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Transcriptome profile of acibenzolar-S-methyl-induced genes in tomato suggests a complex polygenic effect on resistance to *Phytophthora infestans*

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ABSTRACT

Induced resistance by chemicals such as acibenzolar-S-methyl -ASM (commercialized as Actigard by Syngenta Inc) mimics the biological activation of systemic acquired resistance (SAR). ASM takes the place of salicylic acid (SA) in the SAR signal pathway inducing the same molecular markers and range of resistance. The goal of our work was to understand the downstream molecular events by which ASM confers resistance to *Phytophthora infestans* in tomatoes. To accomplish this goal we assayed gene expression in ASM-treated plants using a microarray with more than 12,000 tomato ESTs. As many as 300 genes were responsive to ASM. Of these, 117 were detected in most of the biological replications. Basal defense associated genes as well as SAR and disease resistance genes (R-like) involved in induced resistance and effector-triggered immunity were highly expressed. We attempted to determine the phenotype of 13 of these genes by virus induced gene silencing (VIGS). These 13 genes were selected on the basis of previous implication in plant defense response and by reliability of induction by ASM. VIGS was partially successful for three of the 13 genes, but this partial silencing did not lead to a significant reduction in the effect of ASM. The ethylene pathway was also activated in response to ASM, but a tomato mutant not responsive to ethylene remained responsive to ASM. It seems most likely that the ASM effect is complex and polygenic, depending on the effect of several genes.

1. Introduction

Plants have evolved mechanisms to detect and respond effectively to an array of pathogens by constitutive or inducible defenses. Recognition of a pathogen through the detection of products of pathogen-encoded effectors (initially termed Avirulence-Avr-genes) by plant resistance (R) genes is often associated with a rapid localized programmed cell death called the hypersensitive response (HR). Following the HR, the plant accumulates salicylic acid (SA) and establishes a systemic acquired resistance (SAR) where uninfected parts of the plant develop enhanced resistance to further infection by some pathogens [1,2]. During SAR, SA is required for pathogen resistance and induction of pathogenesis related (PR) genes [3].

Induced resistance can be stimulated by chemicals mimicking the biological activation of SAR. This provides new opportunities to control plant diseases and to investigate disease resistance mechanisms in plants [4]. Two different chemicals 2,6-dichloro isonicotinic acid (INA) and its derivatives [5] and the acibenzolar-S-methyl (ASM), are the best studied resistance activators and its derivatives have been commercialized as ACTIGARD™, BION® and BOOST® [6].

It has been shown that in dicotyledonous plants such as tobacco and Arabidopsis, systemic translocation of these activators can take the place of SA in the SAR signal pathway, inducing the same molecular markers and range of resistance [7–12]. However, in wheat, ASM treatment activated a set of genes different from the set of genes activated by either the non-host pathogen *Erysiphe graminis* f. sp. *hordei* [13] or the pathogen *Fusarium graminearum* [14].

In tomato plants ASM treatment induced systemic acquired resistance (SAR) [4,15] and significantly suppressed late blight, caused by *Phytophthora infestans* [16]. ASM completely suppressed this disease on petunia while it had no detectable effect on potatoes [16].

Late blight is a devastating disease in tomatoes and potatoes worldwide, causing millions of dollars in losses and control costs annually [17]. Despite the efforts to control this disease via resistance genes in both potatoes and tomatoes this organism has been

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consistently shown to break down R gene resistance fairly rapidly [18–20]. The pathogenicity of this oomycete is the subject of intense investigation. It is now known that effectors are secreted and injected into host cells [21], and there may be more than 700 cytoplasmic effectors in the genome [22].

Currently, the control of late blight is mainly achieved by the use of fungicides. On a worldwide basis, these chemicals cost several billions of dollars annually [17]. Additionally these chemicals may be detrimental for the environment [18]. Because of all these factors, it has been proposed that the most efficient method to control this disease is integrated management [19]. An enhanced SAR could have a role in integrated management; therefore, knowledge of the mechanism by which ASM enhances resistance in tomatoes should facilitate efforts to protect plants against *P. infestans*.

The goal of our work was to understand the molecular mechanism by which ASM confers resistance to *P. infestans* in tomatoes. We used gene expression detected via a microarray in induced vs. non-induced plants. Our approach was to identify biochemical pathways that were differentially expressed during induced resistance. A comprehensive overview of the changes in ASM-treated plants and the plausible mechanisms of induced defense are discussed here. Genes detected via this process were then analyzed by either mutant plants (ethylene mutant – never ripe) or by limiting gene induction via virus induced gene silencing.

2. Materials and methods

2.1. Plant material and ASM treatment

Four-week-old tomato plants (*Solanum lycopersicum,* cultivar Sunrise) were used for the microarray experiment. Plants were grown in a greenhouse, with 12 h light and temperatures maintained between 24 and 29 °C.

On the fourth week after sowing, plants were separated into two sets, each set consisting of 9 plants. One set of plants was sprayed with water as control. The other set was sprayed with ASM (37 mg/L; label-recommended rate). Plants were sprayed to run-off with a hand held sprayer. One week after the first ASM treatment, a second ASM spray (37 mg/L), was applied to the same 9 plants while control plants were again sprayed with water. Immediately after the second treatment, plants were transferred to an inoculation chamber at 15 °C and 12 h light at 100% relative humidity (RH), maintained by an automatic humidifier (Trion model 500 Hummert International, Earth City MO). Two days after transfer to the chamber, all the leaflets of 3 plants per treatment were collected and frozen in liquid nitrogen. The remaining six plants were then inoculated with *P. infestans* to determine the effect of ASM on the outcome of the plant–pathogen interaction (see below). This experiment was repeated four times for a total of five biological trials.

2.2. Ethylene mutant (never ripe) tomato plants

To assess the role of ethylene in ASM-treated plants, four-week-old tomato plants of cultivar Ailsa Craig (wild type) and never ripe (ethylene mutants in the Ailsa Craig background) were used [23]. Plants were grown in a greenhouse under the same conditions as described above for cultivar Sunrise. On the fourth week after sowing, plants were separated into two sets, each set contained three plants per genotype (3 Ailsa Craig and 3 never ripe). ASM treatment was done as described above for the cultivar Sunrise. This experiment was repeated twice for a total of three biological trials.

2.3. Inoculum preparation and P. infestans isolate

Sunrise tomato plants were inoculated with the *P. infestans* isolate US970001, which is a member of the US-17 clonal lineage and kept in an inoculation chamber at 15 °C with 100% RH to induce sporulation. Leaflets with sporulating late blight lesions were detached from the plant and rinsed in 100 mL of distilled water to collect the sporangia; the concentration of sporangia in water was determined by using a hemacytometer and then adjusted to 20,000 sporangia per ml. Subsequently, the sporangia were incubated at 4 °C for 1 h to release zoospores. This mixture of sporangia and zoospores was applied to plants with a hand held sprayer until run off. Plants were kept in the inoculation chamber for the next 7 days and were evaluated for disease daily.

2.4. RNA extraction, probe preparation and hybridization on microarrays

RNA was extracted from Sunrise tomato plants in each of five independent biological trials. The RNA from each trial was analyzed independently. All the leaflets of three plants in each trial were pooled together at the moment of collection and immediately flash frozen in liquid nitrogen. Pooled plant tissue from each trial was ground in liquid nitrogen using a cold mortar and a pestle. Total tomato leaf RNA was extracted using the hot-phenol protocol by Perry and Francki [24] as modified by Gu et al. [25]. mRNA was isolated using Dynabeads® mRNA Purification Kit (Dynal-Biotech) following the manufacturers' instructions.

cDNA was synthesized from 0.4 to 2.0 μg of mRNA by reverse transcriptase and subsequently labeled using SuperScript™ Indirect cDNA labeling Core kit (Invitrogen) following the manufacturers' instructions. To avoid potential dye-related differences in labeling efficiency the same procedure was followed for the correspondent dye-swap Cy5™ (NO ASM) and Cy3™ (ASM) probes.

2.5. Gene expression via cDNA microarray analysis

Gene expression was analyzed using microarray technology. Tomato cDNA was hybridized on a cDNA microarray (TOM1) with approximately 12,000 tomato EST (BTI: www.sgn.cornell.edu). The MIDAS computer program [26] was used to perform dye-swap filtering on GenePix results previously converted to TAV files with the CONVERTER program (www.tigr.com). Data were normalized using the local regression technique LOWESS (Locally Weighted Scatterplot Smoothing) with the MIDAS software (www.tm4.org/midas.html). To identify genes with statistically significant changes in gene expression we analyzed the data using Significant Analysis of Microarrays (SAM) [27]. The threshold chosen was 1.5 at a delta value 0.193 with a false discovery rate between 0 and 4%. Genes were considered to be differentially expressed if they were selected by SAM in at least three of the five experiments [27].

2.6. Expression profiling of differentially expressed genes in ASM-treated tomato plants

Differentially expressed genes were classified according to their functional categories derived from Swiss-Prot (http://ca.expasy.org/sprot/) and Blast2GO interface [28] which uses the http://www.ncbi.nlm.nih.gov/BLAST/and the Gene ontology project (http://www.geneontology.org/GO.slims.shtml).

2.7. cDNA microarray validation using northern blots

We used northern blots to validate the up-regulation of several genes. Total RNAs (10 μg) from two of the biological replicates were

separated electrophoretically on a 1.2% formaldehyde-agarose gel for 3 h and transferred to a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). Hybridizations were performed using Puregene Hyb-9 hybridization solution (Gentra Systems, Plymouth, MN, U.S.A.).

DNA probes were obtained by Polymerase Chain Reaction (PCR) amplification from pBluescript SK + plasmid. PCR conditions were 1× PCR reaction buffer (Invitrogen), 50 mM $MgCl_2$, 10 mM dNTPs, 2 μM T7 primer, 2 μM T3 primer, 5U/μL Taq in a final volume of 30 μL with 25 ng of DNA template. PCR amplification conditions included an initial denaturation at 95 °C for 5 min, then 35 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min and 72 °C for 5 min. PCR products were sequenced at the Cornell University BioResource Center to confirm insert amplification. Homology of the sequences was determined at the SGN (http://solgenomics.net) and NCBI (www.ncbi.nih.gov) websites.

2.8. Cluster analysis

The gene expression data were grouped using hierarchical clustering algorithm in MeV software [29] in order to identify genes and pathways with similar expression profiles. Heat map was generated using Java-MeV program.

2.9. Virus induced gene silencing (VIGS)

The tobacco rattle virus (TRV)-based vectors (pTRV1 and pTRV2, kindly supplied by PBL) were used to investigate the role in ASM-induced resistance of the genes of interest. For the silencing vectors, we used fragment sizes of the genes of interest that ranged from 300 to 800 bp. At least two different fragment sizes and two different regions were assayed per gene to obtain the best silencing vector (data not shown).

TRV vectors were constructed using the GATEWAY® technology system (Invitrogen, USA) [30]. Once the gene of interest was cloned into TRV2, each vector was then transformed individually into *Agrobacterium* strain GV3101 and one colony selected for further experiments.

Agrobacterium infiltration was done as described in [31]. After Agroinfiltration, plants were kept at 18 °C with 12 h light. Generally, silencing was accomplished within 20 days after the inoculation with *Agrobacterium*.

We used a randomized complete block design with five replications. Each block contained ten plants. Each plant was inoculated with the pTRV2: gene of interest vector or was a control inoculated with an empty-vector or no vector. There were two plants per vector or per control. Two weeks after Agroinfiltration, one of each of the two plants/vector or control was sprayed with ASM and the other was sprayed with water. Plants carrying a vector with the *Phytoene desaturase* (PDS) were used as a visual guide to determine when gene silencing was achieved.

Once silencing was accomplished as determined by the PDS phenotype (usually at about 20 days after treatment) we collected the two youngest leaflets from each treatment plant in each replication. Each pair of leaflets was flash frozen in liquid nitrogen. This tissue was used to determine if gene silencing had occurred by semi-quantitative reverse transcription PCR. Tissue from each biological experiment was analyzed separately.

Immediately after collecting the tissue, the whole plants were hand spray inoculated until run off with the *P. infestans* isolate US970001 (US-17) at a concentration of 5000 sporangia/mL. The resulting growth of the pathogen was assessed (as described in 2.11) to determine the effect of gene silencing.

A general linear model was used to determine the effect of ASM and gene silencing in plants. Statistical analyses were carried out using MINITAB version 15.

2.10. Semi-quantitative reverse transcriptase (RT) PCR to confirm gene silencing

To determine the degree of gene silencing we used semi-quantitative RT-PCR. RT-PCR of the tomato actin gene was done as control. The primer sets used are described in Table S1. DNase-treated RNA (1 μg) was used for cDNA synthesis, using the ImProm-IITM Reverse Transcription System (Promega), following manufacturer's instructions. PCR was carried out with 2 μL of the cDNA synthesis reaction in a 30-μL volume containing 0.2 mM each of the four dNTPs, 2 μM each of the primers, and 0.5 U *Taq* polymerase. PCR conditions consisted of 1 cycle of 95 °C for 5 min, then 20, 25 or 30 cycles of: 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, and a final step of 5 min at 72 °C.

2.11. Pathogen quantification

2.11.1. Macroscopic quantification of late blight

In all experiments, plants were monitored daily for disease development and were rated visually for the percentage (0–100%) of leaves with disease symptoms for up to 7 days after inoculation (DAI). The quantitative ratings were compiled and a general linear model was performed to analyze the data using MINITAB version 15.

2.11.2. Real time quantitative PCR (rtq-PCR) for pathogen quantification

As an additional technique for pathogen quantification we used rtq-PCR, comparing the amount of pathogen in plants demonstrated to be silenced (see below) with that in control plants. We harvested three inoculated leaflets per plant at four days after inoculation – before the appearance of macroscopic symptoms. The pooled leaflets from each plant were assayed individually.

Total DNA (plant and pathogen) was extracted from inoculated tissue (ASM-treated silenced plants and ASM-treated non-silenced control plants). Tissue was harvested into liquid nitrogen four days after inoculation, and subsequently ground using a mortar and a pestle in liquid nitrogen. DNA was extracted using CTAB (cetyltrimethylammonium bromide) extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris at pH 8, and 20 mM EDTA at pH8 with 0.2% β-mercaptoethanol). DNA concentration was measured using a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany). All samples were diluted to 100 ng/μL before being used for the rtq-PCR with a total of 300 ng used per sample.

The ITS region of the pathogen rDNA was used to generate the rtq-PCR primers and probe Table S2 [32,33]. Conditions for rtq-PCR were done as in [32]. Total transcript levels were determined by rtq-PCR using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.).

A general linear model was used to determine the effect of gene silencing on *P. infestans* growth quantified by rtq-PCR. Statistical analyses were carried out using MINITAB version 15.

3. Results

3.1. Differentially expressed genes

ASM treatment induced many genes in tomato. We detected 300 genes in each biological replication that were differentially expressed. Of these, 117 were detected in at least three of the five trials (Table S3). Of the 117, three were down regulated while 114 were up-regulated after ASM treatment (Table S3).

3.2. Confirmation of microarray results

To confirm the reliability of our microarray results we performed a northern blot analysis for some of the differentially expressed genes. In Fig. 1 we show the differential expression for three of these genes: cysteine protease, PR1-a and acidic chitinase II. Each gene was used as a probe to determine its expression after ASM treatment in comparison to the water control (NO ASM). These northern analyses demonstrate that the expression of each gene was greater in the ASM-treated plants than in the plants not treated with ASM (NO ASM) (Fig. 1). To confirm that the difference in gene expression between the ASM-treated plant and the water-treated control was not due to unequal RNA loading, we show underneath the radiograph an ethidium bromide-stained gel showing equal loading of the RNA samples (Fig. 1).

3.3. Characterization of differentially expressed genes

Most of the 114 ASM-induced genes were involved in either metabolic processes or stress response as classified by Blast2GO (Fig. 2). Each sequence may belong to more than one biological process and this accounts for the total of 175 genes represented in Fig. 2. To analyze the differentially expressed pathways induced by ASM we used Mapman (http://mapman.gabipd.org). Mapman is a tool that allows us to visualize large datasets onto diagrams of metabolic and stress response pathways. In red are the up-regulated genes after ASM application (Fig. 3). Of these, approximately 65% are involved in metabolic processes (primary, secondary or cellular). The remaining genes are involved in response to biotic stress, response to other organisms and defense response, including several defense related genes, protein kinases and WRKY transcription factors (Fig. 3).

As expected, ASM did not induce the accumulation of SA, since it induces SAR either at the same site or downstream of SA; however, we have evidence that SAR was induced because the SAR marker PR1 was highly expressed [11] (Fig. 3). In addition, the ethylene pathway was highly induced (Fig. 3).

In order to discover gene patterns that might explain the effect of ASM in tomato, we performed data mining using herarchical cluster analysis [29]. A heat map was generated using Java-MeV program (Fig. 4), where up-regulated genes were grouped into cluster 1, whereas down-regulated genes were grouped into cluster 2. Up-regulated genes in cluster 1 were also grouped in sub-clusters according to their differential expression level. Genes involved in basal defense such as transcription factors of the WRKY family and ACC-oxidase grouped together in sub-clusters containing genes differentially expressed but just above the 1.5 fold threshold [34](Fig. 4). Whereas several pathogenesis related genes (PR) genes and cyclophilins, proteins involved in a wide variety of cellular processes from protein folding to protein degradation and signal transduction [35,36], as well as many genes with unknown function were sub-clustered together with genes differentially expressed above 2 fold. The three genes in cluster 2 that showed a decrease of $\leq$1.5 fold in their expression after treatment corresponded to unknown proteins.

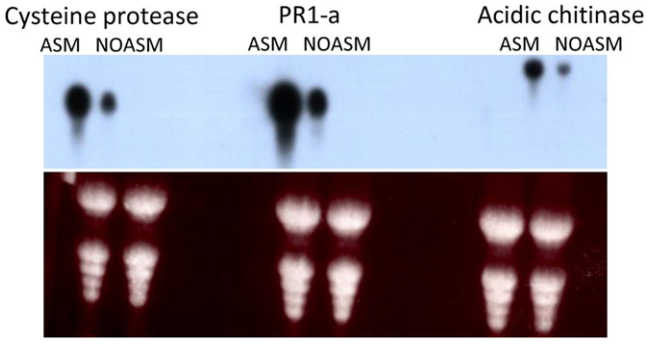

Fig. 1. Northern analysis of cysteine protease, PR1-a and chitinase gene expression in ASM-treated and control tomato plants (No ASM). For each gene, expression was higher following treatment with ASM compared to the no-ASM controls. Loading of the RNA samples is shown by the ethidium bromide stained image below the northern blots.

3.4. Determining the role of ethylene in the defense response after ASM treatment

Because the ethylene pathway was highly induced by ASM, we wanted to test whether ethylene was an essential component in the tomato response to ASM against *P. infestans*. For this purpose, we used the Never Ripe (NR) tomato mutant which is insensitive to ethylene and is therefore unable to respond to either exogenously applied or endogenously generated ethylene [23].

ASM was able to reduce significantly ($p = 0.001$) *P. infestans* growth on both NR mutants and the wild type control. We obtained similar results in all of three biological replications.

3.5. Silencing of genes of interest using VIGS

To test the role of other genes in the ASM response against *P. infestans*, we chose 13 differentially expressed genes for further investigation via VIGS. We had several criteria for choosing these genes (Table 1). Five genes were chosen because they were up-regulated in all five biological trials. These were cysteine protease, tryptophan biosynthetic process, calmodulin, cyclophilin and putative alpha-coat protein. Six genes were selected because they had been previously implicated in defense response against other pathogens. These included ethylene induced PR gene, PR1-a, acidic chitinase II, lipid transfer protein PR-14, glutathione S-transferase, and response to oxidative stress. The high mobility group protein was chosen because it showed the highest increase expression in response to ASM. Finally, an unknown protein that showed a high increase in expression after ASM treatment was also chosen (Table 1).

To predict the timing of silencing, we silenced the *Phytoene desaturase* (PDS) gene which leads to a photo-bleached phenotype [37]. VIGS in tomato produces a mosaic of silenced and not silenced tissue, and therefore the effect is only partial [30,31,38], as illustrated by the bleaching mosaic pattern produced on the tomato leaflets (Fig. 5). Therefore, on a whole leaf basis, we could only expect partial silencing of any gene.

We observed that the plants containing the empty vector or containing the vector with the gene of interest grew less vigorously than the no-vector control plants, suggesting that the virus itself produces symptoms in tomato plants. Perhaps as a consequence of this, the plants with an empty vector or a vector with the gene of interest sometimes appeared to be more susceptible than the plants with no vector. This is depicted in Fig. 6.

We were successful in reducing the ASM-induced up-regulation of three of the 13 genes. These were cysteine protease (SGN-E370972), Pathogenesis-Related 1-a (PR1-a) (SGN-E371639) and acidic chitinase II (SGN-E391165) (Fig. 7). Silencing was detected via semi-quantitative Reverse Transcriptase (RT)-PCR. After 25 cycles, cysteine protease was clearly detected in two empty-vector plants, but was still undetectable in the silenced plant (Fig. 7A). For PR1-a, strong bands were detected in two empty-vector plants after 25 cycles, but in the silenced plant, strong bands were not detected after 30 cycles (Fig. 7C). Finally, for acidic chitinase II strong bands were detected after 30 cycles in two empty-vector plants, but not in the silenced plant (Fig. 7E). The reduced expression of these three

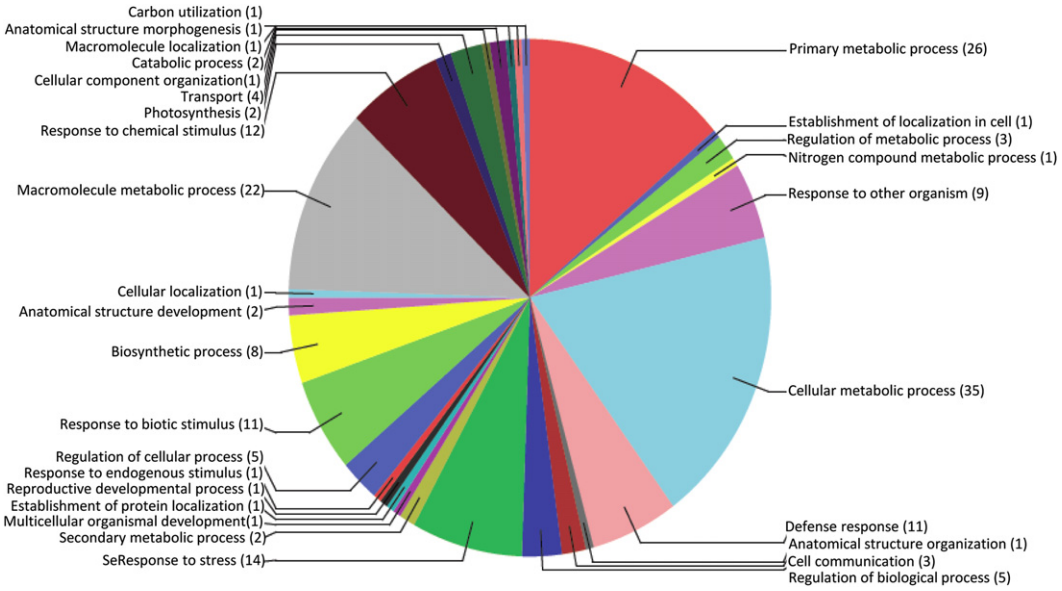

Fig. 2. Predicted biological processes of 114 genes up-regulated by ASM in at least three of five trials. The numbers in parentheses indicate the number of sequences belonging to each process. Approximately 65% of the induced genes are involved in metabolic processes and 35% are involved in response to biotic stress, defense response or response to other organisms. Each sequence may belong to more than one biological process and this accounts for the total of 175 genes represented in this chart (Blast2GO).

genes was not due to variation in template amount – the amplification of tomato actin was the same for the silenced and empty-vector plants (Fig. 7B, D and F).

3.6. Assessment of ASM treatment in non-silenced (empty-vector) and silenced plants

The effect of ASM treatment on resistance to *P. infestans* in silenced and non-silenced plants was assessed visually and via rtq-PCR. Both assessments involved non-silenced (empty-vector) control plants and treated plants in which the gene of interest was known to be silenced (as described above). The phenotypes of silenced and non-silenced plants are depicted in Fig. 8. The visual assessment data were analyzed with a general linear model (GLM). There were several results. First, we confirmed that ASM significantly ($p = 0.000$) induced resistance in tomatoes to *P. infestans* so that treated plants had less disease than non-treated plants. However, ASM-treated plants known to be silenced for cysteine

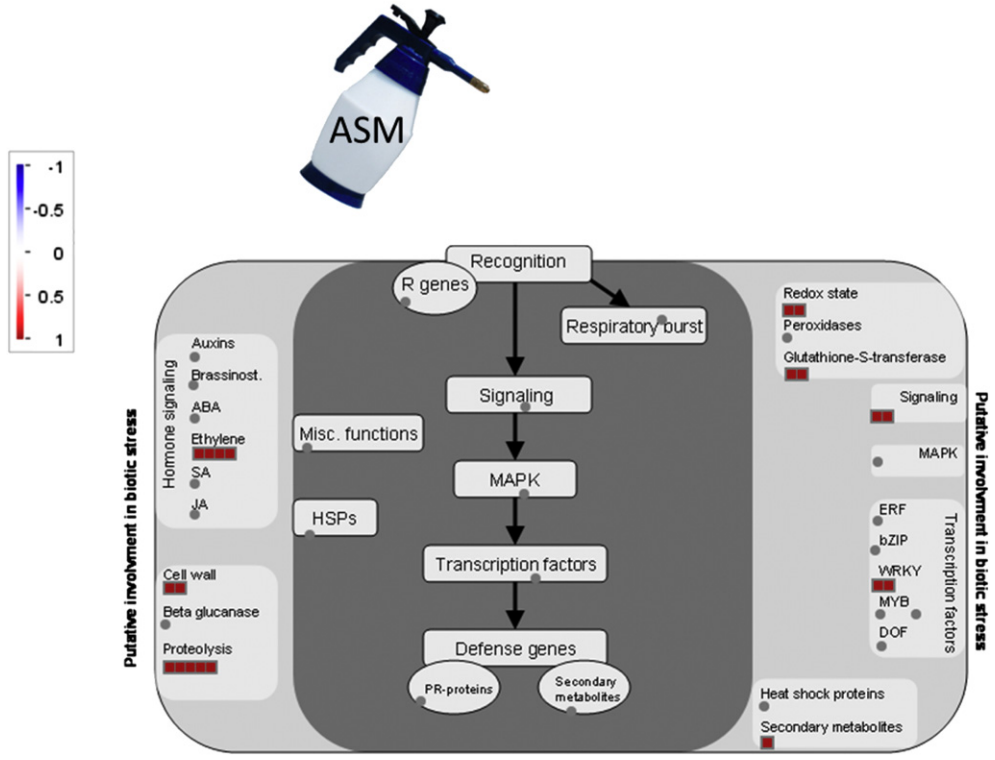

Fig. 3. Mapman (http://mapman.gabipd.org) mapping of pathways induced by ASM. ASM mimics biotic and abiotic stress signaling in plants. In red are genes that are induced by ASM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

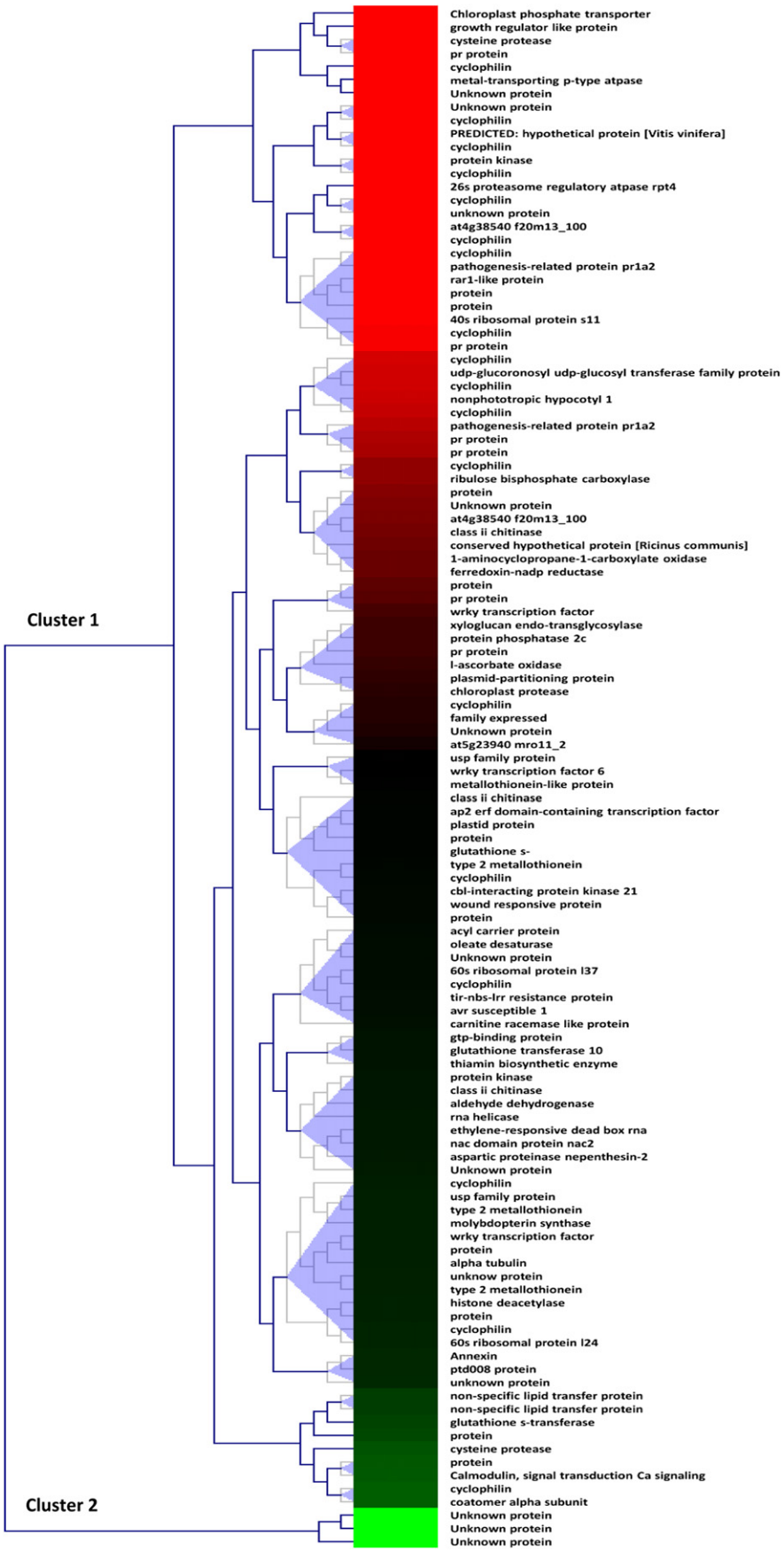

Fig. 4. Hierarchical clustering of differentially expressed genes in tomato after treatment with ASM. Microarray analysis revealed 117 genes differentially expressed after ASM treatment in three out of five biological replicas in tomato. From these, 114 were up-regulated and 3 were down-regulated. Heat map was generated using Java-MeV program.

Table 1
List of 13 genes up-regulated in tomato plants treated with ASM selected for further study.

Gene	Selection criteria	Class	Fold change	Clone I.D.
High mobility group protein potato	High fold change	Regulation of transcription	4.11	1-1-2.2.16.4 (SGN-E284306)
Pathogenesis related ethylene induced	Defense response	Defense PR	4.1	1-1-2.3.14.10 (SGN-E397191)
Pathogenesis-related 1-a	Defense response	Defense PR	4	1-1-5.3.4.17 (SGN-E371639)
Unknown protein	High fold change	Unknown	3	1-1-1.4.7.7 (SGN-E391445)
Acidic chitinase II	Defense response	Defense PR	2.58	1-1-1.2.9.4 (SGN-E391165)
Unspecific lipid transfer protein PR-14	Defense response	Defense PR	1.8	1-1-6.4.15.14 (SGN-E376842)
Glutathione S-transferase-like protein	Defense response	Defense	1.75	1-1-6.3.1.10 (SGN-E373053)
Response to oxidative stress	Defense response	Defense	1.7	1-1-6.4.18.17 (SGN-E395385)
Cysteine protease	Induced in all 5 biological trials	Response to stress	1.6	1-1-2.4.4.13 (SGN-E370972)
Tryptophan biosynthetic process	Induced in all 5 biological trials	Metabolic process	1.56	1-1-6.4.17.8 (SGN-E376482)
Calmodulin, signal transduction Ca signaling	Induced in all 5 biological trials	Calcium ion binding	1.54	1-1-8.4.1.6 (SGN-E394748)
Peptidyl-prolyl cis-trans isomerase cyclophilin	Induced in all 5 biological trials	Protein folding	1.5	1-1-3.1.14.14 (SGN-E395242)
Putative alpha-coat protein	Induced in all 5 biological trials	Intracellular protein transport	1.5	1-1-2.1.6.7 (SGN-E371326)

protease, PR1-a, or acidic chitinase II individually were not more susceptible than non-silenced plants ($p = 0.25$) (Fig. 8).

We confirmed the visual assessments of silencing with rtq-PCR using the ITS region of *P. infestans* to quantify pathogen growth in the infected tissue. Again only plants known to have silenced genes were included in the analysis. At least three silenced plants for each gene were evaluated. Pathogen growth in the silenced plants was not different from that in the non-silenced plants. Thus, silencing of cysteine protease ($p = 0.19$) (Fig. 6), PR1-a ($p = 0.25$) or acidic chitinase II ($p = 0.12$) (Fig. 9) did not eliminate or reduce the resistance to *P. infestans* induced by ASM in tomato.

4. Discussion

Our results show a complex response of tomato to ASM treatment. Interestingly, the ethylene pathway was up-regulated after ASM treatment as evidenced by the high induction of ACC-oxidase. By using microarray analysis we provide a more holistic view of the downstream components induced in tomato plants after ASM application, where basal defense seems to play an important role, with the induction of WRKY transcription factors, protein-like kinases and 1-ACC-oxidase. In addition, SAR markers (PR proteins), R-genes (TIR-NBS-LRR), metallothionein-like proteins and glutathione S-transferase were also induced. This is in agreement with previous studies of ASM-induced genes in *Arabidopsis*, tobacco, papaya and rice [7,8,39,40]. To date, studies of ASM-induced response in plants were limited to pre-selected marker genes to determine the metabolic pathways involved in resistance, thus limiting the information outside of the marker genes chosen [7,8,39,40].

The ethylene pathway was highly induced in ASM-treated plants, suggesting a role for this hormone in ASM response. Therefore, we used NR tomato mutants which are blocked in the perception but not the production of ethylene [23], to determine the role of this hormone in ASM-induced resistance. In contrast to our expectation, ASM treatment of NR tomatoes induced resistance to *P. infestans*. This is in agreement with what has been observed for *Arabidopsis* ethylene-mutants which remained responsive to ASM [8], indicating that ASM action is independent of this hormone in tomatoes as well. It appears that ethylene may have a complex role and although ethylene induction after ASM treatment might contribute to resistance against *P. infestans*, ASM action might be independent of this hormone.

Of the 114 genes induced in response to ASM, we selected 13 to investigate further using VIGS to reduce their expression. We succeeded in partially silencing three of these genes: cysteine protease, PR1-a and acidic chitinase. However, in each case, partial silencing of each gene individually did not significantly reduce the effect of ASM.

Fig. 5. Tomato plant showing VIGS with the *Phytoene desaturase* gene (PDS) as a visual marker and positive control for gene silencing. This picture illustrates the mosaic effect of silencing with VIGS, where patches of leaves are silenced while others are not. The image was captured at 20 days post inoculation with the TRV2:PDS vector.

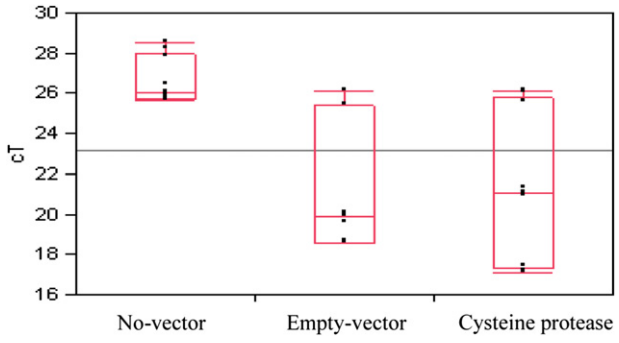

Fig. 6. Box plot representing the quantification of *P. infestans* on plants silenced for cysteine protease and the empty-vector and no-vector controls. A one-way analysis of variance of the threshold cycle (cT) of the real time PCR of the ITS region of *P. infestans* was done. Small cT values indicate more pathogen growth while higher cT values indicate less pathogen on the sample. The median is represented by the horizontal line inside the box. The smallest and largest observations are depicted by the vertical lines from the box blot (in our case we do not see outliers) and the box represents the interquartile range. In this experiment the no-vector control had less pathogen growth than the empty-vector and the silenced plants. This might be due to the effect of the virus in the plants, which causes some symptoms. Therefore, the control which does not carry a vector is less susceptible. No significant differences between the silenced plants and the empty-vector control were found. However, in this experiment, we found significant differences of pathogen growth between empty-vector plants and plants transformed with either the empty vector or the vector with the gene of interest.

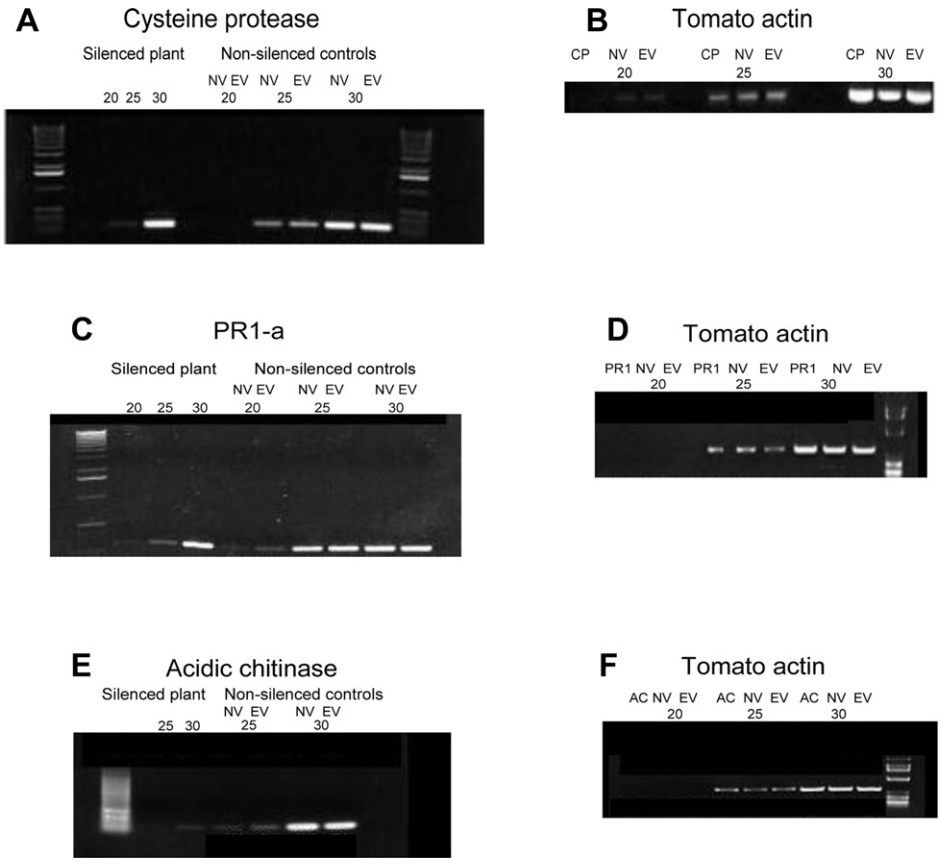

Fig. 7. The product of semi-quantitative RT-PCR at several cycles showing gene silencing for cysteine protease, PR1-a and acidic chitinase. Controls were plants containing no vector (NV) or inoculated with an empty vector (EV). Actin amplification (at 20, 25 and 30 cycles) was used as control to show equal amounts of RNA samples. (A) Amplification for cysteine protease followed by its actin control (B); (C) PR1-a with actin control (D); (E) acidic chitinase with its respective actin control (F).

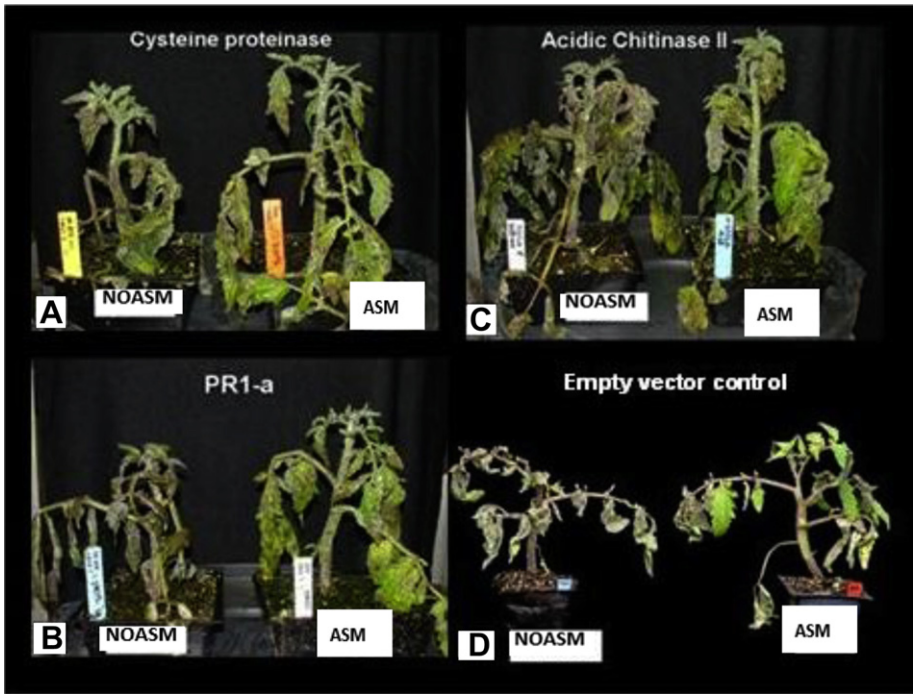

Fig. 8. Box plot representing the quantification of *P. infestans* on plants silenced for acidic chitinase II and PR1-a individually and on non-silenced plants (the empty-vector and no-vector controls). A one-way analysis of variance of the threshold cycle (cT) of the real time PCR of the ITS region of *P. infestans* was done and no significant differences between the silenced plants and their controls were found. The median is represented by the horizontal line inside the box. The smallest and largest observations are depicted by the vertical lines from the box blot (in our case we do not see outliers) and the box represents the interquartile range. In this experiment the virus produced milder symptoms on the transformed plants than in the experiment represented in Fig. 6. There were no differences in pathogen growth between the no-vector controls and the transformed plants (empty-vector, PR1-a or acidic chitinase).

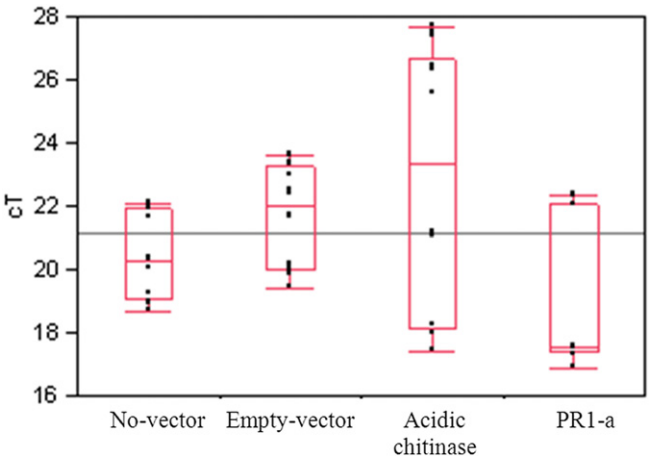

Fig. 9. Box plot representing the quantification of *P. infestans* on plants silenced for acidic chitinase II and PR1-a individually and on non-silenced plants (the empty-vector and no-vector controls). A one-way analysis of variance of the threshold cycle (cT) of the Real time PCR of the ITS region of *P. infestans* was done and no significant differences between the silenced plants and their controls were found. The median is represented by the horizontal line inside the box. The smallest and largest observations are depicted by the vertical lines from the box blot (in our case we do not see outliers) and the box represents the interquartile range. In this experiment the virus produced milder symptoms on the transformed plants than in the experiment represented in Fig. 4. There were no differences in pathogen growth between the no-vector controls and the transformed plants (empty-vector, PR1-a or acidic chitinase).

There are several factors that might have contributed to the continued ASM-responsiveness in the silenced plants. First, the effect of VIGS in tomato is incomplete leaving a mosaic of silenced and not-silenced tissue in a leaflet. It may be that leakiness between silenced and not-silenced tissue reduces the effect of the silencing. Second, the resistance induced by ASM may result from the action of many genes, each of which has a small effect, so that (partial) silencing of the gene would have a very small effect, which is very difficult to detect. Third, it is also possible that the three genes that we silenced may not have been involved in the induced resistance. Finally, it is also possible that redundancy from other members of a gene family compensated for the partial silencing of one member. Redundancy for cysteine protease and for chitinase has been reported in tomato [41–43].

Thus, we propose that the enhanced resistance phenotype observed after ASM treatment might be due to the effect of several genes with relatively small effects on disease resistance acting together instead of a single gene having a major effect on pathogen growth. Examples of the quantitative nature of resistance against *P. infestans* have been previously reported for both tomato and potato. The quantitative nature of partial resistance to *P. infestans* in wild *Solanum* species is correlated with the timing at which HR occurs as well as the quantity of PR genes expression [44,45]. In highly resistant *Solanum* cultivars the HR occurred upon inoculation and the level of PR genes expression was highest when compared to partial resistant cultivars where the HR was delayed up to 46hai and the PR gene expression was lower than for the highly resistant cultivars. Susceptible cultivars showed the lowest PR gene expression and HR was induced only occasionally [44,45]. In the case of tomato, Smart et al. [46] hypothesized that partial resistance in tomato against *P. infestans* is likely to be quantitative and a QTL for resistance on chromosome 6 was identified. Therefore, the silencing of several genes at the same time might be required to change the response of tomato to ASM.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2012.11.006.

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