

# Monitoring the expression patterns of potato genes associated with quantitative resistance to late blight during *Phytophthora infestans* infection using cDNA microarrays

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## Abstract

To elucidate the molecular events of potato quantitative resistance to *Phytophthora infestans*, we performed a comprehensive transcriptional analysis using cDNA microarrays, containing 1009 ESTs from a subtractive library. Leaves of a moderately resistant potato clone were inoculated with *P. infestans* and sampled at nine time points ranging from 2 to 72 h after inoculation. A total of 348 *P. infestans*-responsive genes were identified. These functional genes are mostly related to metabolism, plant defense, signaling and transcription regulation, involving the whole process of plant defense response to pathogens. Based on the general expression patterns of these genes at different time points, we discriminated distinct stages of potato defense against *P. infestans* and revealed genes participating in each stage. To further understand the dynamics of *P. infestans*-induced gene expression, hierarchical clustering was used to illustrate their various expression profiles during the time course, including early, mid and late gene induction as well as early gene repression. Interestingly, some genes involved in the hypersensitive response were also identified, suggesting that a same or similar defense system may exist in both race-specific and race-nonspecific resistances. In addition, 114 novel genes with unknown functions were isolated.

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**Keywords:** cDNA microarray; Expression patterns; Potato; *Phytophthora infestans*; Quantitative resistance

## 1. Introduction

Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is one of the world's most destructive plant diseases and was responsible for epidemics that resulted in the European potato famine in 1845. A century and a half of research has failed to subdue the highly adaptable organism, which has acquired new traits that make it more threatening than ever and virulent, fungicide-resistant strains have appeared throughout the world [1]. Although chemicals targeted against *P. infestans* provide

some level of disease control, worldwide losses due to late blight and measures for its control are estimated to exceed US\$ 5 billion annually [2]. Consequently, it is a major pathway to improve the genetic resistance to *P. infestans* in breeding new cultivars of potato.

Traditionally, genetic resistance of potato against late blight is classified into two different types: the qualitative (race-specific) and quantitative (race-nonspecific) resistance. Qualitative resistance is mediated by R genes that lead to a race-specific hypersensitive response. Generally speaking, these R genes only provide short-lived resistance in the field as new virulent races of the pathogen rapidly overcome the resistance encoded by single race-specific resistance genes [3]. In contrast, quantitative resistance is controlled by many interacting genes that do not prevent infection, but slow down the development of the pathogen at individual infection sites on the plant, and hence, lasts longer [4]. Up to

**Abbreviations:** EST, expressed sequence tag; hpi, hours post-inoculation; HR, hypersensitive response; PR, pathogenesis-related

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now, a number of quantitative trait loci (QTLs) for late blight have been mapped in many experimental populations of potato, which are identified on most of the 12 potato chromosomes [5], but the molecular basis of this phenomena is poorly understood. In addition, studies indicate that the HR plays a crucial role in all forms of resistance, including race-specific, race-nonspecific and non-host resistances [6,7]. As a result, the traditional description of qualitative and quantitative resistance has been challenged.

When potato and *P. infestans* come into contact, a complex and dynamic communication occurs between the two organisms; this activates the defense mechanism of potato and many specific genes may be induced. Several studies have demonstrated that the challenge with *P. infestans* leads to transcriptional activation of specific genes in potato [8–11]. Furthermore, the activation of potato defense responses by *P. infestans* causes the differential expression of numerous genes. A comprehensive analysis of differentially expressed genes could contribute to a better understanding of the molecular processes involved in the plant–pathogen interactions. Therefore, a powerful method, suppression subtractive hybridization (SSH), has been used to construct cDNA libraries enriched for genes that were up-regulated in the compatible or incompatible interaction between potato and *P. infestans* [12–15]. However, progress in understanding the molecular mechanism involved in potato late blight resistance is still limited, and very few studies have been performed on the dynamics of *P. infestans*-induced gene expression on a large scale. The emerging technology of cDNA microarray hybridization offers the possibility of providing a rapid, high-throughput method to screen the SSH library for identifying differentially expressed genes [16]. Although this technique has been used to examine various stress or defense responses such as chemical treatments [17], wounding [18] and pathogen infection [19], very little information is available for that of potato–*P. infestans* interaction.

Based on the SSH cDNA library containing 1009 differentially regulated expressed sequence tags (ESTs) associated with quantitative resistance to *P. infestans* [14], we performed a comprehensive transcriptional analysis at nine time points with the cDNA microarray technique to elucidate, to some extent, the expression patterns of genes related to potato quantitative resistance to late blight during *P. infestans* infection. Thus, our study will provide an effective platform for further investigation of potato defense process against *P. infestans*. The possible involvement of the identified genes in potato response to the pathogen is discussed.

## 2. Materials and methods

### 2.1. Plant materials and pathogen inoculations

The potato clone, 386209.10, was kindly provided by the International Potato Center (CIP), and it has a field

resistance rating of 5 according to the CIP 9-scale late blight resistance criterion and does not carry any of the *Solanum demissum* R-genes, *R1–R11*. Potato plants were grown from tubers in 20 cm × 25 cm plastic pots containing a sterile mixture of soil:peat-based compost (1:1, v/v) in a greenhouse under an average of 14-h day length, with a mean temperature of 20 °C by day and 15 °C by night. Six-week-old potato plants were utilized for experiments, and the fourth and fifth fully expanded leaves, beginning from the youngest leaf on each plant, were used to inoculate as described previously [8,11] with some modifications. Briefly, leaf-petiole cuttings of potato were inoculated from the abaxial side by spraying with a suspension of freshly isolated zoospores at  $2 \times 10^5$ /mL, which was a mixture of the *P. infestans* races 1, 3, 4, and 1.3, whereas each isolate contributed equally to the mixture. Control samples were sprayed with water. Subsequently, the leaf-petiole cuttings were incubated in plastic trays containing wet filter paper and covered with vinyl bags to maintain high humidity. The trays were placed in a growth chamber at 18 °C under a 16-h photoperiod. Leaf samples were harvested at 2, 4, 6, 8, 12, 24, 36, 48, and 72 h after inoculation, and then snap-frozen in liquid nitrogen, and stored at –80 °C until RNA extraction. Corresponding control leaves were also harvested at the same time. A number of treated and control leaves were kept for 4–5 days to confirm that the infection had been successful.

### 2.2. cDNA microarray construction

The ESTs used for cDNA microarray construction are derived from the SSH cDNA library constructed by Tian et al. [14]. In short, total RNA was extracted from leaves at 48 hpi with *P. infestans* (tester) and uninfected leaves (driver) of potato clone, 386209.10. The synthesis of double-strand cDNA and subsequent suppression subtractive hybridization was performed using the Clontech PCR Select cDNA Subtraction Kit (Clontech, USA) according to the manufacturer's instructions. The resulting PCR products were inserted into pBluescript M13 SK (+) plasmid. The ligation mixture was then transformed into *Escherichia coli* and cultured on a LB media plate containing ampicillin and X-Gal/IPTG. White clones were selected to generate a subtractive library. Excluding the two-banded and short-fragment clones, a total of 1009 ones were selected. All these ESTs were PCR amplified in 50 µL reaction mixture containing 1.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 0.4 µM of each primer (T3 and T7), 1 × reaction buffer, 2.5 U Taq DNA polymerase (Takara, Japan), and 2 µL bacterial culture template. PCR was performed as follows: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min followed by 72 °C for 5 min. The PCR products were purified on Multiscreen filter plates (Millipore Corp. Bedford, MA). In addition, the potato *POTHR-1* and *NtPRp27*-like genes that were responsive to *P. infestans* [20,21] were applied to positive controls. Two DNAs derived from the human

transferrin receptor (TFR) gene (DTX806; Takara, Dalian, China) and pUC19 (D3219; Takara, Dalian, China) were used as negative controls to assess nonspecific hybridization. The potato genes encoding actin [22],  $\beta$ -tubulin [23], aconitase/aconitate hydratase (GenBank accession no. X97012), hexokinase (GenBank accession no. X94302) and ribulosebiphosphate carboxylase (GenBank accession no. M76402) were used as internal controls whose expression levels were stable and invariant in our experimental conditions. As an external control, a PCR-amplified fragment from the  $\lambda$  control template DNA fragment (DTX803; Takara, Dalian, China) was applied to equalize hybridization signals generated from different samples [24]. Construction and use of microarrays were performed in compliance with the minimum information about a microarray experiment (MIAME) standards [25]. The microarray slides were printed at Takara Biotechnology (Dalian) Co., Ltd. (Takara, Dalian, China). All these purified ESTs and control cDNA clones were resuspended into 50% dimethyl sulfoxide (DMSO) and 50% MiliQ water to give a final concentration of 0.1  $\mu\text{g}/\mu\text{L}$ , then arrayed on the glass slides (DTX704; Takara, Dalian, China) by an Array Spotter Generation III (Amersham Pharmacia Biotech, Uppsala, Sweden). The ESTs were spotted in duplicate and control cDNA clones were printed 48 times randomly in different locations on each slide. After printing, cDNA was UV cross-linked to the slides (60  $\text{mJ}/\text{cm}^2$ ) and stored in a light-tight box at room temperature until use.

### 2.3. RNA preparation and fluorescent labeling of probes

Total RNA was extracted from inoculated and control potato leaves as described previously [26]. At least two independent biological replicates were applied in each of the nine experiments performed at nine different time points. For each replicate, all leaves from six leaf-petiole cuttings were collected to use for independent RNA preparation and labeling reactions. Each labeling reaction product was used in separate array hybridizations. Total RNA was applied to synthesize Cy3- or Cy5-conjugated dUTP-labeled cDNA probe by using the RNA Fluorescence Labeling Core Kit (M-MLV Version) Ver. 2.0 (Takara, Dalian, China) and following the manufacturer's instructions. The successfully labeled cDNA sample pairs (one with Cy3 and the other with Cy5) were combined and precipitated with ethanol. Pellets were dissolved in 25  $\mu\text{L}$  hybridization buffer (6  $\times$  SSC, 0.2% SDS, 5  $\times$  Denhardt solution, and 0.1  $\text{mg}/\text{mL}$  denatured salmon sperm DNA).

### 2.4. Microarray hybridization, washing, and scanning

The ready probe solutions were denatured for 2 min at 95  $^{\circ}\text{C}$ , left at room temperature for 5 min, and then used for hybridization. These solutions were placed onto the center of the array. A coverslip was placed over the entire array surface to avoid the formation of bubbles. The slides were placed in a sealed hybridization cassette (Takara, Dalian, China) and

submerged in a 65  $^{\circ}\text{C}$  water bath for 12–16 h. After hybridization, slides were washed at 55  $^{\circ}\text{C}$  in 2  $\times$  SSC/0.2% SDS for 5 min, then in 0.1  $\times$  SSC/0.2% SDS for 5 min, and finally in 0.05  $\times$  SSC for 5 min. The slides were immediately centrifuged to dry (2 min at 2500  $\times$  g). Hybridized microarray slides were scanned for Cy3 at 532 nm and Cy5 at 635 nm with an Affymetrix 428<sup>TM</sup> Array Scanner (Affymetrix, Santa Clara, CA, USA). Two separate TIFF images were generated for each channel. The hybridization, scanning, and following data extraction were done at Takara Biotechnology (Dalian) Co., Ltd. (Takara, Dalian, China).

### 2.5. Data analysis

Image analysis and signal quantification were performed with ImaGene<sup>TM</sup> 4.2 software (BioDiscovery, San Francisco, USA). Briefly, grids were predefined and manually adjusted to ensure optimal spot recognition, discarding spots with dust or locally high background. On the basis of measurement quality parameters produced by the image analysis software, spots were individually quantified by using the ImaGene fixed circle method. The sample signal value was measured with the mean of pixels within a circle surrounding the spot, and the background signal value was calculated on the basis of the fluorescence intensity of the negative control genes. All the poor-quality spots were removed from the analysis and spots with signal intensity lower than the background plus 2 standard deviations (S.D.s) were also excluded. To remove as many systematic errors as possible, two different methods (housekeeping genes and external control) were used to compute the normalization factor to equalize hybridization signals generated from different samples. For the final analysis, average ratios and standard deviations were calculated for at least three replicates. The differentially expressed genes were chosen by pair-wise comparison of the inoculated and control samples from the same time point. Based on the report as described by Yang et al. [27] and our own self-self hybridization experiment, we selected a two-fold threshold as the criteria of differentially expressed genes, i.e., genes with ratios, after subtraction of one unit of standard deviation, equal to or more than 2.0 were selected. Furthermore, we found that the average coefficient of variation (CV) of average ratios for all identified genes at each time point is less than 10%. Thus, to increase reliability, only those genes altered 2.2-fold at least at one time point were accepted as differentially regulated by *P. infestans* and clustered using a self-organizing maps algorithm followed by average linkage hierarchical clustering [28]. In addition, identified genes were also categorized according to their functions as described below.

### 2.6. EST sequencing and analysis

The DNA sequencing was completed at Shanghai GeneCore Biotechnologies Co., Ltd. (Shanghai, China).

In brief, alkaline lysis of plasmid preparations was followed by phenol–chloroform extraction and ethanol preparation to yield template plasmid DNA for automated sequencing. The cDNA inserts in the pBluescript II M13(+)-SK vectors were sequenced on the ABI Prism 3700 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with T3 or T7 primer. Each sequence was edited to correct sequencing ambiguities and remove the vector and adaptor sequences. The edited sequences were analyzed using the BLAST programs at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). ESTs showing significant sequence similarity with an  $E$ -value  $\leq 1e-10$  at their entire length were considered highly homologous, whereas ESTs with  $E$ -value  $> 1e-10$  were considered not statistically significant (no similarities found) and were assumed to be novel. Subsequently, the selected ESTs were assigned to different functional groups based on the information gathered from the MIPS *Arabidopsis* database (<http://mips.gsf.de/proj/thal/index.html>) and the Gene Ontology website (<http://www.geneontology.org>).

### 2.7. RT-PCR analysis

Validation of selected genes was performed using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA (10  $\mu$ g) from each time-point sample as described above was reverse transcribed using Powerscript<sup>TM</sup> Reverse Transcriptase (Clontech Laboratories Inc., Palo Alto, CA, USA) according to the manufacturer's instructions. A 10-fold dilution of the reaction product served as a template and was used for each RT-PCR, which was performed in a total volume of 20  $\mu$ L containing 1.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 0.4  $\mu$ M of each primer, 1  $\times$  reaction buffer, 1 U *Taq* DNA polymerase (Takara, Japan), and 1.5  $\mu$ L template. The cycling parameters of PCR amplification were as follows: the reaction was performed for 26–33 cycles (depending on the transcript's abundance) with an initial 3 min at 94 °C and a final 5 min at 72 °C; each cycle consisted of 30 s at 94 °C followed by 30 s at 50–58 °C (depending on the  $T_m$  values of the primer pairs) and 1 min at 72 °C. Gene-specific primers were synthesized for 9 selected ESTs, and the constitutively expressed gene in potato,  *$\beta$ -tubulin* [23], was used as the internal control standard for each RT-PCR. All the primer sequences are listed in the Supplementary material (Table S1). Each RT-PCR experiment was repeated three times, and 5  $\mu$ L products were visualized on 1.2% agarose gel.

## 3. Results

### 3.1. Identification of *P. infestans*-induced ESTs

Little is known about the expression dynamics of *P. infestans*-responsive genes, therefore, we studied gene expression patterns in *P. infestans*-infected potato leaves over nine time points by using custom-designed cDNA

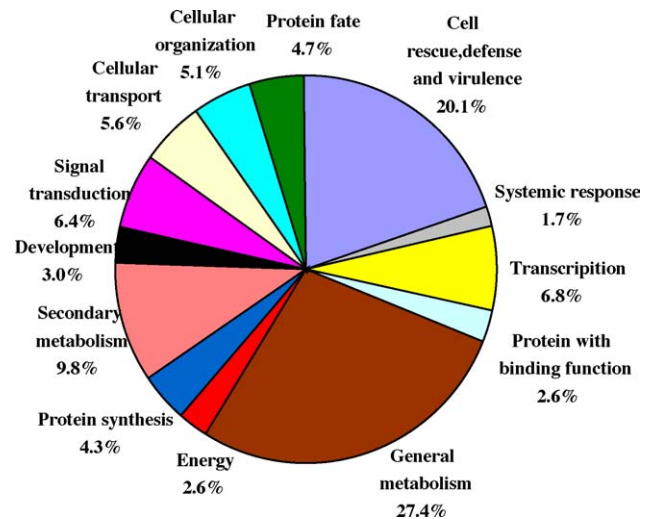


Fig. 1. Pie chart shows the proportion of *P. infestans*-induced genes in each of the functional categories described in MIPS database and Gene Ontology website.

microarrays, which included 1009 ESTs associated with potato quantitative resistance to late blight. To assess the reproducibility of the microarray analysis, two individual microarray slides, each containing two duplicate arrays, were used to analyze the relative mRNA abundance of each sample. One slide was hybridized with one labeled sample pair (one sample RNA labeled with Cy3- and the other labeled with Cy5-conjugated-dUTP); the other slide was hybridized with the same sample pair labeled by swapped dyes. Therefore, at least four array hybridization data sets were generated for each comparison. The ratio of the two fluorescent signal intensities for each cDNA spot on the microarray was used as a relative measure to determine the change of the differentially expressed gene. Only those genes with a normalized expression ratio more than 2.2-fold were selected at least at one time point after inoculation. Thus, a total of 669 *P. infestans*-regulated ESTs were identified.

### 3.2. Sequence analysis of *P. infestans*-induced ESTs

We sequenced all the 669 clones as described above. The sequenced ESTs were used to search by BLASTx, and the redundant sequences which are the same or different segments of the same gene were removed from the analysis. Thus, only 348 different genes were identified. By setting the criterion of  $E$ -value to  $\leq 1e-10$ , 234 ESTs had significant matches. With the information gathered from the MIPS *Arabidopsis* database and the Gene Ontology website, we were able to assign putative functions to these 234 genes. The other 114 ESTs, encoding proteins with insufficient sequence similarity to those with known function, were regarded as unknown-function genes. The genes of known function were classified into 13 categories, whose distribution is represented as a pie chart in Fig. 1. The largest group of genes (27.4%) was allocated to the general



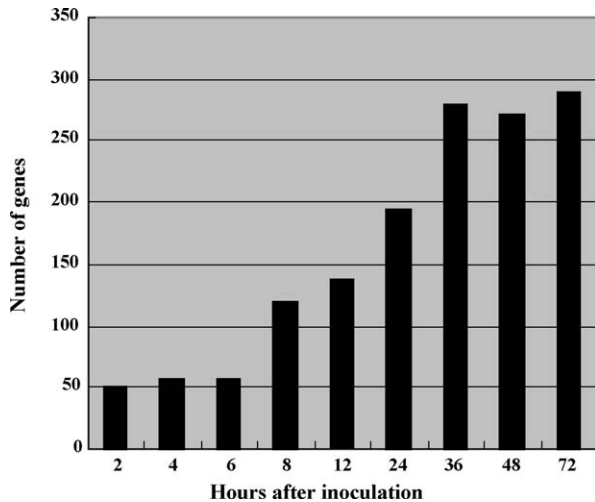


Fig. 2. Variation in the total number of up-regulated genes at the nine time points. A total of 348 genes were assigned.

metabolism category, and genes associated with cell rescue, defense and virulence constitute the second largest group (20.1%). In contrast, genes involved in plant systemic response occupied the smallest proportion (1.7%). Genes participating in secondary metabolism pathway also occupied a larger proportion (9.8%). Moreover, genes related to signal transduction and transcription together comprised 13.3% of all the functional genes. The other functional categories contained fewer genes, ranging from 2.6 to 5.6%. All the differentially regulated ESTs grouped into 13 categories are listed in the [Supplementary material \(Table S2\)](#), and their sequences are available from the corresponding author upon request.

### 3.3. General patterns of gene expression during *P. infestans* infection

Global changes in the transcript levels of 348 genes were catalogued at nine time points from 2 to 72 h post-inoculation (hpi). Fig. 2 presents the total number of up-regulated genes at each time point. Gene expression relatively stabilized from 2 to 6 hpi, and only about 50 genes were up-regulated more than 2.2-fold during this period. That number increased to 120 genes at 8 hpi, 138 genes at 12 hpi, and 194 genes at 24 hpi. At 36 hpi, the number of up-regulated genes increased dramatically to 280, rising to the peak (289) at 72 hpi.

The differentially expressed ESTs shown in Fig. 2 were grouped into 13 functional categories as described above, excluding 114 unknown-function genes. The total number of up-regulated genes in each of the groups (from 1 to 13) at each time point is shown in Fig. 3. The number of differentially regulated genes in the general metabolism group increased slowly from 2 to 6 hpi, but a rapid increase was found until 36 hpi with a slight decline by 72 hpi. A similar variation pattern was observed in the secondary metabolism group except the slight decrease between 2 and

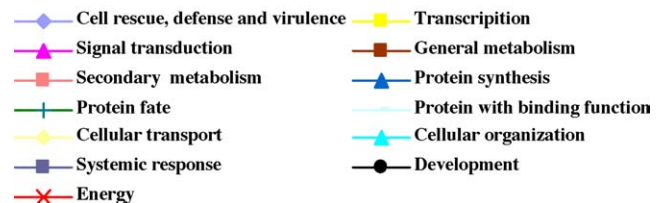
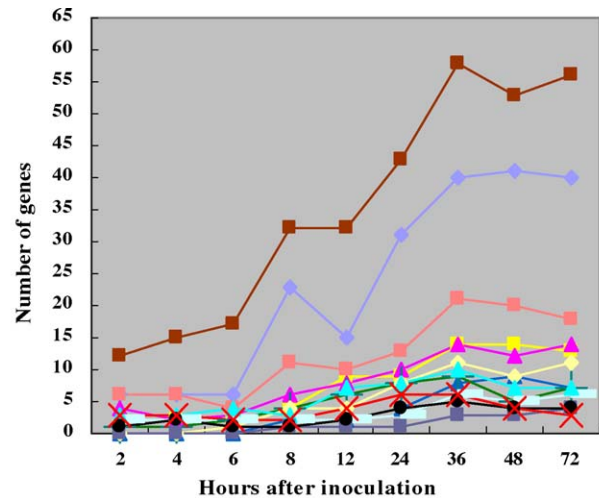


Fig. 3. Variation in the total number of up-regulated genes of 13 functional categories at the respective time point. A total of 234 genes were assigned.

6 hpi. In contrast, the number of up-regulated genes in the group of cell rescue, defense and virulence kept steady in the first 6 hpi, followed by a sharp increase until 36 hpi, and remained at a high level by 72 hpi; the only exception was an obvious decrease at 12 hpi. As a whole, the number of up-regulated transcripts in the transcription and signal transduction groups displayed a gradual increase throughout the 72-h inoculation period. Moreover, there were no up-regulated genes in the protein synthesis and cellular transport groups from 2 to 6 hpi, but their number increased between 8 and 24 hpi, followed by a further increase from 36 to 72 hpi. Meanwhile, the number of up-regulated genes in the groups of protein fate, cellular organization, energy, and protein with binding function showed few differences from 2 to 8 hpi, but there was a larger increase between 12 and 36 hpi and slightly declined from 48 to 72 hpi. In addition, very few genes in the other two categories were up-regulated in response to the inoculation and their proportion showed few differences over the 72-h observation period.

### 3.4. Cluster analysis of microarray data

The temporal program of transcription was studied with *P. infestans*-infected potato leaves. To group genes with similar expression patterns, a hierarchical average linkage clustering program which generates expression profiles organized by both related regulation patterns and expression amplitudes [28], was used to analyze a subset of 348 genes whose expression changed substantially in response to *P. infestans*. All the 348 significantly differentially expressed

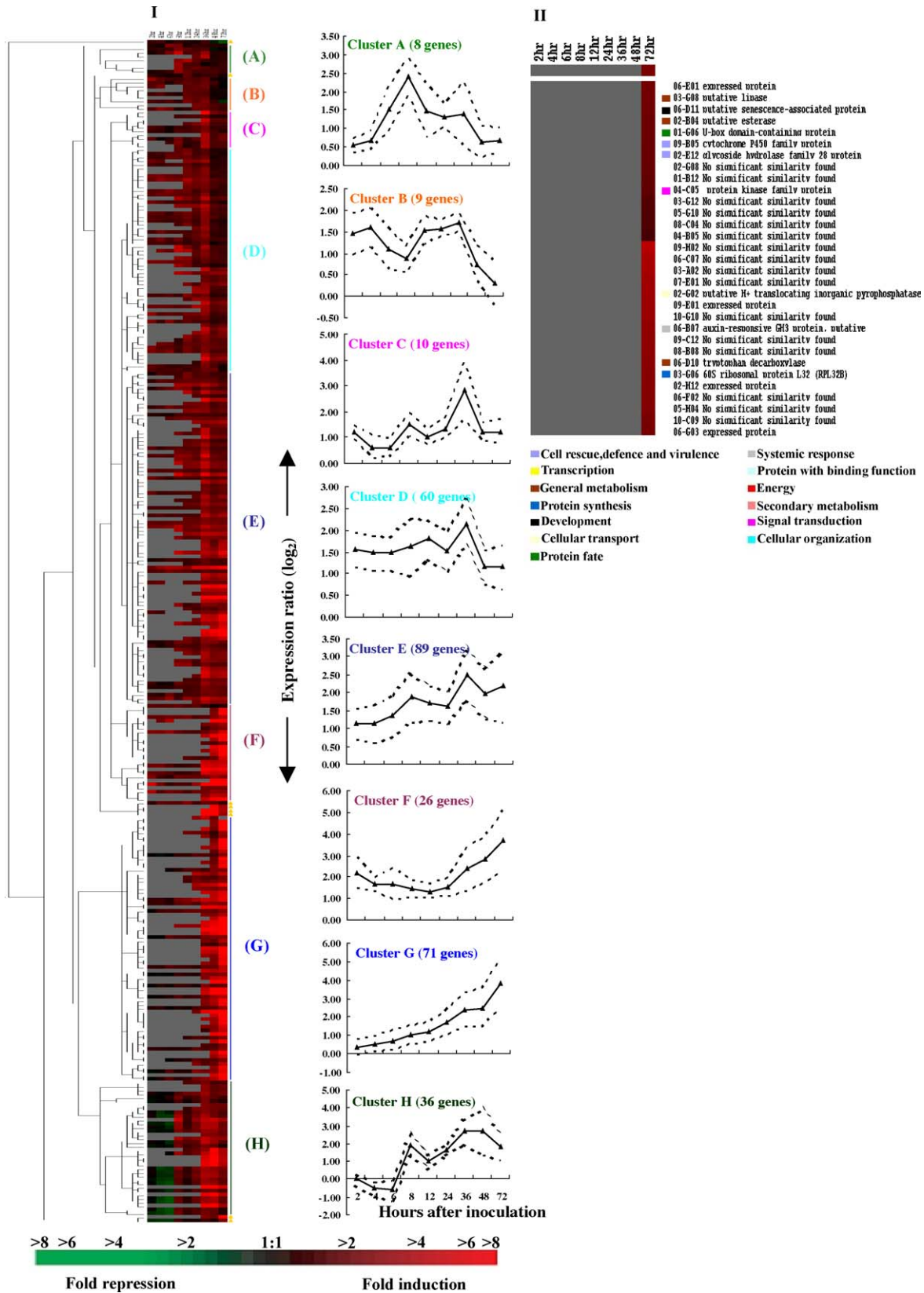


Fig. 4. Clustergram shows the different classes of gene expression profiles. A total of 348 *P. infestans*-modulated genes were subjected to average linkage hierarchical clustering. The main expression patterns are divided into two groups (I and II). Group I is divided into eight clusters (indicated by the letters A–H and color coding). Group I represents the genes only induced at 72 hpi. Each column represents a time point of inoculation with *P. infestans*, whereas each row represents the expression profile of each gene over the nine time points. For each gene, the ratio of mRNA levels in inoculated and corresponding reference

genes were divided into two groups (groups I and II). Group II included 31 genes specifically up-regulated by *P. infestans* only at 72 hpi. Group I contained 317 genes that were primarily grouped into eight clusters. Fig. 4 illustrates various patterns of gene expression during the time course, including early, mid and late gene induction as well as early gene repression. Fig. 5 shows the expanded views of the clusters marked on Fig. 4. For the genes belonging to cluster A of group I, their expression peak mainly occurred at 8 hpi. Cluster B contained genes up-regulated during the earliest period or intermediate time points of the infection. The common feature for the cluster C was the highest expression at 36 hpi. In the cluster D, high up-regulation was mainly observed from 12 to 36 hpi or from 2 to 36 hpi followed by a decrease at late stage of the inoculation (48–72 hpi). The largest expression profile was seen in cluster E that had high expression at mid and late time points or from 2 to 72 hpi with a peak occurring at 36 hpi. Clusters F and G contained genes that were most highly up-regulated at late time points of infection with maximum mRNA levels at 72 hpi. The final cluster, cluster H, contained down-regulated or non-expressed genes during the earlier period. In addition, eight genes showing specific expression patterns were not included in the above clusters. Clone number, GenBank accession number, expression values and extra information are provided as [Supplementary material \(Table S2\)](#).

### 3.5. Data validation

To evaluate the validity of the microarray results in an independent manner, the expression patterns of nine ESTs were further examined by RT-PCR. These ESTs represented different functional categories and regulation patterns by *P. infestans* infection. For example, the gene encoding WRKY family transcription factor (03-F01) was induced throughout the 72-h inoculation period, whereas the polyphenol oxidase gene (10-B03) only showed upregulation at 48–72 hpi. Our results indicated that the overall profiles of gene expression in RT-PCR analysis were similar to that revealed by the microarray data for all the selected genes (Fig. 6).

## 4. Discussion

The focus of this research was to identify the up-regulated genes of potato during *P. infestans* infection and obtain a global overview on the expression patterns of these genes, aiming at further understanding of the mechanism of potato late blight defense system. Since differences in gene expression are responsible for both morphological and

phenotypic diversities as well as indicative of cellular responses to environmental stimuli and perturbations [29], the temporal gene expression profiles of potato during the detailed time course of *P. infestans* infection can provide evidence of genes responsible for the resistance and a basis for further looking into the plant–pathogen interaction and gene function.

### 4.1. Reliability of microarray data

In the present study, two independent biological replicates and two individual microarray hybridizations (technical replicates) were performed for each time point. Our results demonstrated the high reproducibility of the microarray hybridization, which was expressed by the significant correlation coefficient of different replications. For example, the correlation coefficient of the replications between dye-swapped slides was 0.89, whereas that of the replications between arrays on the same slide was 0.97 at 48 hpi, and that of self–self hybridization experiment was 0.98 in uninfected sample. Furthermore, the high expression consistency of the vast majority of genes included in the final data set between adjacent time points was observed, typically, the identified genes showed a related expression trend over the course of multiple time points (Fig. 5), which further supported the good reliability of our data. In addition, the potato *POTHR-1* and *NtPRp27*-like genes used for positive controls on the arrays, which were previously characterized by Northern blotting hybridization during *P. infestans* infection [20,21], displayed a high reproducibility. For instance, the CV of expression ratios of *POTHR-1* and *NtPRp27*-like gene at 48 hpi is 7.3 and 6.9%, respectively (these two genes were printed 48 times in different locations on each slide). To further confirm the quality of our results, we performed RT-PCR experiments and found a good accordance with the microarray data (Fig. 6). Consequently, the obtained data are of sufficient quality, which truly reflect the kinetic expression changes of the *P. infestans*-responsive genes.

### 4.2. Distinct stages of potato defense response to *P. infestans*

Based on the global expression changes of 348 genes throughout the process of *P. infestans* infection (Figs. 2 and 3), the distinct stages of expression changes of these genes could be discriminated, namely early (2–6 hpi), mid (8–24 hpi) and late stages (36–72 hpi), with 6–8 hpi and 24–36 hpi as the turning points of early to mid and mid to late stages, respectively. In combination with morphologic changes of potato leaves during *P. infestans* infection, we

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samples is represented by color, according to the color scale at the bottom. Red, green, black, and grey reflects transcriptional activation, transcriptional repression, no differences, and non-expression (or weak signal), respectively. The cluster tree (on the left) illustrates the nodes of coregulation of gene expression over all nine time points. The graphs show the average expression profiles for the genes in the corresponding “cluster” (indicated by the letters A–H and color coding). Dashed lines indicate standard deviation. Classified function categories are denoted by color-coded squares. Genes not classified into above clusters are denoted by yellow triangles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)





Fig. 5. Enlargement of clusters marked “A–H” in Fig. 4. The average profile for each cluster is represented by the first row below the time-point designations. Gene names are highest BLASTx hits. Clone numbers corresponding to each gene are shown on the right of each cluster. Classified function categories are denoted by color-coded squares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)



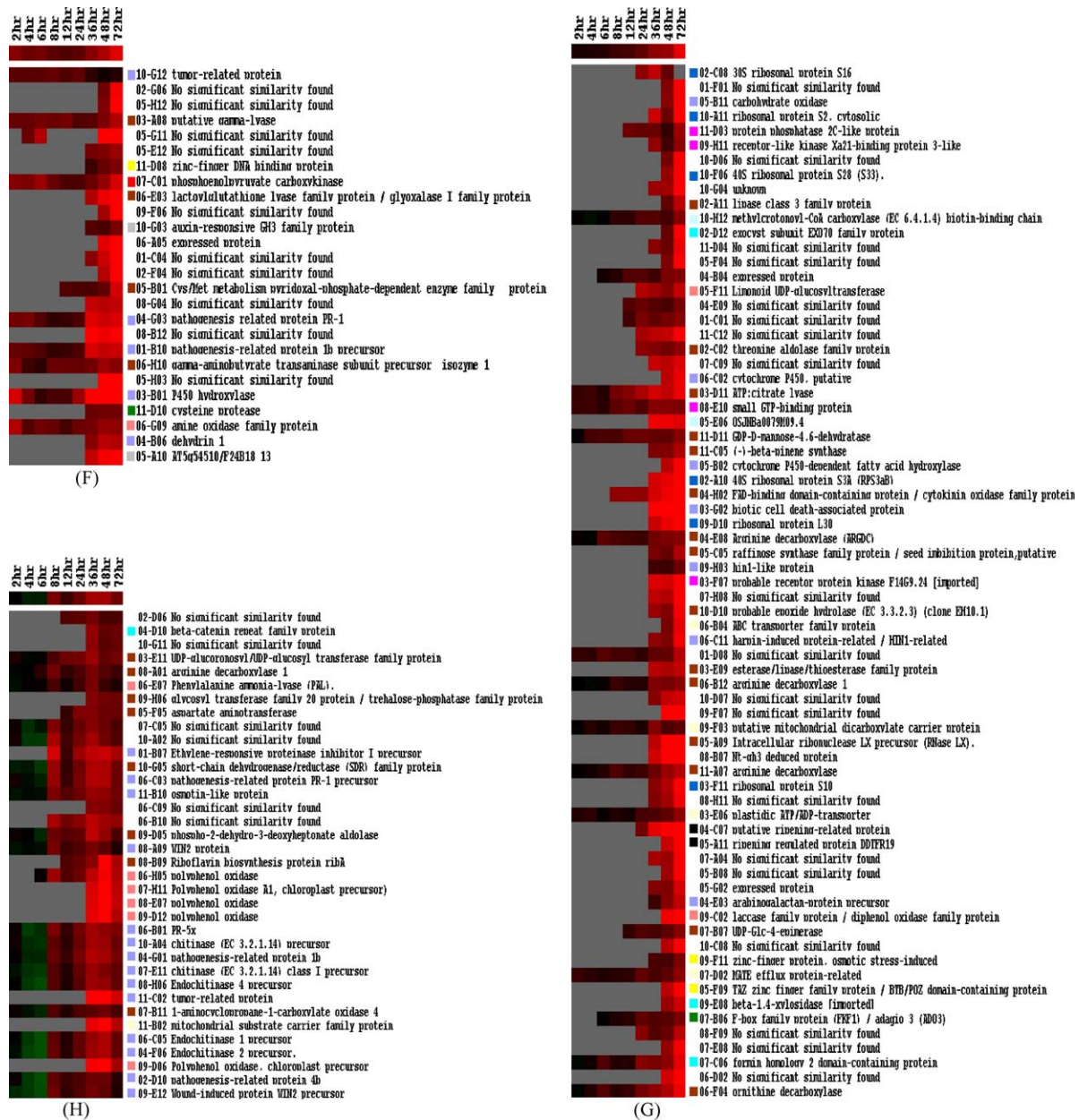


Fig. 5. (Continued).

could speculate preliminarily that the three distinct stages of gene expression might reflect the corresponding distinct stages of potato defense response to *P. infestans*. To our knowledge, this is the first report showing the relationship between the large-scale gene expression patterns of potato and *P. infestans* infection process.

In the early stage, only a low proportion (15%) of the transcripts showed up-regulation; the probable interpretation could be that the first 6 h is a recognitory and adaptive stage of the pathogen by potato plant, which is in accordance with the finding that *P. infestans* penetrates the first cell during 1–2 hpi and the first reaction in the invaded plant cell and surrounding tissues is detectable by 3 hpi [30]. Between 6 and 8 hpi, the number of up-regulated genes increased to

34% of the total, subsequently, the potato defense process might begin turning to the mid stage, and this proportion almost remained at the same level until 12 hpi then increased to 56% by 24 hpi. These results demonstrate that a massive transcriptional reprogramming has occurred within the first 24 hpi, which might correspond to the end of the *P. infestans* biotrophic phase [31]. From 24 to 36 hpi, the proportion of up-regulated genes rose to 80% of the total, after which the compatible interaction of potato–*P. infestans* entered the late stage, and the proportion always stayed at the same level up to 72 hpi. Therefore, a global transcriptional reprogramming was first observed at 36 hpi. This was the time point when the number of up-regulated genes in most of the functional categories rose to the peak (Fig. 3), many of which were

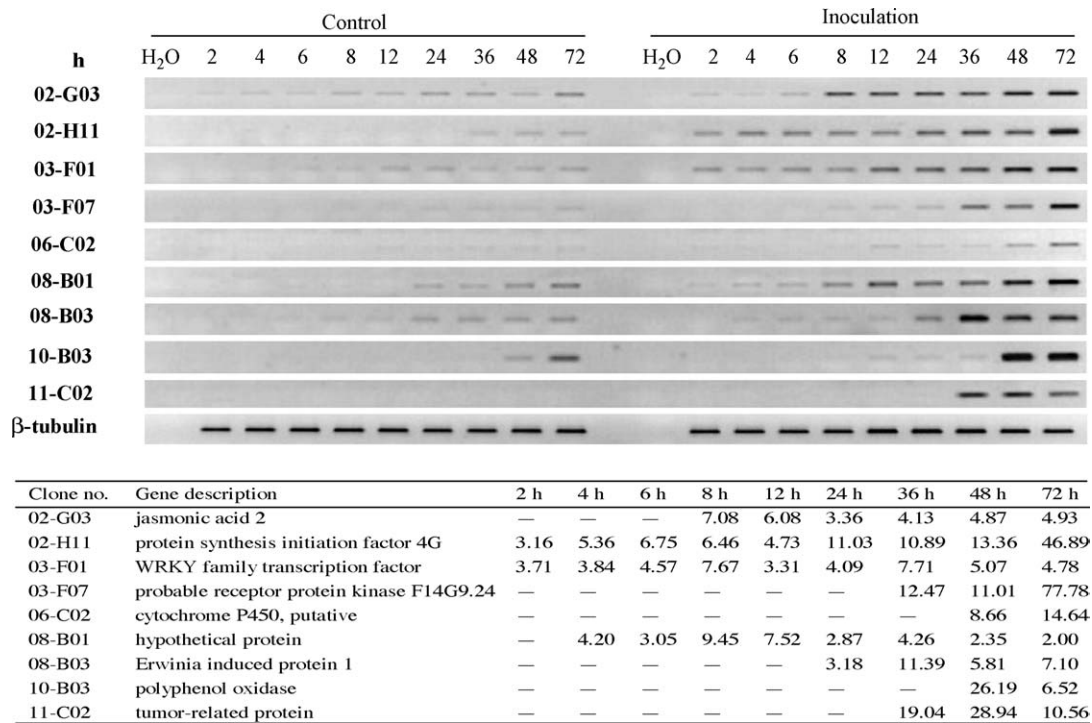


Fig. 6. Verification of microarray results. Nine ESTs selected from the microarray experiments were tested by RT-PCR. Leaves of potato, clone 386209.10, were treated with either water (controls) or *P. infestans*, and samples were taken at 2, 4, 6, 8, 12, 24, 36, 48, and 72 h after inoculation. Amplifications were performed on the reverse-transcribed products from each time point. ESTs tested are indicated on the left. The annealed temperature and cycles of each EST are as follows: 02-G03 (55 °C, 26 cycles), 02-H11 (55 °C, 29 cycles), 03-F01 (50 °C, 28 cycles), 03-F07 (55 °C, 29 cycles), 06-C02 (54 °C, 33 cycles), 08-B01 (54 °C, 29 cycles), 08-B03 (55 °C, 26 cycles), 10-B03 (54 °C, 26 cycles), and 11-C02 (54 °C, 26 cycles). The  $\beta$ -tubulin gene was used to test for equal template presence in all reactions (58 °C, 26 cycles). Primer sequences are listed in Supplementary Material (Table S1). Selected ESTs and expression ratios of microarray are listed in the bottom table. Short line indicates the lack of data because the spots of microarray with signal intensity lower than the background plus 2 S.D.s were removed during data analysis.

highly induced by more than 5- or 10-fold at this stage (Supplementary material, Table S2). It is noticeable that most pathogenesis-related (PR) genes were mainly up-regulated by *P. infestans* in the mid and late stages with an expression peak at 36 hpi (Fig. 5, clusters D, F and H). This expression profiling might correlate with the necrotrophic phase of *P. infestans* infection determined by Avrova et al. [31] that the pathogen entered this stage after 24 hpi. At the same time, we observed the visible traces of fungal inoculation, a morphologic change during the process of *P. infestans* infection, especially at 72 hpi, when all the leaves were chlorotic with spreading water-soaked lesions, further suggesting that the destructive necrotrophic phase has occurred at the late stage of *P. infestans* infection. Interestingly, there were no significant changes in transcripts of genes involved in all the functional categories throughout the late stage (Figs. 2 and 3).

#### 4.3. Transcription factors and different regulation of potato PR genes

The compatible interaction of potato leaves with *P. infestans* results in early induction of some key transcription factors, which can later orchestrate the global switch in expression of downstream target genes. In our study, 16

transcription-related genes were identified, which showed different expression profiles. For example, the MYC gene was obviously up-regulated from 6 to 36 hpi by *P. infestans* (Fig. 5, cluster D), which showed about three-fold induction (Supplementary material, Table S2). As far as we know, this gene may be isolated from potato leaves for the first time, and little is known about its regulation role in plant defense system. Differently, the WRKY gene was highly up-regulated throughout the infection process from 2 to 72 h (Fig. 5, cluster E) with a significant induction more than three- or even seven-fold (Supplementary material, Table S2). WRKYs are a large family of plant-specific transcription factors that bind to the W-box of promoter regions of many PR genes [32], which are thought to be associated with regulating defense responses to both abiotic and biotic stresses [33]. In this study, many genes encoding different PR proteins were identified (Fig. 5, clusters D, F and H). We observed that *PR-1*, which is commonly used as a marker of downstream plant defenses or systemic acquired resistance (SAR) [34], showed basically consistent up-regulation with *WRKY* at transcript level over the 72-h period excluding the 8 and 12 hpi (Fig. 5, cluster F). This pattern is also applied to the *PR-1b* precursor gene (Fig. 5, cluster F). The related regulation of *WRKY* and *PR-1* as well as the *PR-1b* precursor gene in the potato-*P. infestans* interaction further supports

that WRKY transcription factor is a vital component of plant defense signaling pathways. However, a novel finding is that some PR genes such as *PR-1b*, *PR-4b* and *PR-5x* were down-regulated from 4 to 6 hpi (Fig. 5, cluster H), showing contrary expression profiling with *WRKY* at this stage. This may be explained by the most recent viewpoint that WRKY proteins very likely act in a network of mutually competing participants with temporal displacement occurring at defined preoccupied sites by other family members in a stimulus-dependent manner [35]. On the other hand, the expression of PR genes is also regulated by external factors like pathogens. Thus, the further interpretations about down-regulation for PR genes might be that: *P. infestans* is a hemibiotrophic pathogen that stays in the biotrophic phase during the first 24 hpi [10,31]. During this period, *P. infestans* also requires living plant cells for survival like biotrophic pathogens, so it must not only suppress host cell death but also prevent host defenses by producing virulence proteins [36,37]. Therefore, the vital defense genes of potato such as some PRs are temporarily repressed and down-regulated in this stage. In addition, Vleeshouwers et al. [38] reported a positive correlation between the levels of basal *PR-1*, *PR-2*, and *PR-5* mRNA and the resistance of potato cultivars to *P. infestans*. They further indicated that the major resistance responses were affected by the penetration of *P. infestans* [7]. More recently, Ros et al. [15] also found similar results that the expression of *PR-1*, *PR-2*, *PR-3*, and *PR-5* differed in susceptible and moderately resistant potato cultivars. Therefore, we can hypothesize that the levels of PR gene expression, especially at the early stage of *P. infestans* infection, may be applicable to assess the resistance of potato cultivars.

#### 4.4. Activation of HR-related genes in potato race-nonspecific resistance

In general, the hypersensitive response (HR) is an important feature in race-specific resistance. Nonetheless, we observed that some HR-related genes were also induced in the potato race-nonspecific resistance to *P. infestans*. For example, the *Hin1*-related gene, which was associated with the tobacco mosaic virus-induced HR [39], was strongly up-regulated between 36 and 72 hpi (Fig. 5, cluster G) with an alteration of over 4- or even 10-fold (Supplementary material, Table S2). Moreover, the gene encoding cysteine protease was also up-regulated at this stage (Fig. 5, cluster F). The involvement of cysteine protease gene in the HR of potato was reported previously that it was up-regulated at 15 hpi in the incompatible potato–*P. infestans* interaction [9]. Recently, the ubiquitin–proteasome pathway was shown to be important in the implementation of plant defense response, including HR [34,40]. Tör et al. [41] also reported that ubiquitin-mediated proteolysis might act as an essential regulatory mechanism in the R gene-mediated plant defense response. In our study, two genes encoding F-box proteins, the important components of the ubiquitin–proteasome

pathway [42], were up-regulated from 8 to 36 hpi, whereas the other two showed induction between 24 and 72 hpi (Fig. 5, clusters A, D, E, and G; Supplementary material, Table S2). All these results indicate that certain HR-related genes may play a similar role in both race-specific and race-nonspecific resistances.

More interestingly, a homologue to the R13 resistance gene cluster in soybean was identified in the present research. Although the presence of R gene does not contradict the statement that the potato clone used in our experiments does not possess any known R genes from *S. demissum*, *R1–R11*, this gene showed strong induction from 36 to 72 hpi (Fig. 5, cluster E) with an alteration of more than 10-fold (Supplementary material, Table S2), which is different from a rapid and localized HR in R gene-mediated resistance. Moreover, we observed that 15–30 black and brownish granular speckles (HR symptom) appeared in each infected leaflet after 48 hpi, subsequently, the surrounding tissues gradually became water-soaked lesions (data not shown). Previously, Vleeshouwers et al. [7] reported that the HR was fast and occurred within 22 h in fully resistant cultivars, whereas a trailing HR was induced within 46 h in partially resistant clones. More recently, Ros et al. [15] also identified HR genes in susceptible and moderately resistant potato cultivars after *P. infestans* infection. All these observations further support the previous viewpoint that the HR was the major defense response because it was associated with all types of resistant interactions [6,7]. Furthermore, in the race-specific resistance, the R genes are thought to encode specific receptors that recognize elicitors and initiate signal transduction cascades resulting in the HR [43]. Although a major feature of the HR is a rapid and local cell death, many defense-related genes that are not involved in cell death are also activated and may play a more important role in preventing further spread of the specific pathogen [36]. Despite the lack of known R genes in the race-nonspecific resistant clones of potato, they may contain all of the other genetic components of the HR pathway. Therefore, it is further inferred that the trailing HR in partially resistant clones may result from a broad-spectrum recognition of the pathogen by the product of unknown R gene(s) or R gene analogues, or that a same or similar defense system, especially the downstream signaling components such as kinases, phosphatases and other defense genes, may exist in both the race-specific and race-nonspecific resistances, but the discrimination lies in the different expression patterns of these genes.

#### 4.5. *P. infestans* regulation of potato metabolism-related genes

Upon recognition of the *P. infestans* by host potato, a series of signaling pathways are switched on, which leads to the metabolism reprogramming of the host plant. In the present study, approximately 37.2% of all the known *P. infestans*-responsive genes were identified with a general or



secondary metabolism function (Fig. 1). This finding presumably reflects the high metabolic activity of potato that accompanies *P. infestans* infection. We observed that many genes that encode enzymes participating in the defense-related metabolic pathways such as the biosynthesis of phenylpropanoids and alkaloids were activated by *P. infestans*. Most intriguing is the activation of the phenylpropanoid pathway that produces numerous secondary metabolites like lignins and flavonoids [44]. A gene encoding phenylalanine ammonia-lyase (PAL), catalyzing the first committed step in the phenylpropanoid pathway, was identified. This gene showed an interesting expression pattern: it was induced at 8 hpi, returned to a normal level at 12 hpi, and then up-regulated again from 24 to 36 hpi with less induction at 48 hpi (Fig. 5, cluster H). Owing to the importance of PAL in plant secondary metabolism, the typical expression profiling of PAL gene might partially reflect the change of potato from housekeeping metabolism to defense metabolism after inoculation with *P. infestans*. By contrast with *PAL*, the gene encoding tryptophan decarboxylase which takes part in terpenoid indole alkaloid (TIA) [45] showed a different expression profile that it was obviously up-regulated only at 72 hpi (Fig. 4, group II). To some extent, the induction of tryptophan decarboxylase gene suggests a possible involvement of TIA biosynthesis pathway in potato defense response to *P. infestans* attack.

## 5. Summary and conclusions

As far as we know, this is the first study of monitoring large-scale gene expression of potato along all the process of the compatible potato–*P. infestans* interaction, involving the biotrophic and necrotrophic phases of pathogen infection. In the present research, we identified 348 *P. infestans*-responsive transcripts. The functional classification of these genes and their expression profiles at nine time points after inoculation provide useful information on quantitative resistance of potato to late blight. We discriminated distinct stages of potato defense against *P. infestans*, which not only unfolded the natural process of the pathogen infection but also revealed the timing of the events and the genes participating in each stage. These functional genes are involved in the whole process of plant defense responses to pathogen attack, including transcriptional regulation, signaling, activation of defense genes participating in HR and SAR, switch of defense-related metabolism pathways, and cell wall modification. In addition, an exciting observation is that more than 100 of the differentially transcribed genes fall into the unknown group. Their functional identification could broaden our understanding of potato quantitative resistance mechanism to *P. infestans*. Although the fact that a gene shows increased expression in response to pathogen infection does not directly indicate an exact biological function in defined defense pathways, expression-profiling information does provide a

useful starting point for a more in-depth analysis of plant defense response. Our results would serve as a platform for further investigation of *P. infestans*-induced genes, and transcriptional changes listed in this study can provide clues about regulatory mechanisms, broader cellular functions and biochemical pathways.

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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.2005.07.020.

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