples of genes whose expression is increased in response to viral, bacterial and fungal inoculations. Some of these gene products have been called pathogenesis-related proteins (PR) and examples in *Capsicum* have also been described [16]. In addition to gene expression responses commonly observed in plant pathogen interactions, there are genes expressed in the

* Corresponding author at: Department of Plant and Environmental Sciences, MSC 3Q, PO Box 30003, Las Cruces, NM 88003, USA. Tel.: +1 575 646 5172; fax: +1 575 646 4681.
E-mail address: moconnel@nmsu.edu (M.A. O'Connell).

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hypersensitive response (HR), which is associated with a simply inherited disease resistance phenotype. In many cases, the dominant gene product associated with this phenotype is a leucine rich repeat, and again examples of this type of gene and expression pattern have been described for the *Xanthomonas–Capsicum* interaction [17]. Examples of genes commonly expressed in pathogen challenged plants either via the HR signaling route, reactive oxygen species signaling, or as PR proteins have been reported for *Capsicum* [18,19]. Specific characterization of novel gene products induced in response to *Phytophthora* challenge in *Capsicum* includes characterization of phenolic compounds and antioxidant enzymes [20] and nitric oxide generation and enzymology [21].

Global gene expression profiling, or microarray analysis has been used to characterize the alterations in expression in many plant pathogen interactions [22], including potato and *Phytophthora infestans* [23,24] and soybean and *Phytophthora sojae* [25]. No equivalent study has been performed on the *Capsicum–P. capsici* interaction. This study was initiated to utilize cDNA microarray technology to identify genes, which are specifically and/or differentially responsive to a challenge inoculation by *P. capsici* on the roots of susceptible and resistant cultivars of *C. annuum* at various times post-inoculation. The results of this study will identify genes whose expression is associated with root rot resistance in *Capsicum*.

2. Materials and methods

2.1. Plant and pathogen sources

A highly virulent *P. capsici* isolate, PWB-24 [3] was used exclusively for these studies. *P. capsici* was cultured and inoculum prepared essentially as described in [12] with the exception that a filter and heat sterilized extract of *P. capsici* infested soil was used to stimulate sporangia formation. The extract was prepared by stirring 20 g of soil from Leyendecker Farm, NMSU, in 1 L water overnight. The extract was centrifuged (10,000 × *g* for 10 min) prior to vacuum filtering the extract through a 0.22 μ filter. The extract was then autoclaved. Zoospores were quantified using a hemocytometer.

Four genotypes of chile (*C. annuum* L.) were used in these analyses: New Mexico 6-4 (NM6-4), a susceptible line; Criollo de Morelos-334 (CM334), a resistant line; and 01C 1688 a resistant line developed as a backcross with CM334 and *C. annuum* cv. Early Jalapeno [3,26,27]. Plants were grown in washed sand with occasional fertilization using Peters Professional 20-20-20 fertilizer (Scotts) at 0.5 g per L.

2.2. cDNA library and microarrays

CM334 plants were grown as described until the eight leaf stage. Six plants were gently removed from the sand and immersed in a 150 mL beaker containing *P. capsici* zoospores (2×10^5/mL). Following a 2 h incubation at room temperature, the plants were gently replanted in sand for 4 h after which the roots were harvested immediately into liquid nitrogen.

Total RNA was isolated as described in [28] and mRNA subsequently isolated using Ambion's Poly A Pure kit. A lambda ZAPII cDNA library was prepared from the mRNA according to the manufacturer's instructions (Stratagene). The initial library complexity was approximately 1×10^6; the amplified titer was 3.1×10^{10}/mL. Individual plaques were transferred from agar plates into 500 μL SM [0.1 M NaCl, 0.05 M Tris–HCl, pH 7.5, 0.1 M $MgSO_4$. 0.01% (w/v) gelatin]. Chloroform (50 μL) was added to each phage stock to inhibit microbial growth. PCR was used to amplify the inserts from ~9500 unique recombinant phage using the M13 forward (−20) and reverse (−24) primer sites (Qiagen) on the ZAPII vector sequence. Following amplification, the fragments were analyzed by agarose gel electrophoresis, then the amplification reaction was precipitated with an equal volume of isopropanol overnight at −20 °C. Precipitates were collected by centrifugation (1850 × *g* for 30 min), rinsed with 70% ethanol and dried. Samples were resuspended in 20 μL Microarray Spotting Solution (ArrayIt) and stored at −20 °C.

Two sets of microarrays were printed. The first microarray set, the 10K array, was comprised of 9312 amplicons derived from the CM334 root library as well as buffer and air blanks and ~1000 cDNA amplicons from either a *Phaseolus acutifolius* drought-stressed root library [29] or a *C. annuum* drought-stressed leaf library [30]. The 10K array was printed using a Gene Machines microarray printer on polylysine-coated microscope slides. A second array, the 2K array, was prepared containing a subset of the responsive cDNAs identified on the 10K array. The 2K array was comprised of 1783 cDNAs each printed in duplicate on the array slide and had additional spots printed with buffer and air blanks. The 2K array was printed using a OmniGrid 100 Arrayer (GeneMachines, San Carlos, CA, USA) with SMP4 printing pins (TeleChem, Sunnyvale, CA, USA) on polylysine-coated microscope slides, as described in [31]. Both microarrays were post-processed as described by [32].

2.3. Sample preparation

For the initial screening experiments, the plants were grown in sand with occasional fertilization. When plants had reached the 8 leaf stage, they were carefully removed from the sand and immersed in either a zoospore containing solution or water. After a 1 h room temperature incubation, the plants were repotted in sand for either 3 h or 23 h. Plants were removed form the sand and their roots immediately frozen in liquid nitrogen as above. For 0 h samples, plants were removed from the sand and roots immediately frozen. All tissues were stored at −80 °C until RNA isolation.

Cy3- and cy5-labeled cDNAs were generated from total RNA (60 μg) using the aminoallyl-dUTP (aadUTP) dye incorporation method [33]. The dTTP:aadUTP ratio employed in all experiments was 1:1. Hybridizations were carried out in microarray cassettes (ArrayIt), which were immersed in a 42 °C water bath covered with aluminum foil to limit light-induced bleaching of the dyes. Hybridizations were typically carried out for 16 h after which time the microarrays were washed twice with 1× SSC, 0.1% (w/v) SDS; twice in 0.1× SSC, 0.1% (w/v) SDS; and three times with 0.1× SSC, all washes were also at 42 °C. The slides were then centrifuged briefly to dry, and then scanned using a Genomic Solutions GenTac UC4 microarray scanner.

2.4. DNA sequencing

DNA sequences for the clones were determined by cycle sequencing the PCR amplicons that were also printed on the arrays. A T3 primer site was used for the cycle sequencer reaction. Products were analyzed on an ABI 3100 DNA sequencer. For a limited number of clones, DNA sequences were obtained after rescuing the phage into a plasmid form (Stratagene), in those cases the recombinant plasmids were sequenced using dideoxy sequencing methods and read on a LI-COR automated DNA sequencer.

2.5. Data analysis

Spot signal intensities were quantified using Genomic Solutions GeneTac Integrator 4.0 scanner. For the 10K array, the data analysis was performed essentially as described earlier [29]. For the 2K array, data was exported to Microsoft Excel with the XLStat plugin

(www.xlstat.com) for further analysis. Target total intensity values were used for all calculations. Data from four or six hybridizations and utilizing at least two independent RNA samples were compiled for each treatment. Values were normalized by performing non-linear regression and Lowess transformations. A *z*-test was performed on the resulting data to identify highly variable ("bad spots") values. Data with *z*-scores with in 0.01–0.99 were included in subsequent analyses. Discarded values nearly always correlated with prior visual identification of "bad spots" on the microarrays.

Ratios and *p*-values were calculated for the following seven comparisons: (1) CM334, 0 h vs NM64, 0 h; (2) CM334, 4 h *P. capsici* vs CM334, 0 h; (3) CM334, 4 h *P. capsici* vs CM334, 4 h mock inoculation; (4) CM334, 4 h *P. capsici* vs NM64, 4 h *P. capsici*; (5) CM334, 24 h *P. capsici* vs CM334, 0 h; (6) CM334, 24 h *P. capsici* vs CM334, 24 h mock inoculation; (7) CM334, 24 h *P. capsici* vs NM64, 24 h *P. capsici*. Significant differential gene expression was identified as repressed when ratios less than 0.5 were calculated with corresponding *p*-values less than 0.06; and as induced when ratios greater than 1.9 were calculated with corresponding *p*-values less than 0.06. Similar comparisons were conducted with the additional resistant genotype, 01-1688. Since limited seed was available for this genotype, only replicate data using single RNA isolations was obtained for most of the time points.

2.6. Blot hybridizations

Total RNA (10 μg), isolated from roots of plants inoculated with *Phytophthora* as described above, were electrophoresed, transferred to nylon membranes and hybridized with ^{32}P-labeled probes as described earlier [34].

3. Results

3.1. Identification and characterization of cDNAs to be monitored by microarray analysis, 10K array

Three different experiments were performed to identify differentially expressed genes among those printed on the 10K array. A comparison of the gene expression changes in the resistant line, CM 334, at time points following *P. capsici* challenge (1, 4, and 24 h); changes in the susceptible line, NM 6-4 following challenge; and finally a comparison of the two genotypes for expression levels at the same time point. Fig. 1 presents a plot of the normalized expression ratios for the genes that were differentially expressed in the comparison between CM334 and NM 6-4. Each line represents the pattern of expression of a single gene. The lines are colored based on the ratio of expression at $t = 0$ between CM334 and NM6-4. Altogether 335 genes from the 10,000-element array were identified as differentially expressed in one or more of the three different experiments.

A wide range of statistical approaches is available to analyze the data generated by microarrays, including dimension reduction tools such as principal component analysis (PCA), unsupervised clustering like Hierarchical Clustering (HC) or K-means and supervised tools [35]. We have compared the results of different data analysis tools on data acquired from an interaction between *Capsicum* and *Phytophthora* [36]. The results of the PCA analysis are presented in Fig. 2; PCA allowed us to interpret global elements of the gene expression changes in the resistant and susceptible lines. Only those genes that were differentially expressed were included for PCA analysis.

For CM334, 87 genes were differentially expressed in response to *P. capsici* inoculation; the behavior of these genes was projected by PCA (Fig. 2A). Genes exhibiting high levels of the first principal component showed neutral transcription levels after 1 h of *P. capsici* infection, and they were differentially induced after 4 and

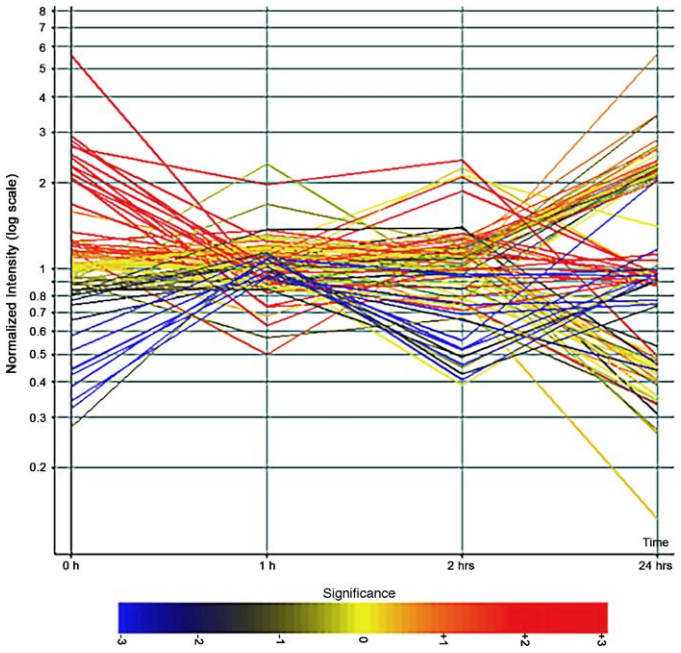

Fig. 1. CM334 vs. New Mexico 6-4 dataset, 10K element microarray. Semi-log plot of normalized ratios for transcript levels (CM334/NM 6-4) at time points 0, 1, 4 and 24, post-inoculation with *Phytophthora capsici*. Clones are colored by their significance levels at time point 0, based on the scale showed on the bottom.

24 h of infection (red line Fig. 2A). Genes that contribute to principal component two had neutral values at 1 h, highly repressed at 4 h and were close to differentially induced values after 24 h of *P. capsici* infection (light blue line). The third principal component represented genes that were highly repressed at 1 h and then neutral transcription levels at 4 and 24 h (green line).

In the susceptible line, NM6-4, 207 genes were differentially expressed in response to *P. capsici* inoculation; the behavior of this response was projected by PCA (Fig. 2B). Genes that contributed to PC1 had neutral expression levels at 1 and 4 h, while they were

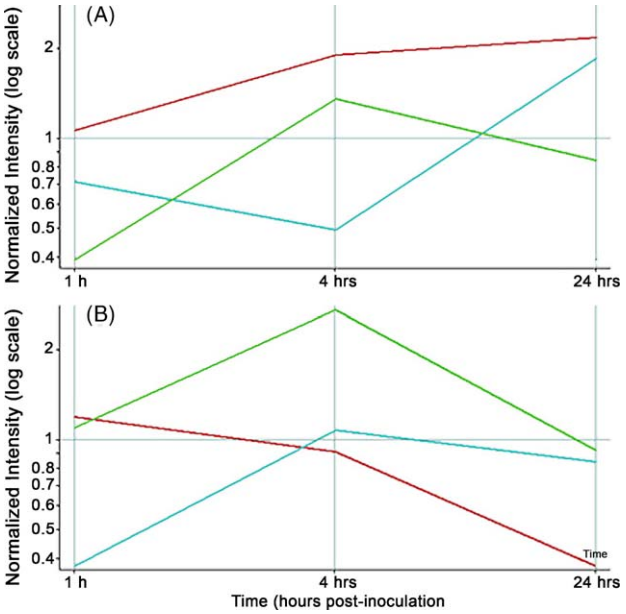

Fig. 2. Principal component analysis (PCA) of differential expression (10K element array) as a function of time post-inoculation with *P. capsici*. (A) Differentially expressed genes from CM334 samples represented in a semi-log plot of ratios at time points 1, 4 or 24 h; red (PC1), light blue (PC2) and green (PC3). (B) PCA of NM6-4 dataset.

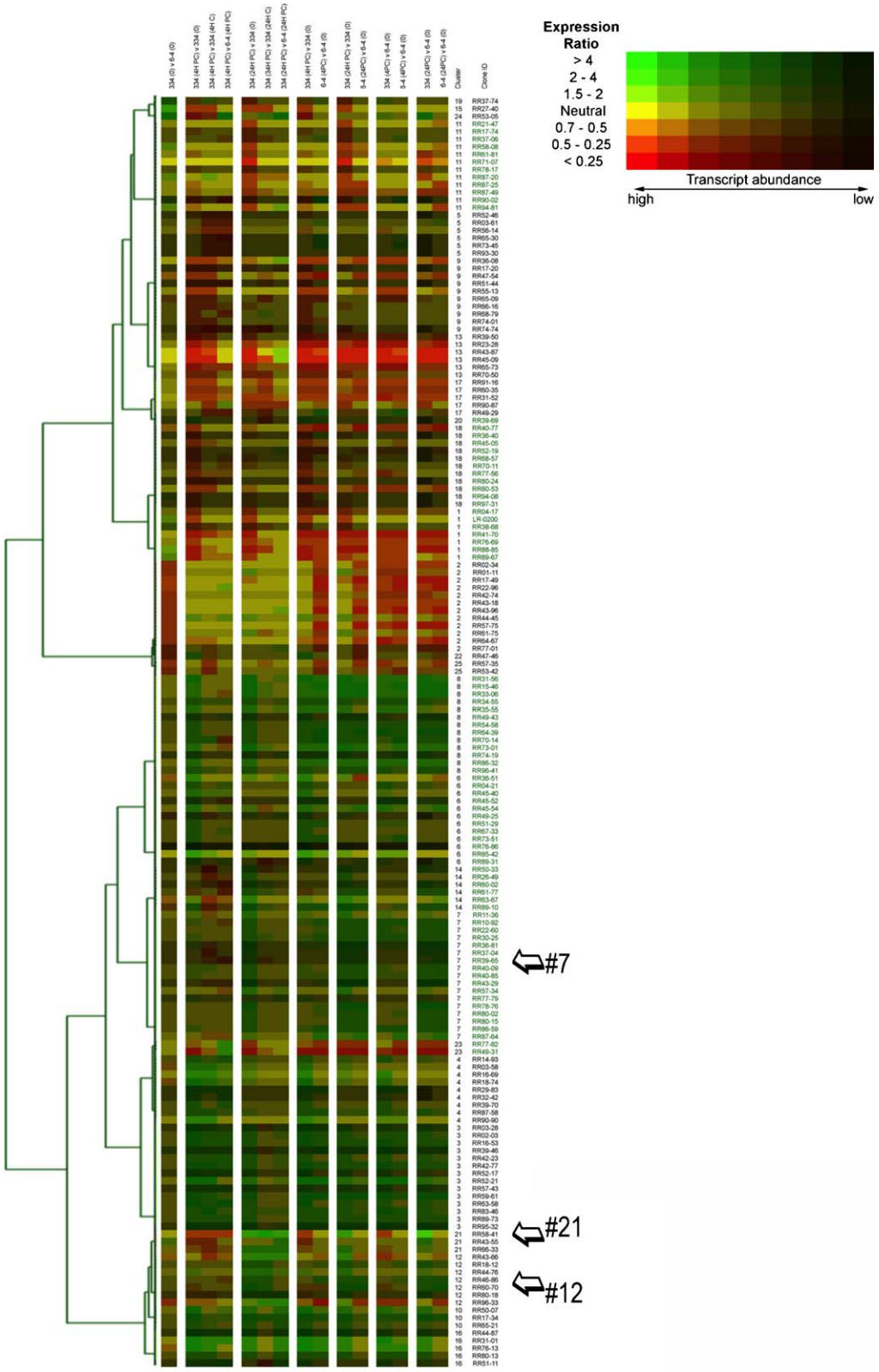

Fig. 3. K-means cluster analysis of differentially expressed genes (2K element array) in root samples from *Capsicum* lines. The expression patterns for the 168 genes (rows) in comparisons between RNA samples (columns) are presented for the following combinations (columns left to right)—col 1: CM334, 0 h vs NM64, 0 h; col 2–4: CM334, 4 h *P. capsici* vs CM334, 0 h or vs CM334, 4 h mock inoculation; or vs NM64, 4 h *P. capsici*; col 5–7: CM334, 24 h *P. capsici* vs CM334, 0 h; or vs CM334, 24 h mock inoculation; or vs NM64, 24 h *P. capsici*; col 8–9: CM334, 4 h *P. capsici* vs CM334, 0 h; NM64, 4 h *P. capsici* vs NM64, 0 h; col 10–11: CM334, 24 h *P. capsici* vs CM334, 0 h; NM64, 24 h *P. capsici* vs NM64, 0 h; col 12–13: CM334, 4 h *P. capsici*; or NM64, 4 h *P. capsici* vs NM64, 0 h; col 14–15: CM334, 24 h *P. capsici* or NM64, 24 h *P. capsici* vs NM64, 0 h. The transcript expression level and ratio for the competitive hybridization is indicated in color, with the key for the color hue and intensity presented in the upper right. The largest cluster, #7 is indicated with an arrow; two clusters #21 and #12 presented in Fig. 4 with gene function annotated are marked with arrows.

differentially repressed after 24 h (red line Fig. 2B). PC2 represented genes that were differentially repressed at 1 h and remained neutral after 4 and 24 h (blue line). PC3 represented genes whose transcription pattern was neutral at 1 and 24 h, and differentially induced at 4 h (green line).

The lines called PC1, PC2 or PC3 in either of the panels in Fig. 2 do not represent a specific gene but rather a trend in expression levels. So in CM334, there was an increase in expression at time 4 and 24 h post-inoculation, while in NM6-4 the primary distinguishing response was a reduction in expression by 24 h (red lines in Fig. 2A and B). Based on PCA, the analysis of CM334 samples showed the most significant transcription induction at time points 4 and 24, while in NM6-4 the predominant variability was by genes repressed at time point 24.

3.2. Differential gene expression in response to P. capsici challenge, 2K microarray

In order to perform a robust analysis of the root gene expression response in CM334 to *P. capsici*, a smaller array printed with more than one copy of each gene was created. All of the recombinant phage clones identified on the 10K array as responsive to *P. capsici* challenge were converted to plasmid form and their DNA sequence obtained. This set of clones was expanded to include ~two hundred clones that were not responsive to *P. capsici* challenge as well as a few hundred clones that were ever observed to have altered expression in response to *P. capsici* regardless of whether this pattern was reproduced in replicate samples. This resulted in ~1700 amplicons that were printed in duplicate on the 2K array. Prior to printing, all of these clones were rescued to plasmid forms and DNA sequence determined for each one. The GenBank EST accession numbers as well as the predicted gene function for these clones is provided in the supplemental table (Supplementary File 1). The annotation of these clones was based on their match to other sequences in GenBank with an expectation value (*E*-value) $<10^{-10}$.

Several different hybridizations were performed with the 2K array to identify those genes that were responsive to *P. capsici*. As described in Section 2, this included seven different dual labeled competitive hybridizations, performed with independent replicate RNA samples. Ratios and *p*-values were calculated for the following comparisons: CM334, 0 h vs NM64, 0 h; CM334, 4 h *P. capsici* vs CM334, 0 h; CM334, 4 h *P. capsici* vs CM334, 4 h mock inoculation; CM334, 4 h *P. capsici* vs NM64, 4 h *P. capsici*; CM334, 24 h *P. capsici* vs CM334, 0 h; CM334, 24 h *P. capsici* vs CM334, 24 h mock inoculation; CM334, 24 h *P. capsici* vs NM64, 24 h *P. capsici*. The *p*-values and averages for these ratios were calculated based on replicate spots per slide and independent replicate hybridizations; this data is provided in Supplementary File 2. Those genes that had a statistically significant differential expression in any one of these comparisons were then included in a set to be characterized in detail. This reduced the set of genes to 168 genes. It should be noted that for several of these genes, they were represented in multiple copies on the array.

The normalized ratios obtained for these genes were then used as input values for K-means clustering tool. This algorithm organized the set of 168 genes into 25 clusters (Fig. 3). The largest cluster, #7, had 17 genes; the prominent expression feature of this cluster was an increased expression of these genes in CM334 24 h post-inoculation. There were five clusters with only one gene: #15, 19, 20, 22 and 24. The upper half of the figure contains clusters whose gene expression pattern represents relative repression of expression in response to *P. capsici* challenge, as many of the cells are colored shades of red. This includes the clusters: 1, 2, 5, 9, 11, 13, 15, 17–20, 22, 24, and 25, which together account for 76 genes. The lower half of the figure represents clusters of genes whose expression pattern is induced by challenge with *P. capsici*, as many of the cells in this portion of the figure are colored shades of green. These include the clusters: 3, 4, 6–8, 10, 12, 14, 16, 21, and 23, which together account for 92 genes. A more detailed representation of two of these clusters, #12 and 21, is presented in Fig. 4. These two clusters contain genes whose expression level is induced in CM334 at 24 h post-inoculation with modest or no induction in NM6-4 by comparison. Inspection of the green intensity in the set of columns (5–7) comparing CM334 24 h PC vs CM334 0 h, or vs CM334 24 h mock inoculation, or vs NM6-4 24 h PC, indicates this pattern of induction.

3.3. Functional characterization of P. capsici responsive genes

All of the responsive genes on the 2K array, detected as significantly induced or repressed in comparison of 24 and 4 h time points, were classified into one of 16 functional groups based on the predicted protein sequence of their DNA sequence. The

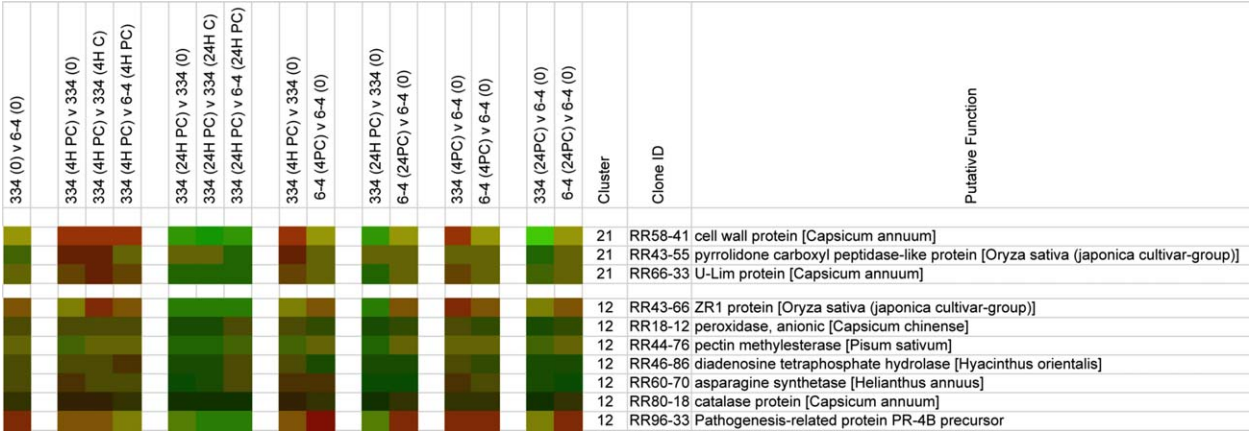

Fig. 4. Expression patterns of two clusters, 12 and 21, organized by K-means cluster analysis. The expression patterns for the 10 genes (rows) in clusters 12 and 21 are presented. Column headings indicate comparisons between RNA samples (columns left to right): col 1: CM334, 0 h vs NM64, 0 h; col 2–4: CM334, 4 h *P. capsici* vs CM334, 0 h or vs CM334, 4 h mock inoculation; or vs NM64, 4 h *P. capsici*; col 5–7: CM334, 24 h *P. capsici* vs CM334, 0 h; or vs CM334, 24 h mock inoculation; or vs NM64, 24 h *P. capsici*; col 8–9: CM334, 4 h *P. capsici* vs CM334, 0 h; NM64, 4 h *P. capsici* vs NM64 0 h; col 10–11: CM334, 24 h *P. capsici* vs CM334, 0 h; NM64, 24 h *P. capsici* vs NM64, 0 h; col 12–13: CM334, 4 h *P. capsici*; or NM64, 4 h *P. capsici* vs NM64, 0 h; col 14–15: CM334, 24 h *P. capsici* or NM64, 24 h *P. capsici* vs NM64, 0 h. The cluster number, the clone ID and the putative function for the gene based on BLAST similarity are presented in the last three columns. The key for the color code representation for transcript expression level and ratio is presented in Fig. 3.

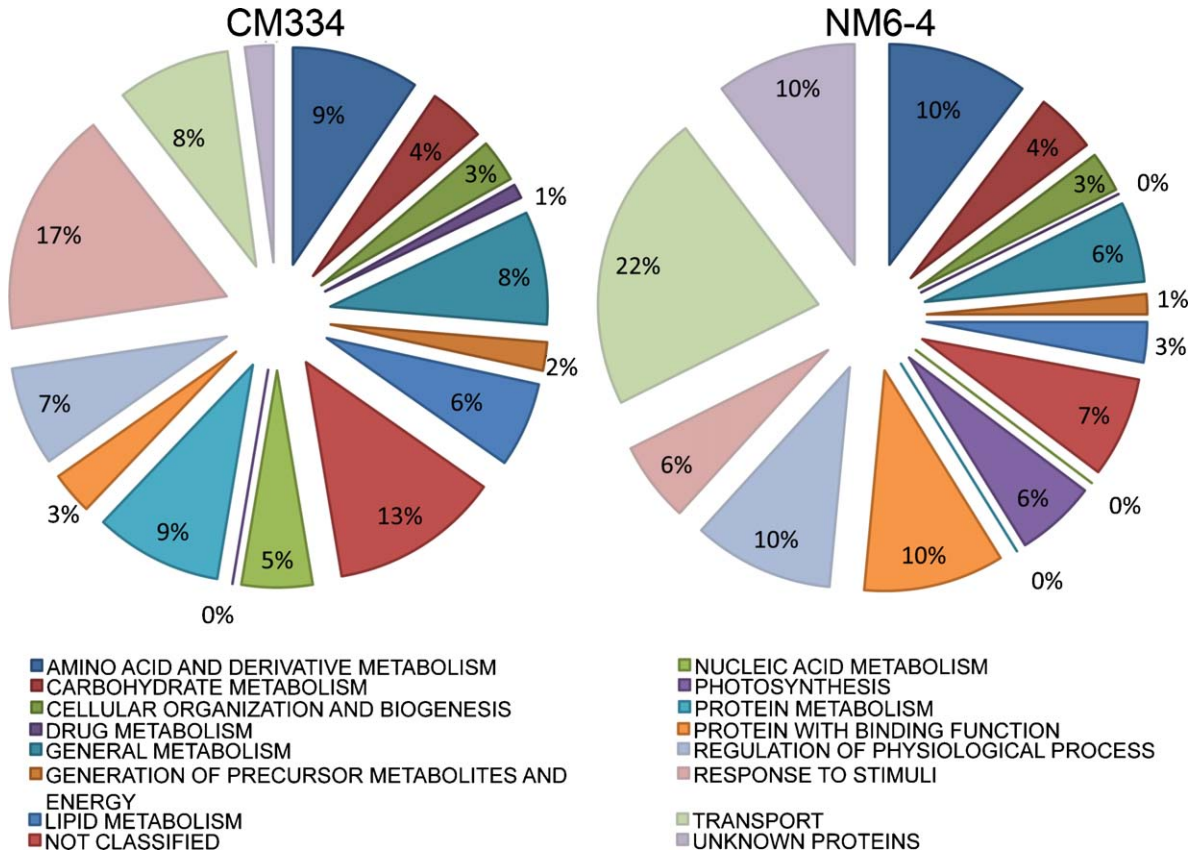

Fig. 5. Functional classification of genes responsive to *P. capsici* inoculation in CM334 and NM6-4. Genes responsive to *P. capsici* at 24 h vs 4 h post-inoculation in CM334 (95 genes) and in NM6-4 (68 genes) were assigned to one of 16 functional classes, listed at the bottom of the panel. The percent of total responsive genes assigned to each class is presented in the pie charts for the indicated *Capsicum* lines.

distribution of these genes into these functional groups is compared for CM334 (95 genes) and NM6-4 (68 genes) in Fig. 5. There were a number of differences between CM334 and NM6-4 in gene population sizes for many of these clusters. For CM334 the functional class with the most gene members was "response to stimuli" with 17% of the responsive genes; for NM6-4 the largest functional class was "transport" with 22%. There were no genes in the class "photosynthesis" for CM334 while this class had 6% of the responsive genes for NM6-4. There were three classes with no responsive genes in NM6-4, "drug metabolism", "nucleic acid metabolism" and "protein metabolism"; these classes had 1, 5 and 9% respectively of the responsive genes in CM334.

3.4. Northern blot confirmation of selected RNA expression patterns

Four clones (RR03-61, RR58-41, RR90-87, and RR80-18) were used as probes on northern blots (Fig. 6). RR58-41, encodes a cell wall protein, is abundantly expressed in *P. capsici* challenged roots of CM334 but not in NM6-4 according to microarray analyses (Table 1). This pattern of expression is confirmed in the northern blot. RR90-87, encodes a tonoplast intrinsic protein predicted by microarray analysis to be induced in NM6-4 in response to *P. capsici*, but not induced in CM334 (Table 2). This pattern of expression was also confirmed by northern blot. RR03-61, encodes an expansin-like protein, expected to be non-responsive in both lines at 4 and 24 h post-inoculation, and this pattern of expression was also observed in the northern blot. RR80-18 encodes catalase, predicted to be slightly induced in both lines (Table 3), and that pattern of expression was also observed by northern blot analysis. These four genes with different disease response patterns determined by microarray analysis were all observed to have the expected qualitative pattern of expression when assayed by northern blot analysis.

3.5. Distinction between P. capsici inoculation responsive genes and wounding responsive genes

RNA samples were collected from roots at 4 and 24 h after a mock inoculation of CM334. The genes that appear differentially expressed in these samples were identified. This data is presented in Supplementary File 2. Only one gene listed in Table 1, RR57-34, the universal stress protein, is also wound inducible in CM334. Two that are listed in Table 2, RR96-41 and RR94-81 are wound inducible in CM334. Seven of the genes listed in Table 3 are also wound inducible in CM334: RR43-29, RR61-77, RR60-02, RR78-76, RR39-65, RR37-04, RR80-02 and RR58-08. The fact that a number of wound responsive genes were detected in the set of genes commonly responsive to *P. capsici* in CM334, NM6-4 and 01-1688 (Table 3) is reasonable. The observation that only one candidate for a wound responsive gene was identified among the set uniquely expressed in the disease resistant genotypes (Table 1) indicates that these genes are very likely associated with the phenotype of *P. capsici* disease resistance.

3.6. Genetic confirmation of disease resistant gene expression profiles

C. annuum line, 01-1688, a backcross line between CM334 and a susceptible line, Early Jalapeno, had *P. capsici* root rot resistance equivalent to CM334 (data not shown). The 2K element array was screened using RNA samples isolated from roots of 01-1688 at 4 and 24 h post-inoculation with *P. capsici*. The average ratios and associated *p*-values for those hybridizations are presented in

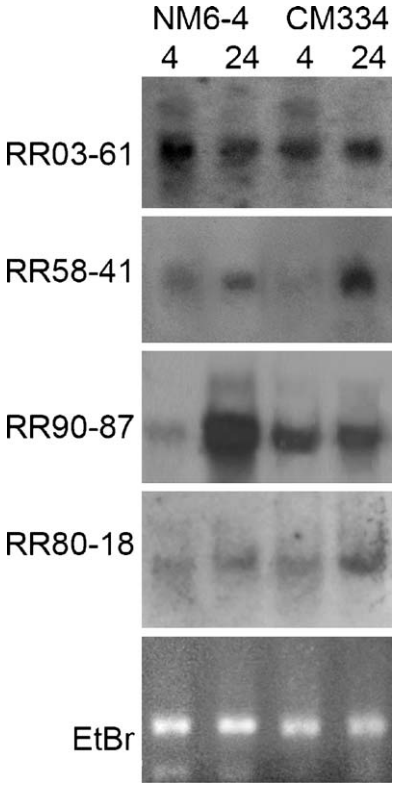

Fig. 6. Northern blot analysis of transcript levels in roots of *Capsicum* lines. RNA samples from roots of CM334 or NM6-4 plants at 4 or 24 h post-inoculation with *P. capsici* were used to prepare northern blots. The blots were hybridized with the indicated ^{32}P-labeled probes for: RR90-87, tonoplast intrinsic protein; RR58-41, cell wall protein; RR03-61, expansin-like protein. The lower panel is a typical ethidium bromide stained image of the gel, demonstrating equivalent RNA loads in each lane.

Supplementary File 3. Those genes that were differentially expressed in response to *P. capsici* in 01-1688 were identified and compared with the genes differentially expressed in response to *P. capsici* in CM334 and NM6-4. This comparison is represented in a Venn diagram in Fig. 7. Eighty genes were differentially expressed at these two time points in 01-1688 roots in response to *P. capsici*. Of those genes, 54 were also expressed in response to *P. capsici* in CM334. Within this set, 32 genes were expressed by all three genotypes, NM6-4, CM334 and 01-1688, in response to *P. capsici*. Twenty-two genes were identified as uniquely expressed in roots of the two resistant lines, CM334 and 01-1688. Lists of these genes, their differential expression level and predicted annotation are provided in Tables 1–3. Among the 22 genes associated with root rot resistance, transcript levels for 19 genes appeared to be induced in response to *P. capsici* challenge while transcription of 3 genes was repressed (Table 1).

Plots of the differential pattern of expression among the three *Capsicum* lines is presented in Fig. 8 for three genes whose pattern of expression is associated with disease resistance, RR66-33, RR43-66, and RR58-41. Increased expression of these three genes is associated with *P. capsici* root rot resistance. For each of these genes, the expression levels at 0 and 4 h were similar among the three genotypes; the main distinction was the marked increase in transcript levels at 24 h in the two resistant genotypes, CM334 and 01-1688. There was a reduction in transcript levels at 4 h in both resistant and susceptible genotypes. However, the resistant genotypes increased transcript accumulation, many fold by 24 h, while transcript levels in NM6-4 remained constant from 4 to 24 h. A similar pattern of expression was observed among a number of other genes, listed in Table 1.

Table 1
Genes responsive to *Phytophthora capsici* challenge only in resistant genotypes CM334 and 01-1688 but not in susceptible genotype, NM6-4. Transcript expression ratios for significant differential expression in response to *P. capsici* inoculation (24 h/4 h ratios, ≤0.55 or ≥1.90 with *p*-values <0.06). Genes are ranked in order of expression ratio in CM334.

Clone ID	Predicted annotation	24 h/4 h		
		CM334	01-1688	NM6-4
Induced in CM334 and 1688				
RR58-41	Cell wall protein	8.57	5.11	0.98
RR68-79	Unknown protein	7.04	3.97	1.84
RR66-33	U-Lim protein	6.80	4.22	1.71
RR57-34	Universal stress protein	5.21	8.67	1.88
RR43-66	ZR1 protein	4.79	3.74	1.10
RR43-55	Pyrrolidone carboxyl peptidase-like protein	3.82	2.30	1.25
RR77-56	Starvation-induced protein	3.75	3.85	1.76
RR80-53	Cyprosin protein	3.49	3.40	1.84
RR89-67	Major latex-like protein	3.33	6.50	1.60
RR18-12	Peroxidase, anionic	3.19	2.06	1.57
RR74-01	Vacuolar processing enzyme-3	3.09	2.89	1.75
RR36-08	Unknown protein	2.77	1.91	1.17
RR90-02	Unknown protein	2.74	4.29	0.32
RR41-70	Invertase inhibitor protein	2.59	4.86	1.08
RR70-50	Unknown protein	2.46	2.11	1.59
RR23-28	Auxin-repressed protein	2.31	3.87	1.39
RR31-52	Major intrinsic protein 2	2.15	2.60	1.87
RR38-68	AKIN gamma	2.13	2.75	1.11
RR40-85	Cytidine deaminase protein	2.05	2.22	1.65
Repressed in CM334 and 1688				
RR42-77	Cytochrome P450 protein	0.60	0.54	0.82
RR39-70	Pectinacetylesterase precursor protein	0.57	0.38	1.25
RR16-53	Membrane protein	0.46	0.34	1.20

Table 2
Genes responsive to *P. capsici* challenge only in susceptible genotype, NM6-4 but not in resistant genotypes, CM334 or 01-1688. Transcript expression ratios for significant differential expression in response to *P. capsici* inoculation (24 h/4 h ratios, ≤0.55 or ≥1.90 with *p*-values <0.06). Genes are ranked in order of expression ratio in CM334.

Clone ID	Predicted annotation	24 h/4 h		
		CM334	01-1688	NM6-4
Induced in NM6-4				
RR90-87	Tonoplast intrinsic protein bobTIP26-2	0.67	1.46	3.41
RR57-43	Double WRKY type transfactor	1.49	0.56	3.04
RR89-73	Harpin inducing protein	0.92	1.82	2.71
RR63-58	Pleiotropic drug resistance like protein	0.76	0.64	2.43
RR51-11	Glutathione S-transferase T1 protein	1.40	1.75	2.36
RR89-31	Unknown protein	0.83	0.58	2.36
RR96-41	SIEP1L protein	1.68	1.45	2.17
RR95-32	Lipoxygenase	1.56	0.50	1.95
Repressed in NM6-4				
RR94-81	Glutathione S-transferase	0.83	1.31	0.51
RR76-13	Omega-6 fatty acid desaturase	0.70	0.96	0.42

Table 3
Genes responsive to *P. capsici* challenge in all three *Capsicum* lines: NM6-4, CM334, and 01-1688. Transcript expression ratios for significant differential expression in response to *P. capsici* inoculation (24 h/4 h ratios, ≤0.55 or ≥1.90 with *p*-values <0.06). Genes are ranked in order of expression ratio in CM334.

Clone ID	Predicted annotation	24 h/4 h		
		CM334	01-1688	NM6-4
Induced				
RR60-70	Asparagine synthetase	26.38	3.28	15.95
RR96-33	Pathogenesis-related protein PR-4B precursor	17.34	9.66	2.34
RR43-29	CPRD2	13.69	4.04	8.17
RR61-77	Phosphatase protein	13.00	6.73	7.45
RR60-02	Unknown protein	10.88	4.38	3.69
RR53-05	Photosystem II oxygen-evolving complex protein 3	9.73	2.19	2.42
RR78-76	Vacuolar H+-ATPase A1 subunit isoform protein	9.63	5.46	11.18
RR39-65	Sodium-dicarboxylate cotransporter protein	7.94	6.55	4.41
RR37-04	O-succinylhomoserine sulfhydrylase protein	7.39	3.09	4.49
RR52-19	Catalase protein	7.29	2.54	2.49
RR65-73	O-linked GlcNAc transferase protein	6.23	4.61	3.56
RR52-46	Elongation factor EF-2	5.78	2.96	2.30
RR80-24	Unknown protein	5.70	2.05	3.04
RR97-31	Ribosomal protein L10a	5.37	3.90	3.08
RR26-49	60S ribosomal protein L1 protein	4.81	1.92	2.20
RR65-09	Acyl-transferase	4.72	7.37	4.18
RR80-02	Xyloglucanase inhibitor protein	4.28	2.15	3.01
RR39-50	Glycogen (starch) synthase	3.90	2.28	2.77
RR80-18	Catalase protein	3.84	2.30	1.99
RR45-05	Vacuolar processing enzyme-3	3.29	4.20	3.28
RR47-54	Histone H1, drought-inducible	3.17	5.17	2.66
RR55-13	Unknown protein	2.49	3.68	2.08
RR01-11	Unknown protein	2.43	1.91	2.07
RR40-77	Beta-tubulin protein	1.80	2.02	1.12
Repressed				
RR49-29	ADH-like UDP-glucose dehydrogenase	0.62	0.23	0.17
RR58-08	MG02641.4	0.61	0.51	0.60
RR16-69	Cinnamic acid 4-hydroxylase protein	0.58	0.30	0.68
RR90-90	Cytosolic acetoacetyl-coenzyme A thiolase	0.53	0.43	0.72
RR31-01	Aldehyde decarbonylase	0.53	0.55	0.52
RR52-21	Tyramine n-hydroxycinnamoyl transferase	0.50	0.46	0.45
RR18-74	Phenylalanine ammonia-lyase protein	0.50	0.31	0.71
RR29-83	Carbonic anhydrase protein	0.26	0.39	0.70

4. Discussion

An especially strong disease resistance source against *P. capsici* root rot, leaf blight, stem blight and fruit rot is found in the landrace *C. annuum*, CM334 [5]. CM334 carries a number of *P. capsici* disease resistances and is resistant to all of the described races of *P. capsici* [37]. Unfortunately, no cultivars have been released that carry the *Phytophthora* disease resistances found in CM334; possibly due in part to the quantitative nature of the resistance trait [10,11]. This study was undertaken to identify any gene expression changes that differentiated resistant and susceptible *C. annuum* responses to *P. capsici*. These gene expression changes might correlate with key genetic elements important in the inheritance of the disease resistance phenotype; these key elements could be used in marker-assisted selection breeding programs. Further, a detailed description of gene expression changes uniquely associated with the resistant phenotype would allow an understanding of the molecular basis of the phenotype that might support breeding efforts to introgress the trait into elite cultivars.

There were a number of genes whose transcript levels significantly increased or decreased in response to *P. capsici* inoculation; Tables 1–3 list these genes grouped as to whether the alteration in expression was unique to a resistant phenotype, unique to a susceptible phenotype, or common to both phenotypes. The genes identified in Table 1 (responsive only in resistant lines) are likely sources of molecular markers for plant breeding purposes. The alleles for these genes from CM334 are presumably distinct in either their coding or promoter regions such that DNA sequence-based markers could be developed. The 22 genes listed in Table 1 are candidate molecular markers for the root rot resistance phenotype. There were four genes on this list for which there was no predicted biochemical function, as they did not match any well annotated genes in a public database. It will be interesting to determine possible functions for these sequences.

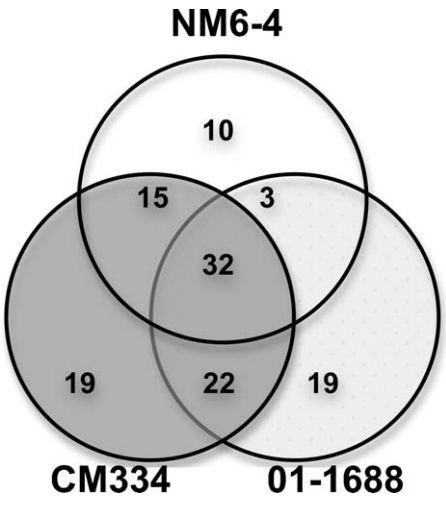

Fig. 7. Venn diagram representation of differential gene expression among three *Capsicum* lines differing in resistance to *P. capsici*. The set of genes identified by microarray analysis to be differentially expressed in response to *P. capsici* in the either the resistant *Capsicum* genotypes, CM334 or 01-1688, or the susceptible line NM6-4 are plotted to reflect their common pattern of differential expression. The list of 22 genes expressed uniquely in the resistant lines in response to *P. capsici* challenge is presented in Table 1; the list of ten genes uniquely expressed in NM6-4 is presented in Table 2; and the list of 32 genes expressed in all three lines in response to *P. capsici* is presented in Table 3.

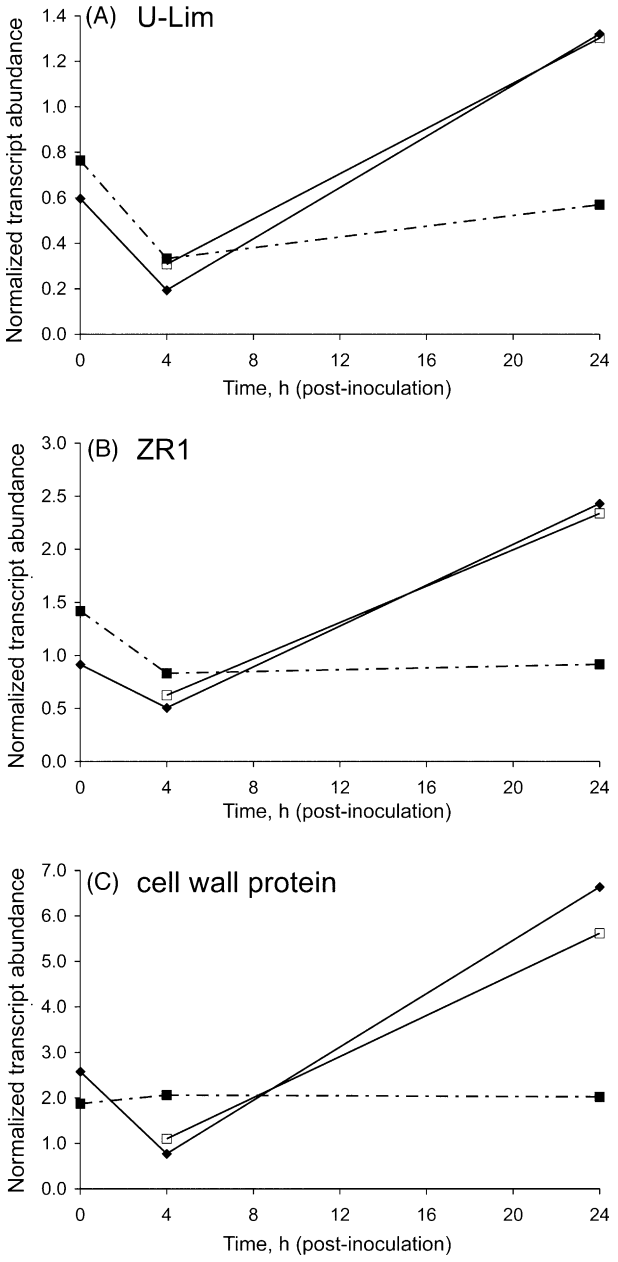

Fig. 8. Time course for expression of three genes in roots of resistant and susceptible *Capsicum* lines. RNA samples from roots of CM334 (solid line, solid box); 01-1688 (solid line, open box); NM6-4 (dashed line, solid box) were collected at 0, 4, or 24 h post-inoculation with *P. capsici*. The normalized transcript abundance detected by microarray analysis for: (A) RR66-33, U-Lim protein; (B) RR43-66, ZR1 protein; (C) RR58-41, cell wall protein.

Only 10 genes were identified as disease responsive uniquely in the susceptible line (Table 2). Further, there was only a modest induction, two- to threefold, for these genes. The microarray was printed with genes from a cDNA library of transcripts from roots of CM334 6 h after *P. capsici* inoculation. Therefore, genes abundantly expressed in a susceptible line might not be present in the set of genes printed on this array.

The largest set of genes (Table 3) listed those identified as disease responsive in both resistant and susceptible phenotypes: 24 induced and 8 repressed genes. Further, this set included the genes with the greatest changes in gene expression, as high as 26-fold increase in expression for asparagine synthetase in CM334 with a similar ~16-fold increase in NM6-4. This list indicated that the resistant and susceptible lines had very similar gene expression profiles for many genes in response to *Phytophthora* challenge.

Transcription of a gene for a cell wall protein was uniquely induced in the resistant *Capsicum* lines in response to *Phytophthora* challenge. The annotation for this gene as a cell wall protein was based on a very high sequence similarity (96%) with a cell wall protein gene induced in *C. annuum* (GenBank AF242730) during the hypersensitive response to tobacco mosaic virus (TMV) [38]. Transcription of the cell wall protein gene was monitored using northern blots of leaf tissue from a *C. annuum* line inoculated with two different TMV pathotypes, one virulent and the other avirulent on the VK-1 host plant. Increased transcription of the cell wall protein is observed only in the hypersensitive response. Transcription of this gene product is associated then with heritable forms of two different disease resistances, TMV and *Phytophthora* in two different organs, leaf and root in *Capsicum*.

Cell wall proteins have been reviewed as components of plant disease resistances against many types of plant pathogens [39]. The roles of this class of proteins include both detection and recognition of the pathogen as well as defense against the pathogen via crosslinking, peroxidation or other chemical remodeling. The role of the *Capsicum* cell wall protein (RR58-41) that appears to be uniquely expressed in the resistant lines in response to pathogen challenge is not known. But this is clearly an abundantly expressed gene whose pattern of expression distinguishes resistant and susceptible lines. Further work on this gene may provide information on the mechanism of *Phytophthora* root rot resistance in *C. annuum* Criollo de Morelos-334.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2009.11.005.

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