Journal of Experimental Botany, Vol. 62, No. 14, pp. 4851–4861, 2011
doi:10.1093/jxb/err147 Advance Access publication 1 July, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Expression profiles of differentially regulated genes during the early stages of apple flower infection with *Erwinia amylovora*

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Received 21 February 2011; Revised 6 April 2011; Accepted 11 April 2011

Abstract

To identify genes involved in the response to the fire blight pathogen *Erwinia amylovora* in apple (*Malus*×*domestica*), expression profiles were investigated using an apple oligo (70-mer) array representing 40, 000 genes. Blossoms of a fire blight-susceptible apple cultivar Gala were collected from trees growing in the orchard, placed on a tray in the laboratory, and spray-inoculated with a suspension of *E. amylovora* at a concentration of 10^8 cfu ml^{-1}. Uninoculated detached flowers served as controls at each time point. Expression profiles were captured at three different time points post-inoculation at 2, 8, and 24 h, together with those at 0 h (uninoculated). A total of about 3500 genes were found to be significantly modulated in response to at least one of the three time points. Among those, a total of 770, 855, and 1002 genes were up-regulated, by 2-fold, at 2, 8, and 24 h following inoculation, respectively; while, 748, 1024, and 1455 genes were down-regulated, by 2-fold, at 2, 8, and 24 h following inoculation, respectively. Over the three time points post-inoculation, 365 genes were commonly up-regulated and 374 genes were commonly down-regulated. Both sets of genes were classified based on their functional categories. The majority of up-regulated genes were involved in metabolism, signal transduction, signalling, transport, and stress response. A number of transcripts encoding proteins/enzymes known to be up-regulated under particular biotic and abiotic stress were also up-regulated following *E. amylovora* treatment. Those up- or down-regulated genes encode transcription factors, signaling components, defense-related, transporter, and metabolism, all of which have been associated with disease responses in *Arabidopsis* and rice, suggesting similar response pathways are involved in apple blossoms.

Key words: Apple, fire blight, gene expression, gene regulation, *Malus*×*domestica*, microarray.

Introduction

Plants have developed various defence mechanisms that are associated with a number of early and late events with the initiation of both biotic and abiotic stresses. These plant defence responses include various physiological, molecular, and cellular events together with transcriptional activation of multiple genes of both known and unknown biochemical functions as well as the accumulation of secondary metabolites (Nakashita *et al.*, 2003; Malnoy *et al.*, 2007). These responses often entail the activation of the hypersensitive response (HR) and leading to the development of systemic acquired resistance (Ryals *et al.*, 1996; Dangl and Jones, 2001). In addition, plants are capable of fortifying the extracellular matrix through cutin formation and callose deposition during pathogen

infection (Dangl and Jones, 2001). A large-scale identification and analysis of genes induced upon pathogen inoculation is an essential step toward understanding the mechanisms of the plant defence response, which, in turn, will aid in the development of disease-resistant lines (Venisse *et al.*, 2002; Liu *et al.*, 2008).

Apple (*Malus*×*domestica* Borkh.) is one of the most important horticultural woody plants cultivated worldwide for its value as a fruit crop. The most economically important fruit and ornamental trees, such as apple (*M.*×*domestica*), pear (*Pyrus communis*), peach (*Prunus persica*), cherry (*Prunus avium*), strawberry (*Fragaria* spp.), apricot (*Prunus armeniaca*), almond (*Prunus amygdalus*), and rose (*Rosa hybrida*) all belong to the Rosaceae family (Shulaev *et al.*, 2008).

Fire blight disease is a highly destructive bacterial disease of apples and pears. The disease is caused by the bacterial pathogen *Erwinia amylovora* which infects almost all the aerial parts of the tree including blossoms, fruits, vegetative shoots, woody tissues, and rootstock crowns, resulting in blossom blight, shoot blight, and rootstock blight (Norelli *et al.*, 2003). Due to the wide diversity of host tissues susceptible to infection and the limited number of management tools available to control this disease, it has been difficult to eradicate or slow down the incidence of fire blight epidemics. Effective management includes, but is not limited to, reducing levels of inoculum in order to avoid the incidence of new infections, imposing barriers to the successful establishment of the pathogen, and reducing host susceptibility to infection (Aldwinckle and Beer, 1979). Management practices include the use of a few size-controlling rootstocks that are resistant to fire blight (Norelli *et al.*, 2003) as most dwarfing rootstocks are susceptible to fire blight, and chemical treatments to enhance host resistance (Maxson-Stein *et al.*, 2002). Recent efforts to develop genetically engineered apples expressing T_4 lysozyme and attacin genes and/or other antibacterial genes have been promising (Ko *et al.*, 2002; Malnoy *et al.*, 2007); however, commercial acceptance of such transgenic apples has not been successful.

To date, genome-wide studies on host–pathogen interactions for fire blight disease are not available. Recently, a genome-wide apple EST database has been established (Newcomb *et al.*, 2006; Gasic *et al.*, 2009), and apple microarrays have been created. Large-scale expression studies using microarrays to elucidate the molecular mechanisms of early fruit development (Lee *et al.*, 2007), aroma production and red coloration (Schaffer *et al.*, 2007), and fruit development (Janssen *et al.*, 2008; Soria-Guerra *et al.*, 2011) have been conducted. Analysis of transcriptional changes in apple following *E. amylovora* infection using an apple microarray was recently conducted using 768 ESTs of apple defence-related genes (Pontais *et al.*, 2008). This has provided some overview of those apple genes involved in the response to fire blight infection.

In this study, an apple microarray of 40, 000 genes representing different tissues and conditions, including fire blight challenged tissues, was used to study expression profiles and to identify a set of genes that are regulated by the bacterial pathogen *E. amylovora.*

Materials and methods

Plant material and pathogen inoculation

Blossoms of apple cv. Gala were collected from established trees growing at the Pomology Research Center (Urbana, IL), and immediately placed on wet filter paper in two square aluminium foil trays. Blossoms in one tray were uninoculated (used as controls); whereas, blossoms in the second container were sprayed with a bacterial suspension of *E. amylovora* strain Ea1189. Bacterial cells were grown in Luria–Bertani (LB) medium at 28 °C. The bacterial suspension culture (OD_{600}=0.1 ($\sim 10^8$ cfu ml^{-1}) was sprayed onto apple blossoms.

Both trays were covered with saran wrap, and incubated in a controlled chamber at high relative humidity (>95%) and 24 °C. Flowers were then collected at 0, 2, 8, and 24 h from each of the two trays. It is noteworthy to point out that no disease symptoms were observed on any of the blossoms throughout this 24 h sampling period as disease symptoms would normally appear within 5–7 d following bacterial inoculation and affect over 70% of blossoms, depending on the prevailing environmental conditions.

RNA extraction and quantification

Total RNA was prepared from inoculated and non-inoculated tissues at 0, 2, 8, and 24 h post-inoculation (hpi) according to Gasic *et al.* (2004). Briefly, 1 g of flower tissue was ground into a fine powder in liquid nitrogen using a mortar and pestle, and homogenized with 10 ml of extraction buffer (2% CTAB, 2% PVP K-30, 10 mM TRIS-HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl, and 0.5 g spermidine). After vortexing, the tubes were incubated at 60 °C for 15 min and an equal volume of chloroform-isoamylalcohol (24:1 v/v) was added. Tubes were centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was transferred to a new tube, an equal volume of chloroform-isoamylalcohol (24:1 v/v) was added, and this was centrifuged again. The supernatant was transferred to a new tube, and 7.5 M LiCl was added at 4 °C for overnight precipitation. RNA was visualized in a denaturing formaldehyde 1.2% agarose gel and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

cDNA synthesis and coupling with aa-dUTP to Cy dye ester

A total of 15 μg RNA was used to synthesise cDNA using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Following coupling with aa-cDNA to Cy-dye ester, cDNA was purified using a QIAquic column to remove unincorporated aa-dUTP and free amines. cDNAs were quantified in a NanoDrop ND-1000 Spectrophotometer.

Microarray hybridization, scanning, and quantification

The apple microarray, consisting of 40, 000 sequences, has been created from the assembly and clustering of expressed sequences derived from a total of 34 cDNA apple libraries. These libraries represented different apple tissues, both vegetative and reproductive (at different stages of development), and subjected to various biotic and abiotic stress conditions from a total of 13 different genotypes of apple. From these broadly representative cDNA libraries, 33, 825 unique sequences were selected, together with an additional 6000 sequences used as positive and negative controls. Altogether, these sequences were used to create a 40, 000 70-mer oligonucleotide-based apple microarray (Invitrogen, Carlsbad, CA) (Soria-Guerra *et al.*, 2011). Oligos were resuspended in 150 mM NaPi pH 8.5 to a final concentration of 20 μM, and printed on ultragaps coated slides (Corning, Corning, NY) using a GeneMachine OmniGrid arrayer (Digilab, Inc., Holliston, MA) in a total of 48 blocks.

Slides were briefly rehydrated in a steaming water bath and immediately placed slides on a 70-80 °C hot block until all the moisture had evaporated. These were then cross-linked using a Strategene UV linker at 6000 uj×100, vigorously washed in

0.2% SDS for 2 min, and then gently shaken twice in milliQ water for 1 min. Slides were incubated for 45 min at 42 °C in preheated pre-hybridization buffer (5× Denhardt's, 6× SSC, 0.1% SDS, 25 μg ml^{-1} tRNA, and 20% formamide), washed five times consecutively in MilliQ water for 1 min and isopropanol for 3–5 min, and then immediately dried by centrifugation.

The Cy3- and Cy5-labelled cDNAs probes were mixed, concentrated by using speedVac for 90 min, and brought to a volume of 10 ®μl. After adding 40 μl 2× hybridization buffer (Ambion, Austin, TX), cDNA was denatured at 98 °C for 3 min, and cooled on ice for 30 s. Probes were then added to each slide, and covered with a Maui mixer AO (Bio Micro Systems, Salt Lake City, UT) cover slip. Slides were then placed in a hybridization chamber (Maui, Bio Micro Systems) and incubated overnight at 65 °C. Following hybridization, slides were washed for 10 min with wash buffer I (1× SSC, 0.2% SDS), vigorously washed with wash buffer II (0.1× SSC, 0.2% SDS) for 5 min at room temperature, and subsequently washed twice with buffer III (0.1× SSC) for 5 min at room temperature. After a final wash, slides were dried by centrifugation and scanned at a resolution of 10 μm per pixel for fluorescence emission at 532 nm for Cy3 and at 635 nm for Cy5 using a GenePix 4000B scanner.

Experimental design

Microarray hybridization and data analysis were performed following the MIAME (minimum information about a microarray experiment) guidelines for international standardization and quality control of microarray experiments (Brazma *et al.*, 2001). For each time point analysed, two independent biological repeats were performed with the control and experimental cDNA clones labelled with Cy3 and Cy5 fluorescent dyes, respectively. In addition, a dye-swap was also included to eliminate any bias resulting from the two fluorescent dyes. A total of nine slides were used.

All comparisons were made between *E. amylovora*-inoculated and uninoculated (control) flowers at each of the time points following treatment, including 2, 8, and 24 h.

Data normalization, filtering, statistical analysis, and clustering

For data analysis using GeneSpring GX 7.3.1 (Agilent Technologies, Palo Alto, CA), a genome array file containing all the data for each of the oligos represented in the array was uploaded. A systematic approach was used to catalogue corresponding EST accession number, clone ID, top hit *Arabidopsis* ID and its description, corresponding gene ontologies to top hits, and associated EST IDs. This file was uploaded in GeneSpring GX 7.3.1 and used for analysis as the apple oligo genome.

Data from a total of nine slides, containing intensity values for control and signal channels as well as flag values for present, absent, or marginal spots were uploaded and sample attributes were defined. Data were initially transformed for dye-swap for the appropriate samples, and all intensity values less than 0.01 or that were negative were converted to 0.01 to reset biologically irrelevant or otherwise unacceptable expression values. Following background subtraction and elimination of all spots flagged as marginal or absent, data were normalized using an intensity dependent LOWESS (locally weighted scatter plot smoothing) normalization method to minimize systematic non-biological differences (Yang *et al.*, 2002). Normalized data were then filtered onto expression levels (>2-fold up-regulated or >2-fold down-regulated) to obtain a set of genes with expression values in at least one of the comparisons made. This set was then used for statistical analysis for measurement of confidence (*t* test *P* value). Clustering analysis was performed using *k-means* clustering to grouped genes with similar transcript abundance profiles with 2-fold up and 2-fold down-regulated genes at different time points.

Microarray data validation by quantitative real-time-PCR (qRT-PCR) analysis

A total of five genes with relevant expression profiles from the Oligo array data were randomly selected to be validated by quantitative real-time PCR (qPCR). Total RNA from blossoms was isolated using a modified Cetyltrimethylammonium bromide (CTAB) method (Gasic *et al.*, 2004). Total RNA (2 μg) from each sample was treated with DNase I (Invitrogen, Carlsbad, CA) and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dT) primer using SuperScript III RT kit (Invitrogen) according to the manufacturer's instructions. Specific primers (Table 1) were designed using a Fast PCR program (http://www.biocenter.helsinki.fi). The SYBR Green real-time PCR assay was carried out in a total volume of 25 μl, containing 12.5 μl of 2× SYBR Green I Master Mix (Applied Biosystems, Foster City, CA), 0.2 μM (each) specific primers, and 100 ng of template cDNA. The amplification program consisted of 1 cycle of 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, and 60 °C for 1 min. The fluorescent product was detected on the last step of each cycle. Following amplification, melting temperatures of PCR products were analysed to determine the specificity of the PCR product. Melting curves were obtained by slow heating at 0.5 °C s^{-1}, from 60 °C to 90 °C while continuously monitoring the fluorescence signal. A negative control without a cDNA template was run with each analysis to evaluate the overall specificity. Amplifications were carried out in 96-well plates in a 7300 Real Time PCR System (Applied Biosystems). All samples were run in triplicate. An apple actin gene (Apple_0902.385.C2.Contig633) was used as a constitutive control gene. The results were presented as fold-change relative to the wild type (WT) at 0 h.

Results

Microarray analysis of gene expression in apple blossom following E. amylovora *inoculation*

To obtain an overall view of gene regulation during *E. amylovora* inoculation, microarray analyses were performed. Data obtained from scans of nine oligo arrays (70-mer) containing 40, 369 apple probe sets representing 40,000 transcripts for each of the three time-point comparisons was quantified, uploaded into GeneSpring 7.2 (Silicon Genetics, CA), transformed, filtered, and normalized (Gonzalez and Vodkin, 2007). Normalized data were further filtered for expression levels for quality control to eliminate genes in the

Table 1. Primers used for real-time RT-PCR

Systematic ID number	Forward primer (5′ →3′)	Reverse primer (5′ →3′)	Size	Annealing temperature
MdUI01753	CGATGCAGCTTCGATCCCAACC	TAGAGTCGCAGGCGTAACGTA	206	57.5
MdUI15852	TTGCAGGGAAGCTAAATCGTGGTC	GATTGCGAAGTAGAATCCCATCG	252	57.6
MdUI26615	GTTACCGTGGCGTTCGACAA	AAGAAGCGGCGGAGACGGAG	228	58
MdUI22046	GATCATGAGGAACAAATCGACGGA	CACCACAAAAAGCAAACCAAGC	232	57.6
MdUI00786	GGAGACTCCTCACAAGGTTCTTGC	CAGTATTTAACCACTGGGAGAGCA	264	57.9

array with no values throughout the study. A *t* test analysis of these genes, with a cut-off *P* value of <0.05, identified a collection of 3500 genes that were statistically significant for at least one of the three time points (Fig. 1). Among these, 1561 genes were up-regulated (see Supplementary Table S1 at *JXB* online); while 1939 genes were down-regulated (see Supplementary Table S2 at *JXB* online). These collective groups of genes were then used for cluster analysis by *k-means* clustering and functional classification of individual members. Changes in expression of all probe sets on a representative microarray at different time points are presented in Fig. 1.

Cluster analysis by k-means *and principal components analysis (PCA)*

To visualize the global gene transcription profiles of apple blossoms during interactions with *E. amylovora* and to group genes that share similar profiles, a cluster analysis was conducted using statistically significant genes that were grouped into 12 sets according to their expression profiles using *k-means* (Fig. 2). This algorithm randomly divides genes into a user-defined number of clusters minimizing intra-cluster variability. Genes grouped within one set share a relatively similar expression profile; however, quantitative and qualitative expression differences among members of the same set are likely to occur.

Principal component analysis (PCA), presented as a 3-D scatter plot in which the loadings for the first, second, and third principal components are plotted along the x, y, and z axes, respectively (Fig. 3), has revealed the largest fraction of the overall variability. Each point along the scatter plot corresponds to a single gene. Genes that exhibit high levels of the first principal component and low levels of the second principal component are displayed in the lower right corner of the plot, and genes exhibiting equal levels of the two components lie along the diagonal. Variances explained by the three PCA components are 64.29% (x), 24.48% (y), and 11.23% (z), respectively.

Genes responding to E. amylovora inoculation in apple

Based on the scatter plot diagram (Fig. 1), numbers of both up-regulated (>2-fold) and down-regulated (<2-fold) genes increased following inoculation. Based on the Venn diagram, the numbers of up-regulated genes at 2, 8, and 24 h are 770, 855, and 1002, respectively; while the numbers of down-regulated genes at 2, 8, and 24 h are 748, 1024, and 1455, respectively (Fig. 4). A total of 444 up-regulated genes and 432 down-regulated genes were common between 2 h and 8 h after inoculation. A total of 576 up-regulated genes and 698 down-regulated genes overlapped between 8 h and 24 h after inoculation; moreover, 452 up-regulated genes and 630 down-regulated genes were common between 2 h and 24 h after inoculation.

Functional categorization of identified genes

To capture and assess the expression profiles of genes regulated during the first 24 h following inoculation, a total of 365 up-regulated and 374 down-regulated genes, that were commonly modulated at all three time points (Fig. 4; see Supplementary Table S3 at *JXB* online), were used to identify or assign putative functions for *E. amylovora*-regulated genes. These were found to belong to the following functional categories (Fig. 5): 22% of commonly up-regulated genes and 17.4% of commonly down-regulated genes belonged to the 'metabolism' GO category including protein, lipid, carbohydrate, and primary metabolism; 7.7% of up-regulated genes and 6.1% of down-regulated genes belonged to the 'transcription' category; 5.5% of up-regulated and 2.6% of down-regulated genes belonged to the 'transporter-related' category; 7.1% of up-regulated and 5.9% of down-regulated genes belonged to the 'cellular communication/signaling mechanism' category; 3% of up-regulated and 2.1% of down-regulated genes belonged to the 'defence' category; 1.3% of up-regulated and 3.5% of down-regulated genes belonged to the 'stress-relative' category; 5.2% of up-regulated and 5.6% of down-regulated genes belonged to the 'cell wall related' category; 2.2% of up-regulated and 3.7% of down-regulated genes belonged to the 'cell components' category; while, 44.8% of up-regulated and 52.4% of down-regulated genes were 'unclassified' or were of unknown function proteins (Fig. 5; see Supplementary Table S3 at *JXB* online). The majority of up-regulated genes seem to be related to four major biological changes,

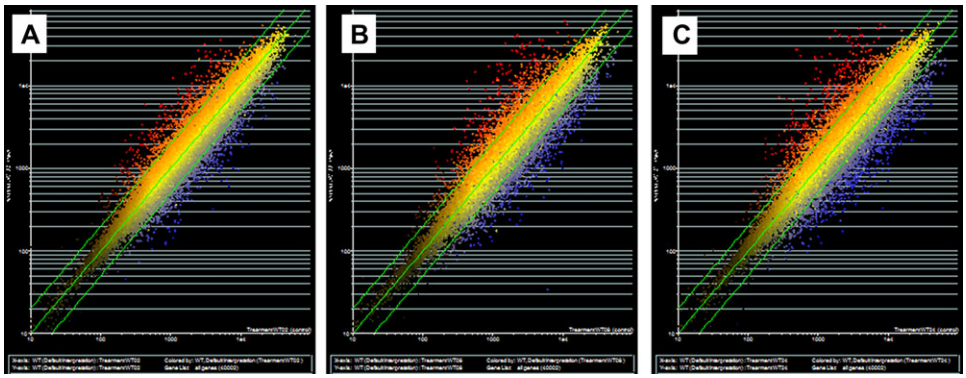

Fig. 1. Scatter plots of expression profiles of apple blossoms treated with *Erwinia amylovora* 2 h (A), 8 h (B), and 24 h (C) following pathogen inoculation. These plots represent the linear scatter distribution of all genes revealing up- and down-regulated genes at 2, 8, and 24 h after treatment. All comparisons were made between inoculated (treated) and uninoculated (control) at each time point.

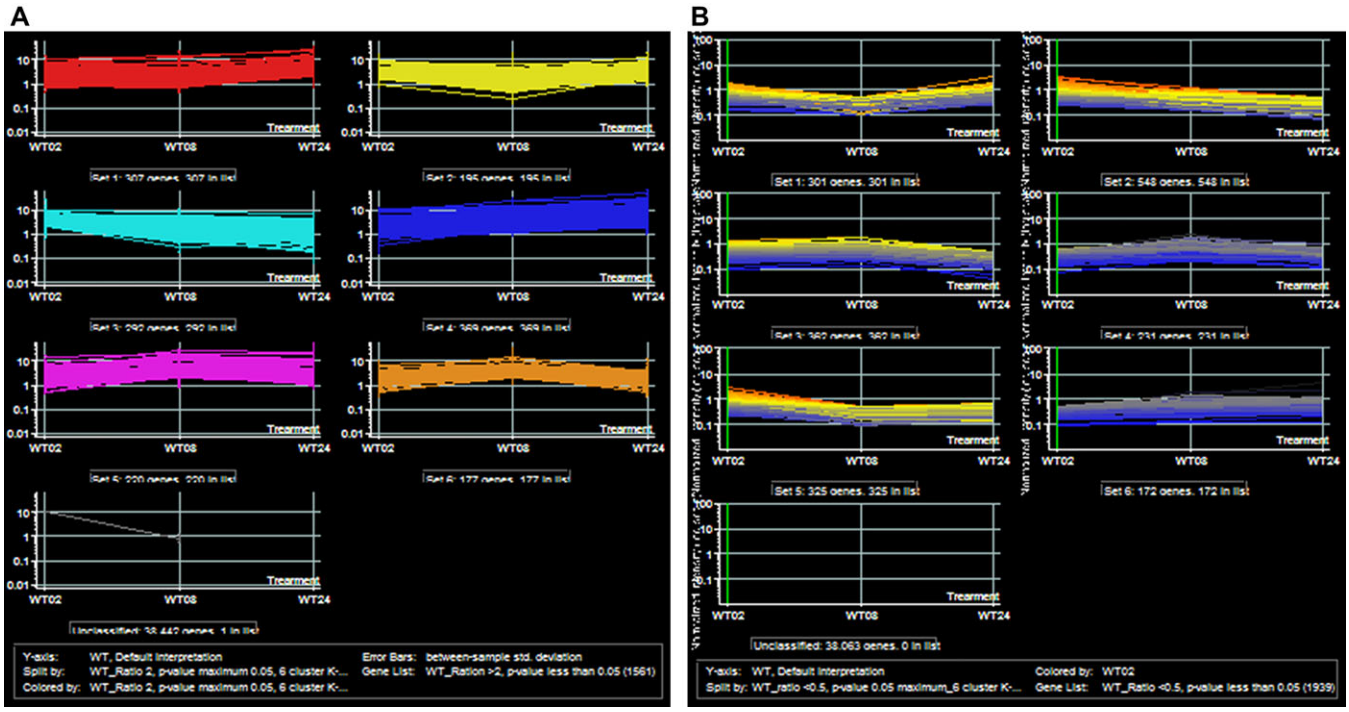

Fig. 2. Cluster analysis of statistically significant genes using *k-means*. Genes are clustered in a pre-determined number of 12 sets using the *k-means* algorithm available from Gene Spring 7.3.1 (Silicon Genetics, CA). Sets are numbered 1 to 6, and the total number of genes per set is recorded. Each set contains the schematic representation of the expression profile for each one of its members. For each diagram, the *x*-axis shows the three time points (2, 8, and 24 h), while the *y*-axis corresponds to fold change in the set of genes. (A) Expression profiles of more than 2-fold up-regulated genes. Set 1 consists of 307 genes; set 2, 195; set 3, 292; set 4, 359; set 5, 202; and set 6, 177 genes. (B) Expression profiles of more than 2-fold down-regulated genes. Set 1 consists of 301 genes; set 2, 548, set 3, 352; set 4, 231, set 5, 325; and set 6, 172 genes.

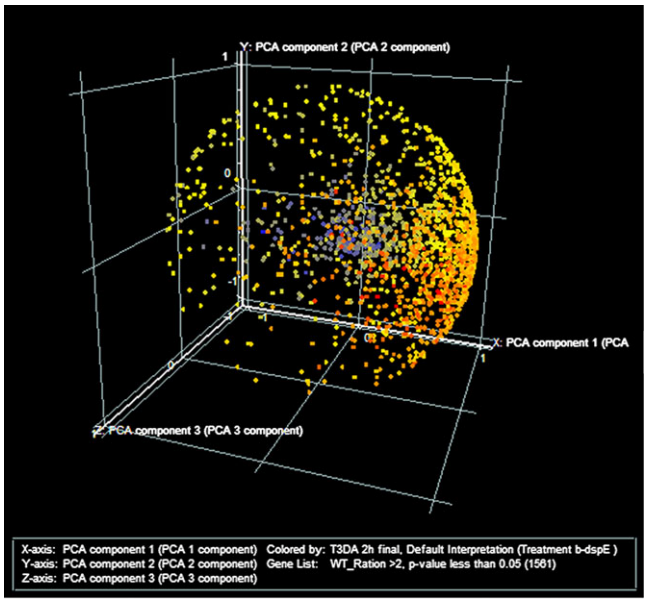

Fig. 3. Visualization of three-dimensional principal component analysis (PCA) of significantly up-regulated genes (1561) under one of the three conditions tested (2 h post-inoculation).

including metabolism (22%), transcription (7.7%), as well as signalling (7.1%) and transport (5.5%) (Fig. 5A), whereas the majority of down-regulated genes are related to three major biological changes including metabolism (17.3%), transcription (6.1%), and signalling (5.8%) (Fig. 5B).

Those up=regulated genes involved in transcription include WRKY DNA-binding protein 18/33, zinc finger (MYND type) and (C3HC4-type) RING finger family proteins, PHD finger transcription factor, BTB and TAZ domain protein 1, myb domain protein 116, NAC domain containing protein 100, basic helix-loop-helix (bHLH) protein-related, and ethylene-response factor 1. In addition, genes involved in transport (e.g. high affinity K^+ transporter 5 and cation/H^+ exchanger 2) as well as those involved in signalling, such as serine/threonine protein kinase, calcium- and calmodulin-dependent protein kinase/kinase, and ethylene response sensor 1, are also up-regulated. Those genes related to enhanced disease resistance include pathogenesis-related family proteins, acidic endochitinase, TIR-NBS class disease resistance proteins, stress response proteins, peroxidases, and cell wall-associated protein β-galactosidase.

Those four largest functional categories, except for the unclassified protein, of the down-regulated genes include those involved in transcription (6.1%), signalling (5.8%), cell wall-related (5.6%), and stress (3.8%). Those down-regulated genes belonging to the transcription category mainly include the myb domain protein, DEAD/DEAH box helicase, zinc finger (DHHC type) family, and KOW domain-containing transcription factor. Those down-regulated genes belonging to the signalling category mainly include those involved in histidine

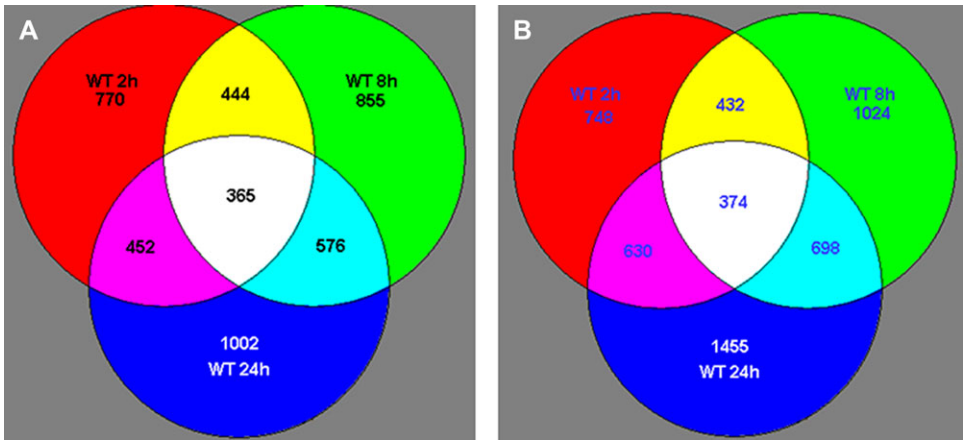

Fig. 4. A generalized Venn diagram with three sets of 2, 8, and 24 h after treatment and their intersections. (A) Up-regulated genes and (B) down-regulated genes for each time point post-inoculation. All comparisons were made between inoculated (treated) and uninoculated (control) at each time point.

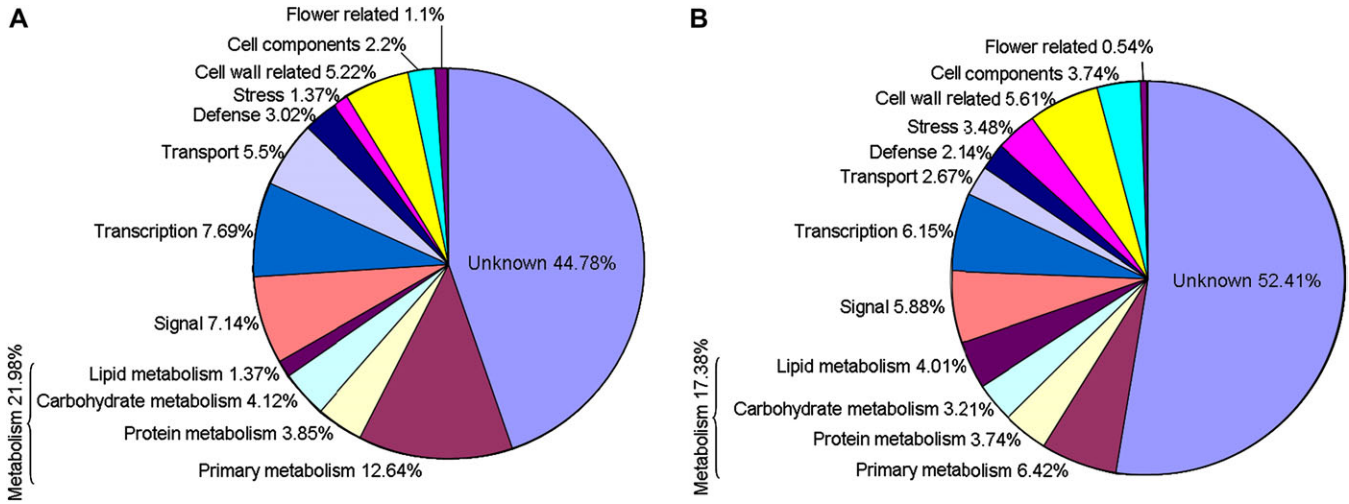

Fig. 5. Pie charts corresponding to frequency of common up-regulated genes (A) and down-regulated genes (B) in each of the functional categories.

kinase, cyclin-dependent kinase, diacylglycerol kinase, leucine-rich repeat transmembrane protein kinase, protein serine/threonine kinase, 2-phosphoglycerate kinase, BRI1-associated receptor kinase, mitogen-activated protein kinase kinase kinase (MAPKKK), and ethylene insensitive 3. Some genes related to cell walls appear to be suppressed during fire blight interaction. These include endo-1,4-beta-glucanase, non-specific lipid transfer protein, and proline-/glycine-rich family protein. Stress-related genes such as early responsive to dehydration and glutathione *S*-transferase, were found to be suppressed in apple blossoms following pathogen inoculation.

Verification of changes in gene expression using qRT-PCR

Several candidate genes were chosen to confirm their transcript abundance profiles throughout the three time points compared with the untreated control. Primers for selected genes were designed exclusively to amplify oligo sequences identified following microarray analysis (Table 1).

Overall, qRT-PCR results for these genes showed similar patterns of expression profiles to those obtained following microarray hybridization (Fig. 6). This indicated that transcript abundance profiles were consistent with those of the oligo microarray results. Therefore, the observed up-regulated and down-regulated gene expression patterns of the microarray analysis were supported by qRT-PCR analyses (Fig. 6).

Discussion

Microarray-based gene expression profiling has been recognized as a powerful tool to observe global gene expression and the physiological processes involved in response to a particular stimulus (Maleck *et al.*, 2001; Schenk *et al.*, 2000). To investigate the molecular controls into the response of apple blossoms to the fire blight pathogen *E. amylovora*, gene expression profiles were analysed using a 40, 000 apple oligo array. During the early stages following inoculation (2, 8, and 24 h), a total of 3500 apple genes (8.75%) were differentially

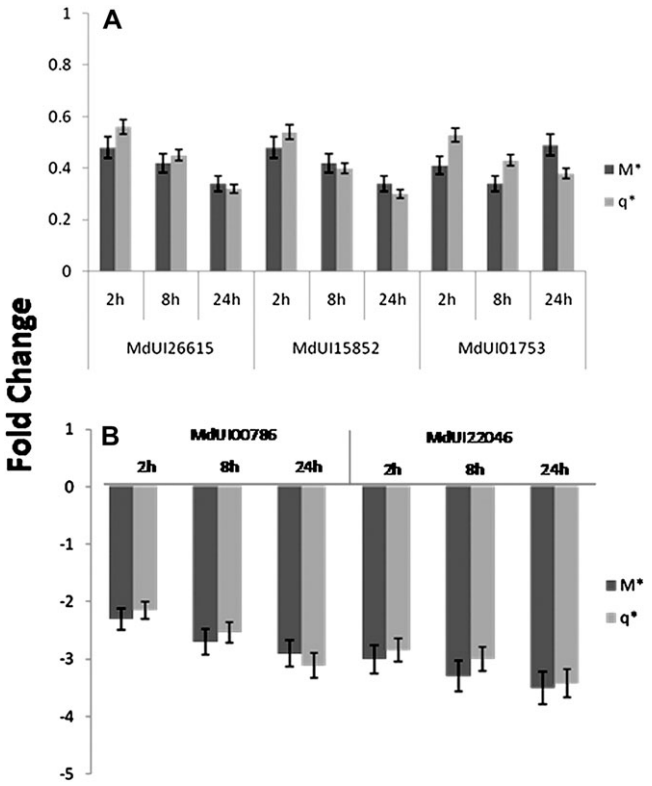

Fig. 6. Real-time polymerase chain reaction (RT-PCR) analysis of mRNA expression of randomly selected genes along with their corresponding microarray results at 2, 8, and 24 h. (A) Up-regulated genes; (B) down-regulated genes.

modulated in response to *E. amylovora*. Previously, Norelli *et al.* (2009) identified a total of 468 non-redundant apple ESTs isolated by suppressive subtractive hybridization (SSH) in response to challenge of leaves of 'Gale Gala' plants with *E. amylovora* strain Ea273 over a period of 96 h post-inoculation. These expressed sequences were also identified in this study during the first 24 h following inoculation.

As the apple cultivar Gala is known to be susceptible to fire blight, expression profiles of genes that are modulated following inoculation could probably serve in aiding the infection process. Therefore, the numbers of modulated genes as well as their levels of response over the 24 h post-inoculation period should provide a global overview of the genetic controls during the early period of fire blight infection.

Regulation of defence-related genes by E. amylovora *infection*

About 3.02% of 365 commonly up-regulated fire blight-responsive genes are classified to have a defence-related function. Approximately 90% of these defence-related proteins are up-regulated by more than 3-fold by 24 hpi. Among those, there are a number of pathogenesis-related (PR) proteins. PR proteins have been reported to be induced in several plant species during infection by viruses, viroids, fungi, or bacteria (Schenk *et al.*, 2000; Cheong *et al.*, 2002; Aswati Nair et al., 2010). Induction of these proteins is not pathogen-specific, but determined by the form of reaction of the host plant. PR proteins form protective barriers against pathogens leading to local or systemic resistance. In this study, transcriptional up-regulation of PR proteins, TIR-NBS class disease resistance proteins, general stress response genes, such as peroxidases, and those possibly involved in antimicrobial metabolite production, such as cytochrome P450, have been detected. All of these proteins can be related to either local or systemic acquired resistance plant responses. Previously, Venisse *et al.* (2002) have reported that many defence mechanisms, including those of PR proteins, are elicited in both resistant and susceptible apple genotypes, although there is a delayed response in a number of genes involved in branch pathways of the phenylpropanoid metabolism in tissues of the susceptible apple genotype, MM 106 apple rootstock, when challenged with a wild-type strain of *E. amylovora.* Moreover, Venisse *et al.* (2002) have indicated that *E. amylovora* is capable of transiently preventing transcription of several isoforms of genes encoding chalcone synthase (CHS), dihydroflavonol reductase (DFR), and flavonol synthase (FLS) in leaves of the apple rootstock MM111.

In this study, induction of peroxidase was observed in the early stages of infection, thus suggesting activation of an oxidative burst. Oxidative burst in plant tissues has been reported to participate in the plant response to fungal and bacterial infection as well as upon treatment with different elicitors, and in response to both oxidative stress and wounding (Schenk *et al.*, 2000; Venisse *et al.*, 2002; Cheong *et al.*, 2002). In fact, it has been reported that oxidative burst in flowers and shoots of susceptible apple tissues is observed during invasion of these tissues by *E. amylovora* (Venisse *et al.*, 2002). Based on these findings, it is noteworthy to suggest that the oxidative burst is an essential component of the infection response in apple blossoms.

Regulation of signal transduction-related genes by E. amylovora *infection*

About 15% of all fire blight-responsive genes are classified to have a signal transduction-related function, such as transcriptional regulators and signalling components. Of these genes, 61% of transcription regulators and 56% of signaling genes are up-regulated by more than 3-fold following *E. amylovora* infection. This indicates that these signalling components are transcriptionally-regulated during fire blight infection in apple blossoms. A gene encoding calcium- and calmodulin-dependent protein kinase is found to be up-regulated in apple blossoms following *E. amylovora* infection, suggesting that the calcium ion may be one of the key signals that initiate stress resistance reactions in blossoms. It has been reported that several CDPKs are likely to mediate biotic or abiotic stress responses. For example, one of the CDPKs has a known target, phenylalanine ammonia-lyase (PAL), which plays an important role in plant defence responses against fungal elicitation in cell suspension cultures of French bean (Allwood *et al.*, 2002); while, a tobacco CDPK, *NtCDPK2*, is involved in defence response against *Agrobacterium tumefaciens* infection (Romeis *et al.*, 2001). Moreover, a non-expressor of *PR* genes, such as the *Arabidopsis thaliana NPR1* gene, has

been identified as a key factor in transducing signals leading to systemic acquired resistance (Malnoy *et al.*, 2007). Over-expression of an apple homologue of *NPR1* in transgenic lines of fire blight-susceptible apple genotypes, cv. Galaxy and the rootstock M26, led to the activation of several *PR* genes and contributed to significantly enhanced resistance to fire blight in transgenic lines of apple (Malnoy *et al.*, 2007).

In this study, some ethylene biosynthesis genes such as ethylene response sensor/receptor and ethylene response 1/two-component response regulator are up-regulated by more than 3-fold following *E. amylovora* treatment. Conversely, ethylene insensitive 3 is down-regulated. Ethylene response sensors/receptors function as negative regulators; therefore, elevated expression of ethylene receptor genes may serve to reduce hormonal responses. A similar observation has been reported in tomato fruit following inoculation with the biocontrol yeast *Cryptococcus laurentii* (Jiang *et al.*, 2009). During biotic and abiotic stress conditions, signalling components have also been shown to undergo transcriptional regulation (Schenk *et al.*, 2000). Interestingly, in this study, WRKY transcription factor and zinc finger (C3HC4-type RING finger and MYND type) family proteins are highly up-regulated in apple blossoms following *E. amylovora* treatment. WRKY proteins comprise a large family of transcription factors. As transcriptional regulators, WRKY factors are likely to act by directing temporal and spatial expression of specific genes, thereby ensuring proper cellular responses to internal and external stimuli (Ulker and Somssich, 2004). It has been reported that *Arabidopsis* plants expressing an *AtWRKY18* showed enhanced levels of resistance to the bacterial pathogen *Pseudomonas syringae* (Chen and Chen, 2002). Thus, the observed up-regulation of WRKY transcription factor and zinc finger family proteins are also involved in the early defence responses of apple blossoms to *E. amylovora* infection.

Regulation of metabolism-related genes by E. amylovora *infection*

In this study, about 22% of commonly up-regulated and 17% of commonly down-regulated fire blight-responsive genes are involved in metabolism-related functions. Many of the genes in this category function as pathogen-responsive genes. This suggests that upon *E. amylovora* challenge, metabolism-related genes are induced and serve as a key defence mechanism in apple. It is interesting to note that genes encoding enzymes that are involved in the mannose-binding lectin pathway are highly up-regulated. The mannose-binding lectin (MBL), also known as mannose binding protein (MBP), appears to be involved in pattern recognition in the first line of defence in the pre-immune host. Specifically, MBL recognizes carbohydrate patterns found along surfaces of large numbers of pathogenic micro-organisms, including bacteria, viruses, protozoa, and fungi, where it can activate its complements (Turner and Hamvas, 2000). Binding of MBL to a microorganism results in the activation of the lectin pathway of the complement system. *E. amylovora* infection strongly increases transcription of curculin-like (mannose-binding) lectin family protein genes in apple blossoms.

In this study, genes encoding enzymes involved in oxylipin signalling are found to be highly down-regulated. Lipoxygenases (LOX) are enzymes that are widely found in higher plants. LOXs catalyse the synthesis of oxylipin which is a potent signalling molecule involved in the defence reactions of both animals and plants (Blee, 2002; La Camera, 2004). This enzyme is involved in the initiation of lipid peroxidation which contributes to the development of a hypersensitive reaction (HR) during an incompatible host–pathogen interaction (Croft *et al.*, 1990). The role of lipid peroxidation has been investigated as a possible early step in the signal transduction mechanism leading to phytoalexin synthesis (Cornille *et al.*, 1998). The primary products of LOX, fatty acid hydroperoxides, are highly reactive, and may cause oxidative membrane damage leading to cell necrosis and premature cell death (Hildebrand, 1998). In some cases, lipid pentadienyl, peroxyl free radicals, or other active oxygen species such as the superoxide anion may also be generated by LOX action (Roy *et al.*, 1994). These reactive bursts occurring during the plant defence response have been reported to promote hypersensitive cell death (Levine *et al.*, 1994).

In this study, genes encoding enzymes involved in protease activity are also highly up-regulated. In plants, proteases play a key role in the regulation of biological processes, such as the recognition of pathogens and pests, and in the induction of effective defence responses. It is interesting to note that, in this study, members of the aspartyl protease family protein are up-regulated more than 3-fold during the early stages of *E. amylovora* infection, and this increases in expression up to 5-fold at 24 h following inoculation.

Based on expression profiles in this study, genes encoding enzymes involved in hydrolase activities are highly up-regulated; thus, they are likely to be involved in the process of fire blight infection. Serine carboxypeptidases (SCPs) are proteins belonging to the hydrolase family (Fraser *et al.*, 2005) that share characteristic structural features, including a signal sequence for intracellular trafficking and/or secretion, multiple N-linked glycosylation sites, and four evolutionarily conserved regions that consist of a substrate-binding domain and three catalytic sites (Vendrell and Aviles, 1999). It has been reported that a rice serine carboxypeptidase-like gene is involved in defence responses against biotic and oxidative stresses (Liu *et al.*, 2008). Therefore, the observed up-regulation of SCPs in apple blossoms challenged with *E. amylovora* is of interest and deserves further investigation.

Regulation of cell wall organization-related genes by E. amylovora *infection*

In this study, approximately 5.2% of *E. amylovora*-responsive genes are related to cell wall related activities. Interestingly, most cell wall organization/modification-related genes are highly up-regulated (more than 3-fold) following *E. amylovora* infection in apple blossoms. Increased expression of cell wall biosynthesis- and modification-related genes has also been observed in *Arabidopsis* following challenge with the fungal pathogen *Alternaria. brassicicola* or following wounding stress (Schenk *et al.*, 2000; Cheong *et al.*, 2003). These

data further confirm the hypothesis that cell wall biosynthesis, modification or fortification is essential in the plant host response to *E. amylovora* infection.

Regulation of hormone signalling-related genes by E. amylovora *infection*

Plant defence against pathogens and environmental stresses involves the regulation of a complex network of signalling cascades that involves several signalling molecules, including salicylic acid (SA), jasmonic acid (JA), and ethylene (Schenk *et al.*, 2000; Cheong *et al.*, 2002). *E. amylovora* infection causes more than a 3-fold increased transcription of genes encoding ethylene response sensors, and, in particular, an ethylene response factor. Conversely, the down-regulation of transcription of an ethylene-insensitive gene is observed in *E. amylovora*-infected apple blossoms. This finding suggests that an ethylene response sensor/factor functions as a regulator to induce expression of ethylene responsive genes. Ruperti *et al.* (2002) have identified four ethylene response genes in abscission zones of young developing fruit of peach that were homologous to thaumatin-like proteins, PR-4 proteins, and to fungal and plant β-D-xylosidases. It is probable that the ethylene response sensor in apple is involved in regulating the expression of ethylene response factors that mediate cellular signalling following *E. amylovora* infection of apple blossoms.

Brassinosteroids (BR) play various roles in disease resistance reactions in tobacco and rice (Nakashita *et al.*, 2003). In this study, a gene coding for the biosynthetic enzyme of BR, namely serine carboxypeptidase, is up-regulated 5-fold following *E. amylovora* infection. This indicates that BR may also be involved in the response to *E. amylovora* infection in apple blossoms.

Previously, Pontais *et al.* (2008) investigated transcriptional changes in apple in response to *E. amylovora* infection using less than 800 EST clones. It was reported that increased numbers of genes related to signal perception and transduction as well as a large number of pathogenesis-related (PR) proteins have been noted to be present in leaf tissues. In this study using an apple microarray comprised of 40, 000 genes, global expression of transcripts regulated by the bacterial pathogen *E. amylovora* in blossoms of apple cv. Gala provided a more comprehensive assessment of gene expression profiles at three different time points following infection. These findings provide an overview of the underlying mechanisms related to modulation and regulation of various biochemical pathways in response to fire blight infection. However, it should be pointed out that some of the genes investigated in this microarray study lack annotation, and therefore their roles in the fire blight disease response are yet to be elucidated, which, in turn, may provide further insights into these findings.

Conclusions

In this study, when blossoms of the apple cultivar Gala are infected with *E. amylovora*, many plant defence genes, including those coding for PR proteins, are elicited within a period of 24 h following infection. Among the up-regulated genes are those involved in transcription, such as WRKY DNA-binding protein 18/33, zinc and RING finger family proteins, and ethylene-response factor 1, as well as those involved in transport, such as high affinity K^+ transporter 5 and cation/H^+ exchanger 2, and those involved in signalling, such as serine/threonine protein kinase, calcium- and calmodulin-dependent protein kinase/kinase, and ethylene response sensor 1. However, these up-regulated groups of genes as well as those of PR family proteins, such as acidic endochitinase, TIR-NBS class disease resistance proteins, stress response proteins, peroxidases, and cell wall associated proteins are either not sustained for long durations following infection or they do not accumulate to high enough levels during the early stages of infection of apple blossoms in fire blight-susceptible apple genotypes, such as 'Gala'. Moreover, it is important to note that genes encoding enzymes involved in oxylipin signalling, *LOX* genes, are highly down-regulated in blossoms of 'Gala' during the first 24 h following *E. amylovora* infection. These signalling genes, together with those involved in modulating role(s) such as those coding for ethylene response sensors/factors, as well as those associated with cell wall hydrolase activities are all contributing to the regulation of the disease response; i.e. a delayed defence response, in apple blossoms during the early stages of infection in the fire blight-susceptible cv. Gala.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table 1. Listing of all up-regulated apple genes following *Erwinia amylovora* challenge of apple blossoms, at 2, 8, and 24 h post-inoculation with fold change and functional categories.

Supplementary Table 2**.** Listing of all down-regulated apple genes following *Erwinia amylovora* challenge of apple blossoms, at 2, 8, and 24 h post-inoculation with fold change and functional categories.

Supplementary Table 3**.** Listing of common up-regulated (A) and down-regulated (B) apple genes following *Erwinia amylovora* challenge of apple blossoms (2, 8, and 24 h) with fold change and functional categories.

Acknowledgements

This work was partially funded by a grant received from the USDA-NIFA-SCRI grant AG 2009-51181-06023 (SSK) and an Illinois C-FAR grant 08I-020-3-Sen (YFZ).

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