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Defense gene expression patterns of three SAR-induced tomato cultivars in the field

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Abstract

Plant activation is an appealing disease management tool, avoiding some of the challenges of traditional chemical control by not directly impacting the pathogen. This study examined effects of acibenzolar-*S*-methyl (ASM), a plant activator that induces systemic acquired resistance, on defense response activation in three field-grown tomato cultivars in New York. Salicylic acid, ethylene and jasmonic acid-mediated responses were monitored by following expression of a marker gene for each signaling pathway using quantitative real-time PCR over the course of two ASM applications. ASM induced salicylic acid and ethylene, but not jasmonic acid-regulated gene expression in all cultivars tested. All three cultivars demonstrated a significantly stronger gene expression response relative to the untreated control following the second ASM application. Implications of these findings on management practices are discussed.

Keywords: Tomato; *Solanum lycopersicum*; Acibenzolar-*S*-methyl; Induced defense responses; Signaling; Real-time quantitative PCR

1. Introduction

Plant activators, compounds that control disease without directly impacting the pathogen, could be useful tools for crop protection. Acibenzolar-*S*-methyl (ASM) is a commercially available activator derived from benzo[1,2,3]thiadiazole (BTH). It is a functional analog of salicylic acid known to stimulate the production of plant defense-related compounds and induce systemic acquired resistance (SAR) [1–3]. SAR is distinguished from other plant defense responses by local and systemic activation of specific pathogenesis-related genes (PR genes) [4].

ASM activates defense responses in a wide range of plants, potentially providing broad-spectrum protection [5]. Within 2 h of application, ASM can be detected in tomato leaves (both treated and distant from the application site) [6]. Under growth chamber conditions, ASM concentration was highest 2 h after treatment and levels decreased to baseline levels by 48 h in distal and 72 h in proximal leaves [6]. Other studies have found raised expression levels of the tomato gene P4 (a marker for SAR) 4 days after BTH application in the greenhouse [1]. One previous study examined the molecular effects of plant activators in the field and found that BTH-treated tomatoes exhibited higher, though not significantly different from the untreated control, levels of P4 mRNA expression 5 days after application [2].

While ASM has been found effective against a wide range of bacterial pathogens of tomato [7–12], little is known about the influence of genotype (plant cultivar), environment and crop nutrition on induced responses. Greater understanding of the possible fitness costs and differences in activation associated with induced resistance, both in the presence and absence of pathogens, is necessary to effectively implement plant activators in a pest management program [13]. The efficacy of ASM to control bacterial diseases of tomato has been assessed in several tomato growing areas in North America, though differences in defense gene activation between cultivars have not been compared [9].

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The goal of this study was to determine if plant defense responses were induced at the same rate and to the same degree in three tomato cultivars under field conditions in New York. Salicylic acid, ethylene and jasmonic acid-mediated defenses were followed using marker genes and expression quantified with quantitative real-time PCR (qRT-PCR). This information is crucial to determine the optimal application regime which will enable the highest level of disease control.

2. Materials and methods

2.1. Plant materials and treatments

Tomato (*Solanum lycopersicum*) cultivars Rutgers, Rio Grande, and Supersonic were used in this experiment. All three cultivars exhibit resistance to Fusarium wilt and Verticillium wilt. Rutgers and Rio Grande are determinate cultivars (both widely used in research studies) whereas Supersonic is indeterminate. Tomato seeds were sown in 24 cell polystyrene flats in Cornell mix, a soil-less peat mixture, with perlite and vermiculite (4:1:1). Fertilizer containing nitrogen, phosphorus and potassium (10–5–10) was added at a rate of 2.67 kg/m^3 of mix. Plants were grown under natural sunlight in the greenhouse with temperatures of 23–26 °C (day) and 20–22 °C (night). The photoperiod in Geneva, NY, is approximately 15 h/day from mid-May to mid-June. One week prior to field planting, seedlings were moved to an outdoor coldframe.

2.2. Field evaluation

Six-week-old tomato plants were planted in early June at 45.7 cm intervals. Plants were fertilized with liquid fertilizer (15:30:15 N–P–K) prior to field planting. Each plot consisted of two plants, with two plants (~140 cm) separating each treatment (ASM-treated and the untreated control) and three replications per cultivar. Tomato fertilization, irrigation and weed control were implemented following typical production practices for NY [14]. Acibenzolar-*S*-methyl (Actigard, Syngenta Crop Protection, Greensboro, NC) was applied twice, on 29 July and 5 August at the highest labeled rate (52 g/ha).

2.3. Tissue collection and RNA extraction

Two leaves (each with five to seven leaflets) were collected from individual field plots for each of three replicates. All tissue collections were made at 7:00 AM. Tomato tissue was collected at four time points relative to each ASM application, immediately prior to the first treatment (designated as day 0) and 1, 2, and 3 days following. Tissue collection preceding the second ASM treatment occurred on day 7, with successive samples taken over the next 3 days (days 8, 9 and 10). Leaf tissue was flash frozen in liquid nitrogen in the field immediately following collection and stored at −80 °C. Total tomato RNA was isolated from leaf tissue using the hot phenol protocol [15] as modified by Gu and colleagues [16] and further DNase treated (Turbo DNA-*free*, Ambion Inc., Austin, TX). RNA (1 μg) was separated electrophoretically on a 1.2% agarose formaldehyde gel to check for degradation.

2.3.1. Quantification of gene expression using qRT-PCR

Primers and fluorogenic probes for *Pin*2 and actin were developed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) (Table 1). Acidic and basic *PR*-1 primer and probe combinations were identical to those described by Block et al. [17]. All primers and probes used in this study are listed in Table 1.

Two-step qRT-PCR was performed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The first step consisted of generating cDNA from total RNA (2 μg) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Each reaction (40 μL) received 1X iScript reaction mix (RNAse inhibitors, oligo(dt) and random hexamer primers) and iScript reverse transcriptase. To check for DNA contamination, controls lacking reverse transcriptase were included. The reverse transcription reaction involved a three-step process: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min. Following the reverse transcription, samples were held at 4 °C. The cDNA product synthesized from each sample was assayed for expression levels of each of four tomato genes (acidic *PR*-1, basic *PR*-1, *Pin*2, actin).

Real-time quantitative PCR reactions were carried out in triplicate in 96-well plates. Each reaction (25 μL) was performed using the iQ Supermix Kit (Bio-Rad Laboratories) and consisted of 1X Mastermix, forward and reverse primers (300 nM final concentration) and 200 nM fluorogenic probe. Reaction parameters consisted of 95 °C for 4 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 1 min, and 72 °C for 30 s. Controls from the cDNA reaction lacking reverse transcriptase and reactions with no cDNA template were also included.

The standard curve method was used to calculate the initial transcript levels (User Bulletin ♯2, ABI PRISM 7700 Sequence Detection System, Relative quantification of gene expression, 2001, Applied Biosystems). Standard curves were produced by performing qRT-PCR on serial dilutions of tomato total genomic DNA [18]. Construction of standard curves was done by plotting the threshold cycle (Ct) against the logarithm of the known concentrations. These curves were used to calculate the absolute quantity of the product in each sample [19,20]. Relative expression values (REVs) were then calculated by normalizing against the tomato actin gene as an internal control [21]. Acibenzolar-*S*-methyl and other salicylic acid-analogs are not known to induce *Pin*2 expression, hence REVs normalized to actin are presented [22].

Analysis of acidic and basic *PR*-1 response to ASM application was determined by calibrating the samples to the mean REV of the three replicates (individual field plots) on days 0 and 7 for both the ASM-treated and untreated

Table 1
Primer and probe sequences used in this study

Gene	Primer sequence (5′-3′) F	Primer sequence (5′-3′) R	Probe sequence (5′-3′)	References
Actin	TTGCCGCATGCCATTCT	TCGGTGAGGATATTCATCAGGTT	6-FAM/CGTTTGGATCTTGCTGGTCGTGATTTAACT/TAMRA	(This study)
PinII	TGATGCCAAGGCTTGTACTAGAGA	AGCGGACTTCCTTCTGAACGT	6-FAM/TGTGGTAATCTTGGGTTCGGGATATGCC/TAMRA	(This study)
PR-1a	GAGGGCAGCCGTGCAA	CACATTTTTCCACCAACACATTG	6-FAM/TGTGGGTGTCCGAGAGGCCAGA/BHQ_1	Block et al. [17]
PR-1b	GGTCGGGCACGTTGCA	GATCCAGTTGCCTACAGGACATA	6-FAM/CAACGGATGGTGGTTCATTTCTTGCA/BHQ_1	Block et al. [17]
PR-1a[a]	CCCAAAATTCACCCCAAGACT	TCAATCCGATCCCACTTATCATT		(This study)

[a]Primer sequences used in PCR to generate northern probe.

control plots. Statistical significance was determined using the log of the REVs for each of the three marker genes tested. Values were then analyzed by analysis of variance (ANOVA) using the Proc Mixed procedure and SAS software version 9.1(Cary, NC, USA). Orthogonal contrasts were used to evaluate treatment differences in expression at each time point and P-values <0.05 were considered statistically significant as previously described by Mittapalli et al. [20]. Thus, each bar in Fig. 1 represents the average REV for the three field plots (a total of 9 qRT-PCR data points) for each cultivar $\pm$standard error.

2.3.2. Northern blot analysis

Tomato PR genes used as probes for northern analyses included basic *PR*-1 and the proteinase inhibitor gene *Pin*2, and were identical to those described by Gu et al. [16]. A probe for northern detection of acidic *PR*-1 was created by PCR amplifying tomato DNA utilizing primers listed in Table 1, followed by amplicon purification using Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, WI). Identity of the fragment was verified via sequencing performed at the Cornell University Bioresource Center. Sample RNA (10 μg) was run on a 1.2% formaldehyde–agarose gel and transferred to Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). The same RNA extractions (described above) were used for both qRT-PCR and northern analyses. Hybridizations were performed as described by Smart et al. [23].

3. Results

3.1. ASM induction of jasmonic acid-mediated gene expression

Induction of wound-inducible, jasmonic acid-mediated defenses was measured by the proteinase inhibitor marker gene *Pin*2 [1,22,24]. As expected, *Pin*2 expression was not induced by ASM application in any of the cultivars tested (Table 2). The *Pin*2 gene was induced sporadically in both treated and untreated plants, usually to a very high level (up to 553-fold increase, Rutgers, control rep 1, day 7). When activated, *Pin*2 expression usually dropped to baseline levels the following day (Table 2). In a few instances, *Pin*2 was induced at low levels for several days (Rio Grande, ASM-treated, rep 2; Rutgers, untreated control, rep 2) or consistently at a high level (Supersonic, ASM-treated, rep 1; Rutgers, control and ASM-treated, rep 1). In these cases application of ASM did not modify expression levels.

3.2. ASM induction of salicylic acid-mediated PR gene expression

Acidic *PR*-1 is a known marker for salicylic acid-mediated responses and SAR in tomato [17,25,26]. Acidic *PR*-1 was induced following application of ASM in all three tomato cultivars tested (Fig. 1, hatched bars). This

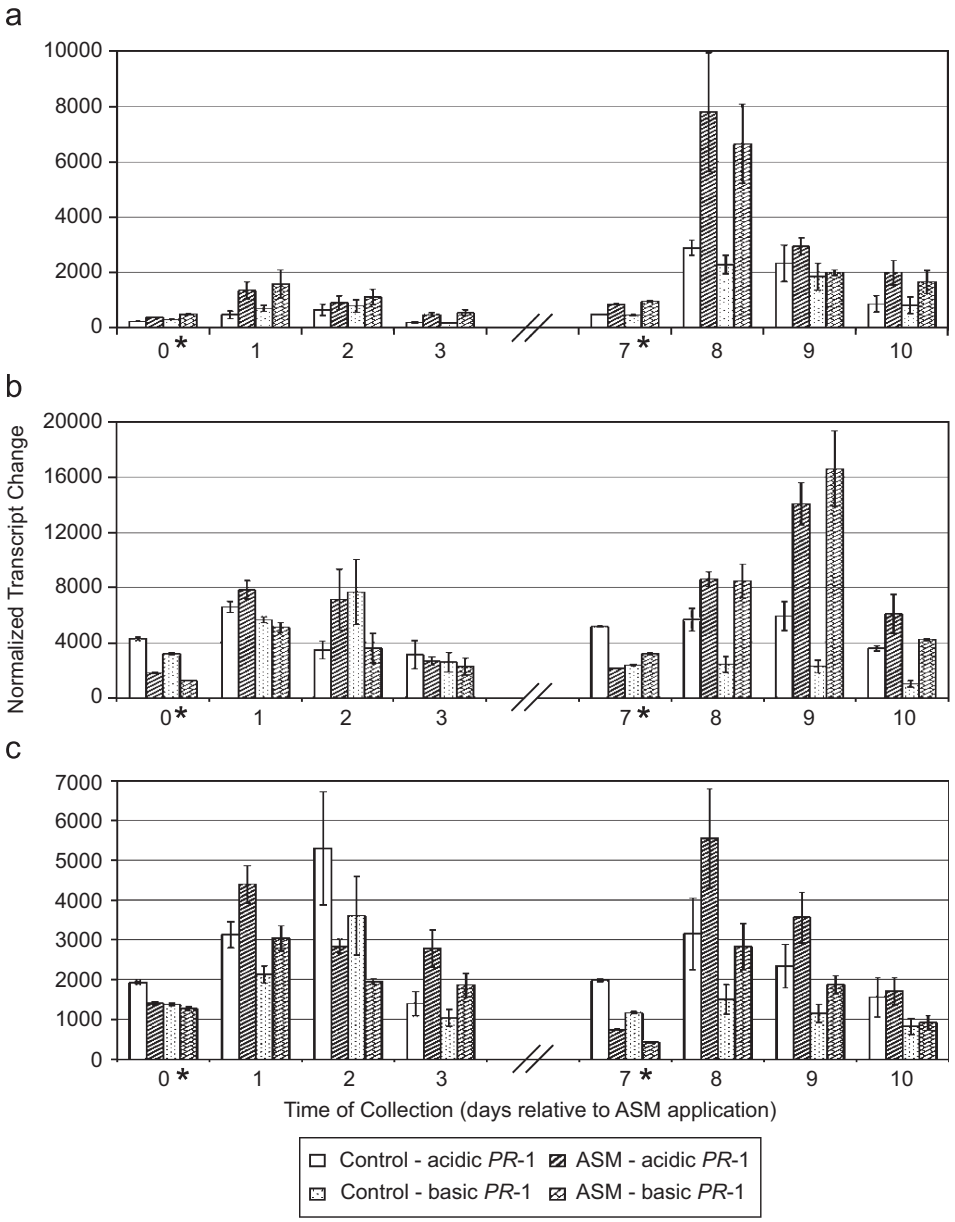

Fig. 1. Expression patterns of two tomato marker genes for signaling pathways in three field-grown tomato cultivars following acibenzolar-*S*-methyl (ASM) treatment. Top (A), middle (B) and bottom (C) panels represent gene expression in Supersonic, Rutgers and Rio Grande tomato cultivars, respectively. Numbers on the *x*-axis represent the collection dates following the two ASM applications. Acidic *PR*-1 expression is represented by solid white bars for untreated plants and hatched bars for plants that received ASM treatment. Expression of basic *PR*-1 is depicted by spotted and cross-hatched bars for untreated and ASM-treated plants, respectively. Asterisks (*) denote when ASM treatments were applied, following tissue collection on days 0 and 7. Transcript levels were calculated from triplicate data using the standard curve method and normalized to tomato actin expression as an internal control. Bars represent average induction (±SE) of gene transcripts in ASM-treated compared to untreated plants averaged over three replicate plots. Gene activation was calibrated to the mean expression value prior to ASM application (days 0 and 7) for each treatment group (ASM-treated or untreated control).

marker gene was induced to a consistently significant level following the second application in all replicates of all cultivars. Fluctuation in acidic *PR*-1 expression was observed in untreated control plants in all cultivars (Fig. 1, white bars).

3.2.1. Induction of Supersonic tomatoes

Expression levels of acidic *PR*-1 increased 1 day following the first ASM application in the Supersonic cultivar and decreased to baseline by day 3 (Fig. 1A). Acidic *PR*-1 expression was 3.6-fold ($P = 0.0019$) higher in ASM-treated plots on day 1 than on day 0. By day 7 of the experiment, acidic *PR*-1 expression remained approximately two-fold higher than initial levels in both ASM-treated and untreated plants. Similar to the response following the first ASM treatment, the Supersonic cultivar showed greatest acidic *PR*-1 expression 1 day following the second ASM application (day 8). Induction of acidic *PR*-1

Table 2
Pin2 relative expression values, normalized to tomato actin gene expression, for ASM-treated and untreated replicate plots of three tomato cultivars

Cultivar	Treatment	Rep	Days relative to ASM application							
			0	1	2	3	7	8	9	10
Supersonic	Control	1	11.75	5.55	10.97	0.56	68.03	16.15	35.55	149.62
		2	8.44	16.79	26.48	6.83	49.05	0.02	5.30	26.84
		3	5.29	0.15	9.79	1.89	37.06	12.05	12.50	0.55
	ASM	1	34.22	35.99	37.68	17.56	191.07	119.88	99.12	129.30
		2	1.77	8.62	2.60	33.64	40.02	0.69	13.18	2.17
		3	2.24	11.66	15.80	6.36	119.37	12.94	0.00	105.24
Rutgers	Control	1	15.97	13.36	6.23	9.50	8827.60	4031.50	2414.90	4870.40
		2	6.13	2536.50	198.00	0.04	115.80	1.74	72.96	31.23
		3	23.19	25.54	17.39	3.38	42.96	7.07	27.61	2.32
	ASM	1	5.33	3.17	3.68	1.86	1518.00	3835.60	5627.30	5021.60
		2	8.37	56.55	44.36	52.28	130.05	164.43	24.18	170.14
		3	2878.70	16.91	18.54	7.35	284.70	5.82	18.47	22.73
Rio Grande	Control	1	15.80	21.85	38.97	98.96	34.42	5.87	26.38	44.40
		2	9.28	822.51	45.18	0.62	28.18	55.40	96.64	217.16
		3	29.67	52.04	19.17	6.84	72.92	126.59	1819.70	62.06
	ASM	1	7.15	59.23	7.89	0.32	40.07	22.92	199.50	31.69
		2	13.81	55.62	65.67	83.53	21.17	2.65	25.76	10.63
		3	3038.70	21.73	28.42	12.06	73.81	14.38	36.68	88.72

was 9.2-fold ($P<0.0001$) higher in ASM-treated plots, than on day 7 (Fig. 1A). Thus, activation of salicylic acid-mediated responses followed similar a pattern between the two ASM applications, with a greater level of induction following the second treatment.

3.2.2. Induction of Rutgers tomatoes

Prior to ASM application (day 0), tomatoes in untreated control plots demonstrated two-fold higher acidic *PR*-1 expression than ASM-treated plots. ASM-treated Rutgers tomatoes showed increased acidic *PR*-1 expression (4.3-fold increase, $P<0.0001$) on day 1, with expression levels slowly decreasing to near-baseline levels by day 3 (Fig. 1B). Expression of acidic *PR*-1 was approximately the same on days 0 and 7, just prior to ASM application. Rutgers tomatoes had a different pattern of acidic *PR*-1 induction following the second ASM application; peak levels (6.6-fold increase from day 7, $P<0.0001$) occurred on day 9 and were characterized by a rapid decrease in expression on day 10 (Fig. 1B). While Rutgers tomatoes demonstrated greater variability than the other two cultivars in acidic *PR*-1 response to ASM, this cultivar also exhibited the highest acidic *PR*-1 expression levels.

3.2.3. Induction of Rio Grande tomatoes

Induction of salicylic acid-mediated responses following the first ASM application to Rio Grande tomatoes was observed on day 1 (3.1-fold increase, $P<0.0001$) and levels decreased slowly (Fig. 1C). The untreated control plots of Rio Grande had a sharp increase in both acidic and basic *PR*-1 expression on day 2. Expression levels of acidic *PR*-1 in ASM-treated plots were significantly lower on day 7, prior to the second application, than observed on day 0. Salicylic acid-mediated gene expression was activated on day 8, one day following the second treatment (7.5-fold increase, $P<0.0001$), with expression levels slowly decreasing after peak activation, similar to the results from the initial spray. Like the other two cultivars, Rio Grande tomatoes showed a greater response to the second ASM application than the first. Of the three cultivars tested, Rio Grande tomatoes demonstrated the most variable acidic *PR*-1 expression in untreated control plots.

3.3. ASM induction of ethylene-mediated PR gene expression

Ethylene-mediated responses, as measured by expression of the tomato basic *PR*-1 gene [17,25,26], was induced in all cultivars tested following application of ASM, generally with lower expression levels than acidic *PR*-1 (Fig. 1, cross-hatched bars). The general trends of activation were similar between salicylic acid and ethylene-mediated responses in all three cultivars. Prior to ASM application, basic *PR*-1 was expressed at low levels in all plants. Basic *PR*-1 expression fluctuated in untreated control plants, though expression changes were usually to a much lesser degree than ASM-treated plants (Fig. 1, spotted bars). In some instances, levels in untreated plots were elevated consistently over several days (Supersonic cultivar, days 7 through 10). In the Rutgers cultivar (days 0 through 3) and Rio Grande cultivar (on day 2 only), basic *PR*-1 expression in the untreated control was greater than in the ASM-treated plots.

3.3.1. Induction of Supersonic tomatoes

Basic *PR*-1 expression increased 1 day following treatment with ASM in Supersonic tomatoes (3.2-fold increase, $P = 0.0269$) and declined to near-baseline levels over days 2 and 3 (Fig. 1A). The expression level was approximately two-fold higher on day 7 then observed on day 0 prior to ASM treatment, similar to that observed for salicylic acid-mediated gene expression. Following the second ASM spray, the highest basic *PR*-1 expression levels were observed 1 day after application (day 8, 7.0-fold increase, $P < 0.0001$), and decreased to levels two-fold higher than day 7 by day 10 (Fig. 1B). As observed with salicylic acid-mediated responses, activation of ethylene-mediated responses followed a similar pattern after each ASM treatment, with a greater degree following the second ASM application.

3.3.2. Induction of Rutgers tomatoes

Activation of ethylene-mediated responses in Rutgers tomatoes increased 1 day following ASM application (4.1-fold increase, $P < 0.0001$), though the untreated plants exhibited higher expression levels than the treated plants on days 1, 2 and 3 (Fig. 1B). Expression of basic *PR*-1 decreased to levels two-fold higher than at time zero by day 3 in ASM-treated plants. As observed with acidic *PR*-1 activation, basic *PR*-1 was more highly expressed following the second ASM application. Peak activation occurred on day 9 (5.2-fold increase, $P < 0.0001$) and declined to levels 1.5-fold greater than day 7 on day 10 (Fig. 1B).

3.3.3. Induction of Rio Grande tomatoes

Induction of ethylene-mediated gene expression in Rio Grande tomatoes occurred 1 day following ASM application (2.4-fold, $P = 0.0011$, Fig. 1C), remained elevated until day 3, then dropped to below baseline levels prior to the second ASM application (day 7, Fig. 1C). Comparable to acidic *PR*-1 expression, basic *PR*-1 was more highly expressed, relative to untreated control plants, following the second ASM application. Similar expression patterns were observed following the second ASM application, with highest basic *PR*-1 activation one day after treatment (day 8, 6.7-fold increase, $P < 0.0001$). Basic *PR*-1 levels remained elevated two-fold above baseline through day 10 (Fig. 1C). As seen with cultivar Rutgers, there was significant variation in expression levels in untreated plants during the first 3 days of the experiment.

3.4. Northern validation of qRT-PCR results

Expression patterns observed using northern blot analyses of RNA extracted from tomato field samples (cultivar Rutgers) support findings from the qRT-PCR study. Data shown are from one replicate field plot of Rutgers tomato tissue; similar results were found in all three replicates. A representative northern is shown in Fig. 2 for tissue collected relative to the second ASM application (days 7 through 10). The greatest induction of salicylic acid-mediated gene expression was observed 2 days following the second ASM application (day 9), with acidic *PR*-1 levels decreasing drastically by day 10 (Fig. 2A). Fluctuations in acidic *PR*-1 expression were observed in untreated plants, though levels were significantly lower than those observed in ASM-treated plants. Ethylene-mediated PR gene expression was also activated by ASM application (as measured by basic *PR*-1 expression), though at lower levels than the salicylic acid-mediated expression (Fig. 2B). Peak expression of basic *PR*-1 was observed 2 days after the second treatment with ASM and markedly decreased by day 10. Untreated plants exhibited low, fluctuating levels of basic *PR*-1. No activation of the jasmonic acid-responsive gene *Pin*2 was observed in treated or untreated plants, except at low levels on day 7 in ASM-treated plants (Fig. 2C). The low level of activation of *Pin*2 at this time point was detectable by qRT-PCR, although was below the threshold of detection by northern blotting. The qRT-PCR results shown in Fig. 2 are not identical to those shown in Fig. 1, as they represent a single field plot replicate whereas Fig. 1 presents the combined data from all three replicates.

4. Discussion

While plant activators have been extensively studied in the greenhouse and growth chamber [27–29], this is the first experiment following activator induction in multiple tomato cultivars over time in the field. Importantly, we demonstrated that a commercially available SAR-inducing compound effectively activated tomato defense responses, though there was some variation in cultivar response.

Field experiments performed by Thaler et al. found BTH consistently induced SAR in field tomatoes 5 days after application, though not to levels significantly higher than the untreated control [2]. Other research has examined plant activator effects in tobacco grown in the greenhouse, determining that acidic *PR*-1 was activated 12 h and basic *PR*-1 three days after application, with both genes maintaining high levels of expression up to 20 days after treatment [30]. Potlakayala et al. [27] found that BTH induced *PR*-1 in greenhouse-grown canola starting 1 day after application, and remained upregulated for up to three weeks prior to pathogen challenge. It is intriguing that ASM-induced defense gene responses in the three tomato cultivars tested in this experiment decreased to baseline levels by 7 days following application (Figs. 1 and 3), a different result from the response of greenhouse-grown canola and tobacco. Levels of ASM were measured in growth chamber-grown tomatoes using HPLC analysis and were found to peak and return to baseline between 2 and 72 h after treatment [6]. It is interesting that despite the variability in activation of salicylic acid-mediated responses among the three cultivars in this study, acidic *PR*-1 expression patterns also peaked and returned to baseline within this time frame.

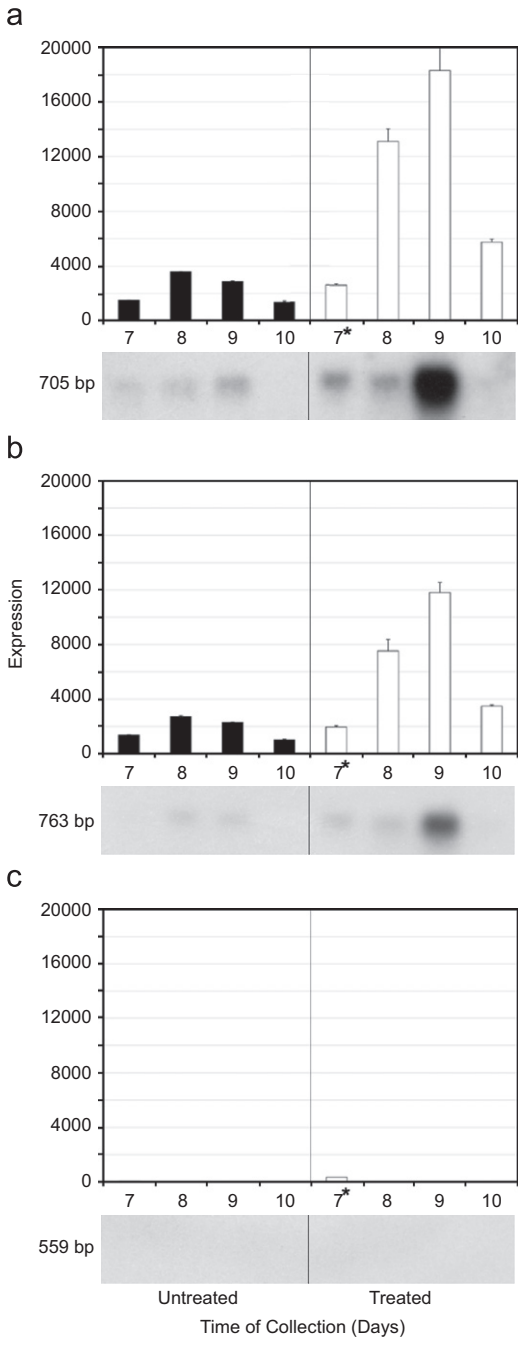

Fig. 2. Validation of qRT-PCR results using northern blot analysis, comparing ASM-treated and untreated tissue (cultivar Rutgers) from days 7 through 10. Transcript levels depicted in the graphs were determined using qRT-PCR and calculated from triplicate data using the standard curve method. Panels A, B, and C refer to the tomato defense response genes acidic *PR*-1, basic *PR*-1, and *Pin2,* respectively. Solid bars indicate results from untreated plants while open bars indicate results from plants treated with ASM. Asterisks (*) denote when ASM was applied, following tissue collection on day 7. Northern hybridizations, using RNA from the same extraction as that used for qRT-PCR, are shown below the graph. The size (in base pairs) of each defense gene mRNA is indicated to the left of each panel.

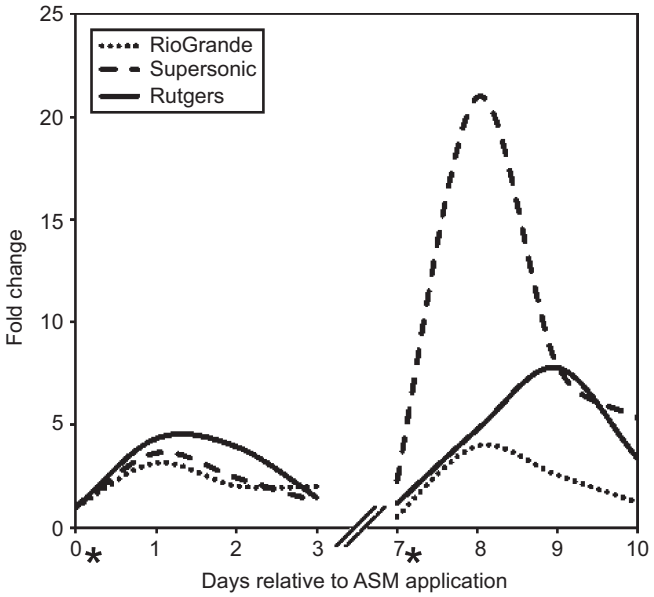

Fig. 3. Compilation of data depicting the fold change of acidic *PR*-1 expression (which serves as a marker gene for salicylic acid-mediated responses) for each tomato cultivar throughout the time course of the experiment. Changes are shown as the fold-change of acidic *PR*-1 compared to untreated plants on day 0. Asterisks (*) denote when ASM treatments were applied (immediately following tissue collection on days 0 and 7).

Environmental conditions in the field were normal over the course of the experiment, with an average temperature of 26.7 °C during the day and 16.7 °C at night. Over the 11 days of the experiment, 10.0 cm of rain fell though there were no storm or hail events. Wound-induced jasmonic acid-mediated responses were not turned on in all plants by any weather event during the experiment. In fact, no pattern was observed in wound-induced (*Pin*2) expression in any tomato cultivar (Table 2). Periodically this gene was activated, presumably due to insect, mammal or environmental damage.

While three tomato signaling pathways were monitored in this study, we were most interested in activation of salicylic acid-mediated responses, which are characterized by an increase in acidic *PR*-1 expression, as ASM induces SAR [17,25,26]. Fig. 3 depicts the fold-change induction of the three cultivars tested (relative to day 0) over the course of two ASM applications in the field. Interestingly, among the three cultivars tested, induction of salicylic acid-mediated PR gene expression exhibited a similar pattern following the first ASM application, with all three cultivars exhibiting 2.5–5-fold increases from day zero, 1 day after treatment (Fig. 3). Although acidic *PR*-1 was clearly induced in Supersonic tomatoes, the effect of ASM on the Rutgers and Rio Grande cultivars was confounded by significant acidic *PR*-1 expression in untreated control plants (Fig. 1). All cultivars exhibited near-baseline expression levels by 7 days after ASM application. Following the second ASM application, all cultivars exhibited a much more significant response relative to untreated control plants. Acidic *PR*-1 was consistently induced in the Supersonic cultivar 1 day following treatment, with drastically higher activation following the second ASM application (Fig. 3). Rio Grande tomatoes responded in a similar fashion independent of spray timing, though there was

much less fluctuation in the untreated control plants after the second ASM treatment. In contrast to Supersonic and Rio Grande cultivars, where activation of SAR followed a similar pattern between the two ASM applications, Rutgers tomatoes responded more slowly, but were induced to a greater level, following the second ASM treatment. Activation was delayed 1 day in the Rutgers cultivar, as peak expression was on day 9.

Real-time monitoring of gene expression in the field was validated with northern blot analysis (Fig. 2). Similar findings between the two methods indicate that the qRT-PCR results are accurate and no cross hybridization occurred. These data also demonstrate that qRT-PCR could detect small fluctuations in gene activation that were below the detection threshold of northern blot analysis (Fig. 2C). This is consistent with previous finding that qRT-PCR is significantly more sensitive than northern analysis [31].

Results of this study provide fundamental information on the defense signaling involved with usage of the plant activator ASM under field conditions. Variability in SAR activation among the three cultivars tested offers insight into the complexities of plant activator–tomato interactions. Tomato cultivar differences have been shown to affect the level of insect damage and degree of insect control from insecticides [32]. In sunflower, ASM-induced resistance to the parasitic plant, *Orobanche cumana* (broomrape), but level of control was cultivar dependant [33]. Knowing that field-grown tomato cultivars differ in both baseline levels of acidic and basic *PR*-1 levels as well as in the level of activation will enable future studies to determine if ASM application will have a larger impact on disease control in cultivars such as Supersonic, with a dramatic fold-increase in acidic *PR*-1 expression following the second treatment. Additionally, studying signaling pathway activation by monitoring marker gene expression following the application of a plant activator and inoculation with a pathogen is necessary to determine if pathogen attacks trigger the re-activation of plant defenses. These pathogen–plant activator interactions will further enhance our ability to integrate plant activators into disease control strategies.

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