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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