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ORIGINAL PAPER

Elevated O_3 reduces the fitness of *Bemisia tabaci* via enhancement of the SA-dependent defense of the tomato plant

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Abstract The effect of elevated O_3 on tomato plants of three different genotypes (wild-type, a jasmonic acid (JA) defense-enhanced genotype (*35S*) and a JA-deficient genotype (*spr2*)) grown in association with the whitefly *Bemisia tabaci* Gennadius biotype B was examined in the field in open-top chambers. We experimentally tested the hypothesis that elevated O_3 tends to reduce the nutrition of tomato plants, and to increase the SA-dependent pathway defenses and the secondary metabolites, and therefore decrease the population fitness of the whitefly. The results show that for all three tomato genotypes, elevated O_3 reduced the soluble sugars and free amino acids, increased the phenylalanine ammonia-lyase enzyme activity and the accumulated salicylic acid (SA), and up-regulated the pathogenesis-related protein (*PR*1), which is commonly considered to be the whitefly-resistance gene product involved in SA-dependent defense. Elevated O_3 did not affect the JA level in any of the three plant genotypes, but it increased the levels of some secondary metabolites, including total phenolics and condensed tannins. Elevated O_3 prolonged the developmental time of whiteflies fed on the three plant genotypes, and it also reduced the fecundity and the intrinsic rate of increase of whiteflies fed on either the *35S* or the wild-type plants. These results suggest that elevated O_3 reduces the nutrition of tomato plants and enhances their SA content, relative *PR* mRNA expression and secondary metabolism, resulting in decreased fitness of whiteflies on these tomato plants.

Keywords *Bemisia tabaci* · Elevated O_3 · Jasmonic acid · Salicylic acid · Tomato

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Introduction

The global atmospheric concentration of ozone (O_3) has risen from less than 10 ppb (parts per billion) a century ago to 40 ppb today, and it is projected to continue to increase at an annual rate of 1–2% (Vinzargan 2004; Jaffe and Ray 2007). Levels of atmospheric O_3 are anticipated to reach 68 ppb by the year 2050 (Wilkinson and Davies 2010).

A growing number of studies have shown that elevated O_3 can affect plant physiology (Ashmore 2005). Elevated O_3 may change levels of primary metabolism and its allocation, leading to decreased nutritional content and relatively increased levels of secondary metabolism in plant tissues (Koricheva et al. 1998; Andersen 2003). Due to alterations in plant biochemistry, especially in the quality and quantity of soluble sugars and total phenolics, the behavioral and life-history parameters of herbivorous insects are also influenced by elevated O_3 levels (Kainulainen

et al. 2000; Jondrup et al. 2002). However, the effect of elevated O_3 on insects via changes in host plant quality vary depending on the insect and plant species investigated, the O_3 level, and the fumigation time (Holopainen et al. 1995; Jondrup et al. 2002; Holopainen 2002). Moreover, the mechanism underlying fitness changes in insects that result from host plant interactions in elevated O_3 conditions remains unclear.

Host plants serve as a food source for herbivorous insects, but they also have developed elaborate induced defense mechanisms over the long-term evolutionary course of their interactions with insects (Kusnierczyk et al. 2008). Salicylic acid (SA) and jasmonic acid (JA) are regarded as the most important phyto-hormonal mediators of the induced defense of plants against herbivores, pathogens and other stressors (Meur et al. 2008). Elevated SA levels can decrease aphid density and population growth (Cooper et al. 2004; Pegadaraju et al. 2005). Expression of the SA-inducible pathogenesis-related (*PR*) protein, which is involved in plant defense, is induced by whitefly feeding (Tamaoki et al. 2003; Kempema et al. 2007; Puthoff et al. 2010). Additionally, JA plays an important role in induced resistance to whiteflies (Zarate et al. 2007). Together, SA- and JA-dependent acquired resistance directly and negatively affects phloem-feeding insects (Cooper et al. 2004; Thompson and Goggin 2006). Elevated O_3 can change SA- and JA-mediated signaling pathways in plants (Langebartels et al. 2002; Kangasjarvi et al. 2005). For example, exposure of tobacco plants to 200 ppb O_3 results in a marked increase in the free SA content of the plants (Pasqualini et al. 2002). O_3 reacts primarily with the plasma membrane, causing alterations in lipid composition and increasing the production of linoleic acid, a precursor of JA biosynthesis (Rao et al. 2000; Creelman and Mullet 1997). Although previous studies have suggested that elevated O_3 impacts defense signaling pathways such as those mediated by SA and JA (Langebartels et al. 2002; Tamaoki et al. 2003), it is still unknown whether SA- and JA-dependent defense pathways exhibit similar responses to elevated O_3 and whether these responses, in turn, affect the performance of herbivorous insects.

The tomato is an important agricultural crop throughout the world and is also an O_3-sensitive species (Oguntimehin et al. 2010). *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is an invasive phloem pest worldwide (Boykin et al. 2007). Unlike leaf-chewing insects, whiteflies puncture leaf tissue with piercing-sucking mouthparts and feed on the phloem (Byrne and Bellows 1991). This pest has particularly afflicted tomatoes in the last decade (Bardin et al. 2008) and is causing increasing damage to tomato crops (Bleeker et al. 2009), especially in China (Jiu et al. 2007). Natural crop defenses are considered to be one of the most important components of integrated pest management (IPM) that are affected by the abiotic environment (i.e., O_3, CO_2) (Kangasjarvi et al. 2005; Sun et al. 2011). For these reasons, it is important to examine the response of the interaction between *B. tabaci* and tomato plants to elevated O_3.

Here, we hypothesize that elevated O_3 will alter the population fitness of *B. tabaci* via O_3-induced nutritional and defensive changes in plant leaves. To test this hypothesis, the effects of elevated O_3 on three tomato genotypes, including wild-type, a JA-defense-enhanced genotype (*35S*) and a JA-deficient genotype (*spr2*), in association with the phloem feeder *B. tabaci* Gennadius biotype B were examined in field open-top chambers. Our specific aims were to determine the effects of elevated O_3 on (1) nutritional content in three tomato genotypes that differed with respect to the JA-dependent defense pathway, (2) the induced defense pathways and secondary metabolites in three tomato genotypes, and (3) the developmental time, fecundity and population parameters of *B. tabaci*.

Materials and methods

Open-top chambers

Experiments were conducted using eight octagonal open-top chambers (OTCs), each 2.2 m in height and 2 m in diameter, at the Observation Station for Global Change Biology at the Institute of Zoology of the Chinese Academy of Science, in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). Four OTCs were used for each O_3 concentration treatment. In the elevated O_3 treatment, O_3 was generated from ambient air by an O_3 generator (3S-A15, Tonglin Technology, Beijing, China) and then transported to the entrances of the OTCs using a fan (HB-429, 4.1 m^3 min^{-1}, Ruiyong Mechanical and Electrical Equipment Company). Mixed air (O_3 and ambient air) was ventilated into each OTC through columniform polyvinyl chloride pipes (inner diameter 11 cm, outer diameter 16 cm). O_3 concentrations were monitored both at the fan output (Shenzhen Yiyuntian Electronic CO. LTD) and within the OTCs (AQL-200, Aeroqual). From July 28 to October 4, 2009, except for 7 rainy days, the OTCs were ventilated with air daily from 9:00 a.m. to 5:00 p.m. through a hemispherical stainless steel sprayer (diameter = 30 cm) situated 0.5 m above the canopy at a rate of approximately 15 m^3 min^{-1}, resulting in approximately two air changes per minute in each OTC. Gas concentrations were measured once every hour in each chamber receiving O_3 treatment (Chang et al. 2011).

The two O_3 concentration treatments employed were as follows: (1) current ambient atmospheric O_3 levels (average value from 9:00 a.m. to 5:00 p.m. on all air-treated days of 37.3 nmol mol^{-1}) and (2) twice the current

ambient O_3 levels (average value from 9:00 a.m. to 5:00 p.m. on all air-treated days of 72.2 nmol mol^{-1}).

Host plants

Three tomato genotypes were selected for the present study: wild-type (Wt) tomato plants (*Lycopersicon esculentum* cv. Castlemart), jasmonate-deficient *spr2* mutant tomato plants (*spr2*) and *35S*: *prosystemin* transgenic tomato plants (*35S*), which were generously provided by Professor C. Li of the Institute of Genetics and Developmental Biology at the Chinese Academy of Sciences. *L. esculentum* cv. Castlemart was the Wt parent for both the *spr2* mutant plants and the *35S* transgenic plants. The *35S*: *prosystemin* (*35S*) JA-biosynthesis mutant transgenic plants overexpress prosystemin, which constitutively activates system defenses in unwounded plants and results in stronger and more rapidly induced resistance. In contrast, the *suppressor of prosystemin-mediated responses2* (*spr2*) mutant exhibits reduced chloroplast *w*3 fatty acid desaturase, which impairs the synthesis of JA (Li et al. 2003; Sun et al. 2010). Following two weeks of growth in sterilized soil, tomato seedlings were individually transplanted into small plastic pots (14 cm diameter, 12 cm height) containing sterilized loamy soil. At approximately 40 days of age, when they were approximately 20–30 cm tall, the plants were moved to the OTCs on July 27, 2009. Each OTC contained 12 plants (4 individuals of each of the three genotypes).

Developmental time, fecundity and adult longevity of *B. tabaci*

The B-type *B. tabaci* was collected from the greenhouse of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (CAAS) in 2000 (Feng et al. 2009). After the generations of breeding, we collected the individuals of *B. tabaci* biotype B (Middle East-Asia Minor 1) (De Barro et al. 2011) on April 5, 2009. The identification of B-biotype *B. tabaci* was investigated by the method of AFLP marker (Zhang et al. 2005). The offspring of these whiteflies were reared on tomatoes. Tomato plants of uniform size were randomly selected from cultivars to each OTC, and three leaves on each plant were each inoculated with 10 pairs of whitefly adults using clip cages (3.5 cm diameter, 1.5 cm height). A total of 12 tomato leaves were thus inoculated with whitefly adults for each cultivar under each O_3 treatment. The adults were removed after 24 h of infestation, and 30 eggs were left per leaf. The developmental status of the offspring (F1) *B. tabaci* was recorded using a microscope daily until adult eclosion. After adult eclosion, pairs of newly eclosed adults from each treatment were transferred to another leaf of the same tomato plant in the same OTC using a clip cage. If a male died, another healthy male from the same treatment was immediately added. Adult longevity and fecundity for each individual whitefly were recorded daily.

Analysis of free amino acids and soluble sugars

Free amino acids were extracted according to the procedure described by Chen et al. (2004). A leaf tissue sample of 0.5 g was homogenized, and 10 ml of ethanol (70%, V/V) was added. Samples were centrifuged at 1,000*g* for 5 min following boiling and cooling. The supernatant was transferred to graduated vessels. Five milliliters of phosphate buffer (pH 8.0) and ninhydrin–ethylene glycol (3%, W/V) were added. This mixture was heated, and 70% ethanol was added to a total volume of 50 ml. This solution was agitated, and free amino acids were then measured at 570 nm with a spectrophotometer (DU 800, Beckman Coulter) using ninhydrin reagent (Sigma). For the calculation of amino acid concentrations, a standard curve was prepared using glycine.

Soluble sugars were extracted according to the procedure described by Irigoyen et al. (1992), with slight modifications. Soluble sugars were extracted from 0.5 g of lyophilized leaves using 15 ml of distilled water. These extracted samples were centrifuged at 1,000*g* for 5 min following boiling and cooling. The supernatant was transferred into a 100-ml volumetric flask and brought to 100 ml with distilled water. Soluble sugars were measured at 620 nm with a spectrophotometer (DU 800, Beckman Coulter) using anthrone reagent. The concentration of soluble sugars was estimated using the anthrone method with glucose as the standard.

PAL measurements

Phenylalanine ammonia-lyase (PAL) was extracted according to the procedure described by Solecka and Kacperska (2003), with slight modification. A 0.1-g sample of fresh leaves was homogenized on ice and extracted with 900 µl of Tris–HCl buffer (pH 7.8, polyvinylpyrrolidone (7%, W/V), 1.67 mM phenylthiourea, 0.3 M KCl, 0.4 mM ascorbic acid). This mixture was centrifuged at 10,000*g* (4°C) for 10 min, and 50 µl of the supernatant was added to 950 µl of 0.02 mol L-Phenylalanine (0.33 g L-Phenylalanine dissolved in 100 ml Tris–HCl (pH 8.8)). The mixture was measured at 290 nm using a spectrophotometer (DU 800, Beckman Coulter).

SA measurements

SA was extracted and quantified as described by Ren et al. (2010), with slight modifications. A sample of 0.5 g of

frozen leaf tissue was homogenized on ice, extracted with 3 ml of 90% methanol and centrifuged at 8,000*g* for 20 min at 4°C. The supernatant was extracted again with 2 ml of pure methanol and centrifuged. The merged supernatant was dried at 60°C in a kettle in a water bath, and 1.5 ml of 5% trichloroacetic acid was added. This mixture was centrifuged at 7,500*g* for 15 min and then extracted three times with a mixture comprising of equal volumes of ethyl acetate and cyclohexane. The organic phase containing the free SA was then dried in a speed vacuum. Following evaporation of the solvent in the collected sample, 500 µl of acetonitrile was added to the residue. The resulting solution was filtered through a 0.45-µm filter, and the filtered liquor was analyzed using high-performance liquid chromatography (HPLC).

The fractions generated during the HPLC analysis of endogenous free SA were collected by injecting 20 µl of the sample into a C_{18} reversed-phase column (5 µm, 250 mm × 4.6 mm). The column was maintained at 40°C with a gradient (0.8 ml min^{-1} flow) programmed as follows: 0/100 (5 min) to 60/40 (30 min) to 80/20 (35 min) to 0/100 (40 min hold) of acetonitrile/H_2O containing 0.5% acetic acid. UV absorption was monitored at 295 nm, and the data were analyzed using ChromQuest. The HPLC system consisted of a G1313A autosampler, a G1313A Quarpump, a G1315B DAD Detector, a G1316A Column temperature box and a G1379A DeGAS Series. SA was measured by comparing the retention time and the scan spectra library of the standard for SA. The retention time of SA was identified through a data system, and the quantitative analysis of SA was completed by plotting the results against the standard curve.

Endogenous JA measurements

Following procedures described by Ren et al. (2010), 0.5 g of fresh leaf tissue was ground to a fine powder on ice, mixed with 4 ml of 80% methanol (V/V) and kept at −20°C for 12 h. This mixture was then added to 6 µl of [9, 10]-dihydro-JA to be used as an internal standard. The total extracted preparation was centrifuged at 8,000*g* for 20 min.

The supernatant was condensed to an aqueous phase after the methanol was vaporized. The aqueous sample was frozen at −20°C and then thawed, and this was repeated three times. After the third thawing, the extract was centrifuged at 3,000*g* for 20 min. The pH of the supernatant was adjusted to 2.5–3.0 using HCl (approximately 2 mol/l). The supernatant was extracted with an equal volume of ethyl acetate and then dried. The dried extract was re-suspended in 0.1 M acetic acid and then loaded onto a C_{18} column (Waters Company). After loading, the C_{18} column was sequentially eluted with a series of solvent mixtures, collecting 5.0 ml of the solvent mixture each time. The series consisted of acetic acid/methanol at 83/17, 60/40 and 40/60, V/V. The last 4 ml eluted in 40% methanol and the first 3 ml eluted in 60% methanol were collected. Following evaporation of the solvent and esterification of the residue with excess diazomethane, the elution sample volume was adjusted to 50 µl with acetic acid and analyzed using gas chromatography/mass spectroscopy (GC/MS).

The GC/MS analysis of the extracts of endogenous JA was conducted using a DB-5-MS column (30 m × 0.32 mm × 0.25 µm, J&W Scientific, Agilent Technologies, USA). Helium was used as the carrier gas, with a constant flow rate of 0.8 ml min^{-1}. For each extract sample, 1 µl was injected in splitless mode. The injector temperature was 280°C, the GC–MS transfer line temperature was 250°C, and the source temperature was 200°C. The emission current was 150 µA, the detector voltage was 500 V, the ionization potential was 70 eV, the scan speed was 0.4 s, and the scan range was 29–450 m/z. The GC was programmed for an initial oven temperature of 50°C, a temperature increase at the rate of 20°C min^{-1} up to 180°C, a 4-min hold, an increase of 10°C min^{-1} up to 220°C and a final 15 min hold.

Endogenous JA and its internal standards (Dihydro-JA) were analyzed using full GC/MS scans. Retention times were identified using Xcalibur 1.2 and the NIST 2003 mass library and retention time. Endogenous JA was measured by GC–MS selected ion monitoring (SIM). The characteristic ions of JA (m/z) and the internal standard [9, 10]-dihydro-JA (m/z) were 151/224 and 153/226, respectively.

Relative *PR* mRNA expression

Following procedures described by Sun et al. (2011), a sample of fresh leaves from each plant was removed and stored at −78°C for real-time PCR. Each treatment combination was repeated for four biological replicates, and each biological replicate contained three technical replicates. The RNeasy Mini Kit (Qiagen) was used to isolate total RNAs from tomato leaves, and 2-µg quantities of the RNAs were used to generate the cDNAs. The mRNA of the single target gene, pathogenesis-related protein (*PR*1), was quantified using real-time quantitative PCR. Specific primers for the target gene were designed from the tomato EST sequences using PRIMER5 software (F: TACGCTA CCAACCAATGTG; R: TCCAGTTGCCTACAG GATC, fragment length = 151 bp, accession numbers: DQ159948). The PCR reactions were performed in a 20-µl total reaction volume containing 10 µl of 2 × SYBRs Premix EX TaqTM (Qiagen) master mix, 5 mM gene-specific primer and 1 µl of cDNA templates. Reactions were carried out using the MX 3500P detection system (Stratagene), with parameters as follows: 2 min at 94°C followed by 40 cycles of 20 s at 95°C, 30 s at 56°C and 20 s at 68°C, and finally one cycle of 30 s at

95°C, 30 s at 56°C and 30 s at 95°C. This PCR protocol produced melting curves that can be used to judge the specificity of PCR products. A standard curve was derived from serial dilutions to quantify the copy numbers of target mRNAs. The relative level of the target gene was standardized by comparing the copy numbers of target mRNA with copy numbers of β-actin (the "housekeeping gene"), which remain constant under different treatment conditions. The β-actin mRNAs of the control were examined in every PCR plate to eliminate any systematic error.

Analysis of total phenolics and condensed tannins

Total phenolics were extracted according to the procedure described by Kujala et al. (2000), with slight modifications. A 0.1-g sample of dried leaves was shaken with 8 ml of boiling water and placed into a water bath for 30 min at 100°C. An aliquot of 1 ml of this mixture was extracted and diluted with 1 ml of distilled water. A 2-ml volume of Folin–Ciocalteu reagent was added to the sample, and this mixture was left for 5 min, added to 4 ml of 20% Na_2CO_3 and then left for 10 min. The absorbance of the supernatant was measured at 730 nm using a spectrophotometer (DU 800, Beckman Coulter). To calculate the concentrations of total phenolics, a standard curve was prepared using gallic acid.

Condensed tannins were extracted according to the procedure described by Terrill et al. (1992), with slight modifications. A 0.1-g sample of dried leaves was shaken with 2.5 ml of methanol–HCl (10:1, V/V) and left for 24 h at room temperature. An aliquot of 1.5 ml of supernatant from this extraction was transferred into a test tube. A 3-ml volume of vanillin/methanol reagent (4% (W/V) in methanol) and 1.5 ml of HCl were added to the test tube, which was then wrapped with silver paper. The mixture was incubated in a water bath for 20 min at 20°C. Condensed tannins were measured at 510 nm using a spectrophotometer (DU 800, Beckman Coulter). For calculating the concentrations of condensed tannins, a standard curve was prepared using catechin.

Population fitness estimation

As an index of fitness, the intrinsic rate of increase (r_m) was analyzed based on the age-stage, two-sex life table model developed by Chi and Liu (1985) and Chi (1988). The jackknife method was used to estimate the means and standard errors of the population parameters (Sokal and Rohlf 1995). The TWOSEX-MSChart computer program (Chi 2004), developed for data analysis and jackknife estimation using Visual Basic in the Windows operating system, was used. This program is available at http://140.120.197.173/Ecology/prod02.htm (Chung Hsing University) and http://nhsbig.inhs.uiuc.edu/wes/chi.html (Illinois Natural History Survey) (Yin et al. 2009).

Statistical analyses

Two-way factorial ANOVAs (SAS 6.12, SAS Institute Inc., USA, 1996) were used to analyze tomato genotypes, O_3 levels and their interactions on the population parameters of *B. tabaci* (e.g., the larval stage, the pupal stage, the combined larval and pupal stages, fecundity and intrinsic rate of increase (r_m)), and the chemical composition of tomato leaves (e.g., soluble sugars, free amino acids, phenylalanine ammonia-lyase (PAL) activity, free SA, JA, relative *PR* mRNA expression, total phenolics and condensed tannins). Differences among means were determined using Tukey's test at $P < 0.05$. Pearson's correlations were calculated in order to analyze the relationships between the developmental time, fecundity and r_m of *B. tabaci* and the soluble sugars, free amino acids, SA levels, relative *PR* mRNA expression, total phenolics and the condensed tannin content of tomatoes grown under the two atmospheric O_3 conditions.

Results

Soluble sugar and free amino acid content of tomato plants

The atmospheric O_3 level significantly influenced the soluble sugar and free amino acid content of the tomato plants (Table 1). Elevated O_3 decreased the soluble sugar content by 37.17% in the Wt genotype ($F_{1,\ 4} = 13.659$, $P = 0.021$), by 45.46% in the *35S* genotype ($F_{1,\ 4} = 22.020$, $P = 0.009$) and by 47.15% in the *spr2* genotype ($F_{1,\ 4} = 22.532$, $P = 0.009$) (Table 1, Fig. 1A). Elevated O_3 decreased the free amino acid content by 24.92% in the Wt genotype ($F_{1,\ 4} = 11.133$, $P = 0.029$), by 29.25% in the *35S* genotype ($F_{1,\ 4} = 53.147$, $P = 0.002$) and by 35.62% in the *spr2* genotype ($F_{1,\ 4} = 23.946$, $P = 0.008$) (Table 1, Fig. 1B). Within each O_3 level, no significant influence of tomato genotype on either the soluble sugar content or the free amino acid content was observed (Table 1, Fig. 1A, B).

PAL activity of tomato plants

O_3 level, genotype and the interaction between these two factors significantly influenced the PAL content of the tomato plants (Table 1). Elevated O_3 increased the PAL content by 27.11% in the Wt genotype ($F_{1,\ 4} = 14.538$, $P = 0.019$), by 69.64% in the *35S* genotype ($F_{1,\ 4} = 118.500$, $P < 0.001$) and by 41.71% in the *spr2* genotype

Table 1 Results of the ANOVA performed to test the effects of O_3 level and plant genotype on the population parameters of *B. tabaci* and the biochemical indices of tomato leaves

Measured indices	O_3		Genotype		O_3 × genotype	
	F	*P*	*F*	*P*	*F*	*P*
Free amino acids	26.83	0.000	2.20	0.174	1.09	0.381
Soluble sugars	50.04	0.002	3.05	0.104	0.61	0.568
PAL[a]	23.78	0.000	89.62	0.001	22.77	0.000
Free SA[b]	111.46	0.000	11.43	0.005	9.43	0.008
JA[c]	7.45	0.058	8.36	0.011	0.36	0.707
PR1[d]	1164.68	0.000	37.93	0.000	33.58	0.000
Total phenolics	36.00	0.004	7.04	0.017	0.95	0.425
Condensed tannins	42.74	0.003	4.58	0.047	2.06	0.190
Larval stage	215.96	0.000	15.33	0.000	6.72	0.001
Pupal stage	2.34	0.177	11.05	0.000	2.67	0.070
Larval to adult stage	173.24	0.000	19.30	0.000	3.32	0.037
Fecundity	10.67	0.017	2.28	0.107	1.17	0.313
r_m^e	6.16	0.040	35.69	0.000	3.45	0.065

[a] Phenylalanine ammonia-lyase

[b] Free salicylic acid

[c] Jasmonic acid

[d] Relative pathogenesis-related mRNA expression

[e] The intrinsic rate of increase

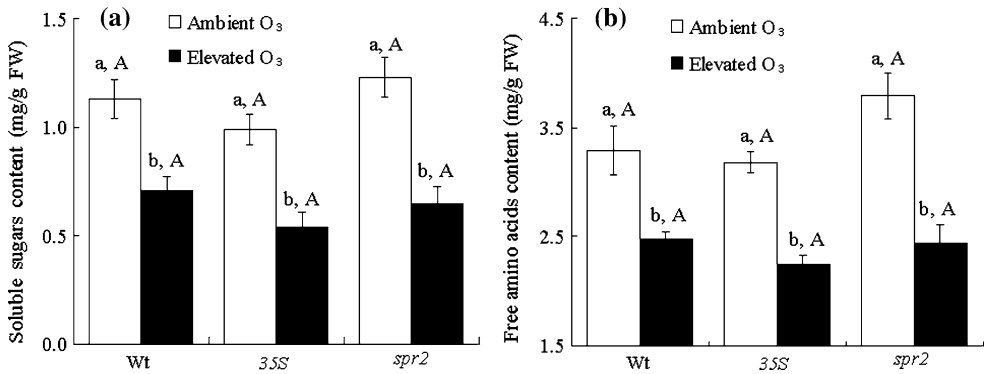

Fig. 1 The measured concentrations of soluble sugars (**A**) and free amino acids (**B**) in the three tomato genotypes (Wt, *35S* and *spr2*) grown in ambient and elevated O_3 levels for three weeks. Each value represents the average (±SE) of three replicates. Different *lowercase letters* indicate significant differences between the O_3 treatments within a tomato cultivar, and different *uppercase letters* indicate significant differences between tomato cultivars within a given O_3 level (Tukey's test: $P < 0.05$)

($F_{1,\ 4} = 98.886$, $P = 0.001$). The PAL content of the *35S* plants ($F_{2,\ 6} = 40.101$, $P < 0.001$) was the highest among the three tomato genotypes under elevated O_3 levels (Table 1, Fig. 2).

Free SA and JA content of tomato plants

O_3 level, genotype and the interaction between these two factors significantly influenced the free SA content of the tomato plants (Table 1). Elevated O_3 increased the free SA content 1.38-fold in the Wt genotype ($F_{1,\ 4} = 101.142$, $P = 0.001$), 2.30-fold in the *35S* genotype ($F_{1,\ 4} = 51.921$, $P = 0.002$) and 1.06-fold in the *spr2* genotype ($F_{1,\ 4} = 23.031$, $P = 0.009$). The free SA content of the *35S* plants ($F_{2,\ 6} = 13.456$, $P = 0.006$) was the highest among the three tomato genotypes under elevated O_3 levels (Table 1, Fig. 3A).

Genotype significantly influenced the JA content of tomato plants (Table 1). Elevated O_3 did not influence JA content in any of the three tomato genotypes (Wt: $F_{1,\ 4} = 3.069$, $P = 0.155$; *35S*: $F_{1,\ 4} = 2.781$, $P = 0.171$; *spr2*: $F_{1,\ 4} = 5.657$, $P = 0.076$). Regardless of the O_3 level, the JA

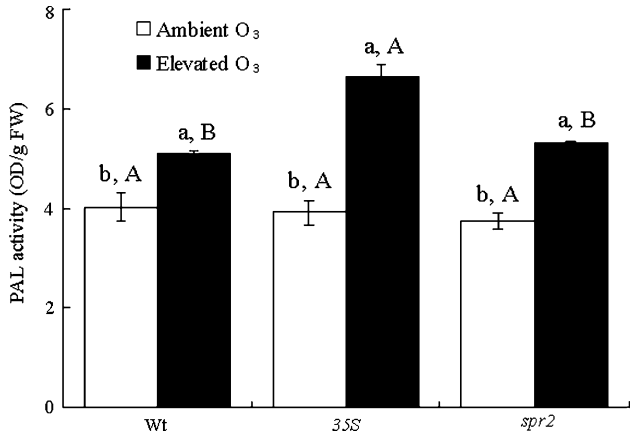

Fig. 2 The phenylalanine ammonia-lyase (PAL) activity measured in the three tomato genotypes (Wt, *35S* and *spr2*) grown in ambient and elevated O_3 levels for 3 weeks. Each value represents the average (±SE) of three replicates. Different *lowercase letters* indicate significant differences between O_3 treatments within a tomato cultivar, and different *uppercase letters* indicate significant differences between tomato cultivars within a given O_3 level (Tukey's test: $P < 0.05$)

content of the *35S* plants was higher than that of the *spr2* plants (Table 1, Fig. 3B).

Relative *PR* mRNA expression of tomato plants

O_3 level, genotype and the interaction between these two factors significantly influenced the relative *PR* mRNA expression in the tomato plants (Table 1). Elevated O_3 increased the relative *PR* mRNA expression 7.03-fold in the Wt genotype ($F_{1,\ 6} = 302.370$, $P < 0.001$), 10.67-fold in the *35S* genotype ($F_{1,\ 6} = 149.976$, $P < 0.001$) and 4.17-fold in the *spr2* genotype ($F_{1,\ 6} = 113.278$, $P < 0.001$). The relative *PR* mRNA expression of the *35S* plants ($F_{2,\ 9} = 48.355$, $P < 0.001$) was the highest among the three tomato genotypes under elevated O_3 levels (Table 1, Fig. 4).

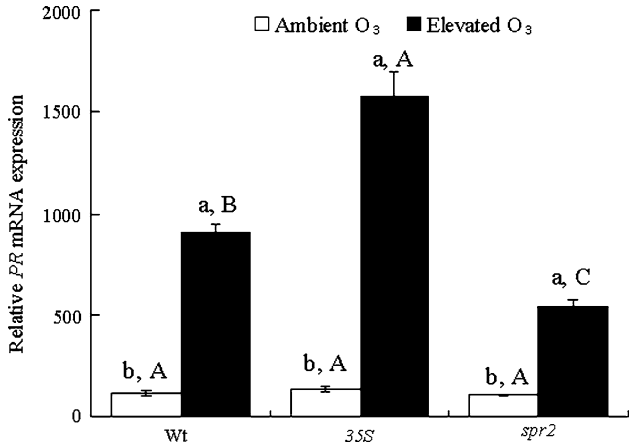

Fig. 4 The relative expression of pathogenesis-related protein (*PR*) mRNA measured in the three tomato genotypes (Wt, *35S* and *spr2*) grown in ambient and elevated O_3 levels for 3 weeks. Each value represents the average (±SE) of four replicates. Different *lowercase letters* indicate significant differences between O_3 treatments within a tomato cultivar, and different *uppercase letters* indicate significant differences between tomato cultivars within a given O_3 level (Tukey's test: $P < 0.05$)

Total phenolics and condensed tannin content of tomato plants

O_3 level and genotype significantly influenced the total phenolics in the tomato plants (Table 1). Elevated O_3 increased the total phenolics by 51.12% in the Wt genotype ($F_{1,\ 4} = 8.357$, $P = 0.045$), by 49.32% in the *35S* genotype ($F_{1,\ 4} = 19.082$, $P = 0.012$) and by 37.28% in the *spr2* genotype ($F_{1,\ 4} = 8.786$, $P = 0.041$). The total phenolics in the *35S* plants ($F_{2,\ 6} = 7.96$, $P = 0.020$) was the highest among the three tomato genotypes under elevated O_3 levels (Table 1, Fig. 5A).

O_3 level and genotype significantly influenced the condensed tannin content of the tomato plants (Table 1). Elevated O_3 increased the condensed tannin content by

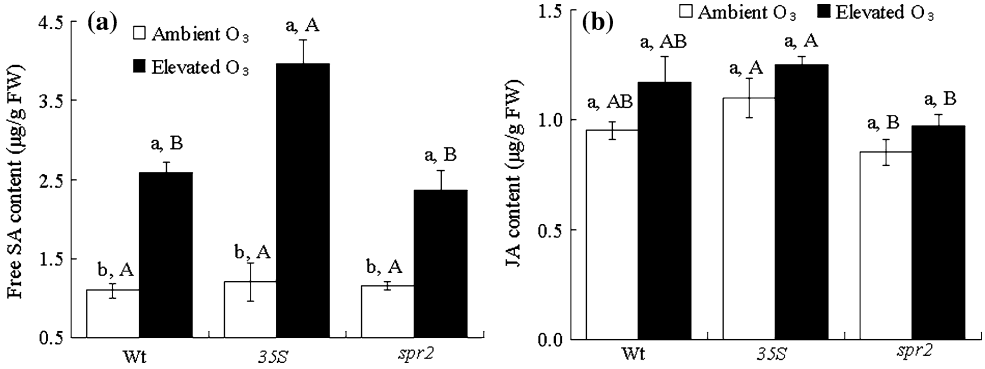

Fig. 3 The measured concentrations of free salicylic acid (**A**) and jasmonic acid (**B**) in the three tomato genotypes (Wt, *35S* and *spr2*) grown in ambient and elevated O_3 levels for 3 weeks. Each value represents the average (±SE) of three replicates. Different *lowercase letters* indicate significant differences between O_3 treatments within a tomato cultivar, and different *uppercase letters* indicate significant differences between tomato cultivars within a given O_3 level (Tukey's test: $P < 0.05$)

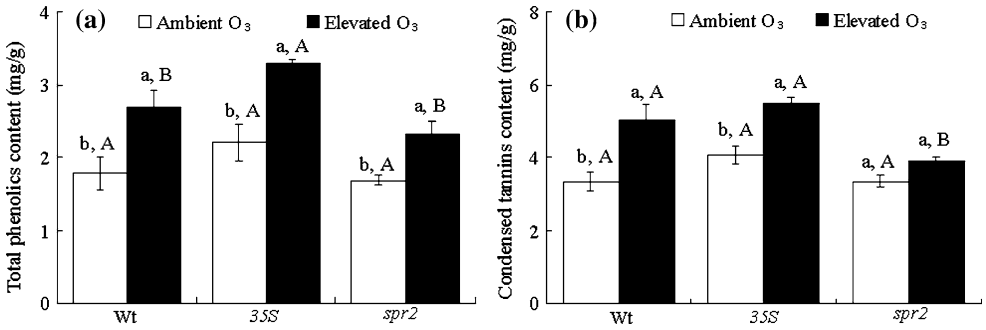

Fig. 5 The concentrations of total phenolics (**A**) and condensed tannins (**B**) measured in the three tomato genotypes (Wt, *35S* and *spr2*) grown in ambient and elevated O_3 levels for three weeks. Each value represents the average (±SE) of three replicates. Different *lowercase letters* indicate significant differences between the O_3 treatments within a tomato cultivar, and different *uppercase letters* indicate significant differences between tomato cultivars within a given O_3 level (Tukey's test: $P < 0.05$)

51.05% in the Wt genotype ($F_{1,\ 4} = 10.724$, $P = 0.031$) and by 34.89% in the *35S* genotype ($F_{1,\ 4} = 24.493$, $P = 0.008$).The condensed tannin content of the *35S* plants was higher than that of the *spr2* plants under elevated O_3 levels (Table 1, Fig. 5B).

Developmental time, fecundity and intrinsic rate of increase (r_m) of *B. tabaci*

Developmental time of *B. tabaci*

O_3 level, tomato genotype and the interaction between these two factors significantly influenced the duration of the larval stage and the total duration of the larval and pupal stages of whiteflies. Tomato genotype significantly influenced the duration of the pupal stage of whiteflies (Table 1).

Elevated O_3 increased the duration of the larval stage of whiteflies by 12.22% for the Wt genotype ($F_{1,\ 142} = 54.920$, $P < 0.001$), by 11.72% for the *35S* tomato genotype ($F_{1,\ 142} = 56.819$, $P < 0.001$) and by 4.64% for the *spr2* genotype ($F_{1,\ 142} = 7.646$, $P = 0.006$) (Table 1, Fig. 6A). Elevated O_3 increased the total length of the larval and pupal stages of whiteflies by 9.65% for the Wt genotype ($F_{1,\ 142} = 56.744$, $P < 0.001$), by 9.71% for the *35S* tomato genotype ($F_{1,\ 142} = 48.844$, $P < 0.001$) and by 5.41% for the *spr2* genotype ($F_{1,\ 142} = 13.162$, $P < 0.001$) (Table 1, Fig. 6C).

Tomato genotype influenced the duration of the larval stage ($F_{2,\ 213} = 18.047$, $P < 0.001$) and the total duration of the larval and pupal stages ($F_{2,\ 213} = 18.222$, $P < 0.001$) of whiteflies under elevated O_3 levels (Table 1, Fig. 6A, C). Tomato genotype also influenced the duration of the pupal stage ($F_{2,\ 213} = 11.070$, $P < 0.001$) and the total duration of the larval and pupal stages ($F_{2,\ 213} = 3.813$, $P = 0.024$) of whiteflies under ambient O_3 levels (Table 1, Fig. 6B, C). Within each O_3 level, the total duration of the larval and pupal stages of whiteflies was longer on the *35S* plants than on the *spr2* plants (Fig. 6C).

Fecundity of *B. tabaci*

O_3 level significantly influenced the fecundity of whiteflies (Table 1). Elevated O_3 decreased the fecundity of whiteflies by 19.33% for the Wt genotype ($F_{1,\ 44} = 5.683$, $P = 0.022$) and by 34.76% for the *35S* tomato genotype ($F_{1,\ 44} = 9.566$, $P < 0.001$). The effect of elevated O_3 on whitefly fecundity was not significant for *spr2* plants. Whitefly fecundity did not differ significantly across tomato genotypes for either of the O_3 levels (Table 1, Fig. 6D).

Intrinsic rate of increase (r_m) of *B. tabaci*

O_3 level and tomato genotype significantly influenced the r_m of whiteflies (Table 1). Elevated O_3 decreased the r_m of whiteflies by 12.20% for the Wt genotype ($F_{1,\ 6} = 7.580$, $P = 0.033$) and by 13.97% for the *35S* tomato genotype ($F_{1,\ 6} = 8.156$, $P = 0.029$) (Table 1, Fig. 6E). The effect of elevated O_3 on the r_m of whiteflies was not significant for *spr2* plants. Different genotypes significantly influenced the r_m of whiteflies under two O_3 levels. Regardless of the O_3 level, the r_m of whiteflies on the *35S* plants was lower than those on the *spr2* plants (Fig. 6E).

Pearson correlations between population parameters of *B. tabaci* and biochemical indices of tomato leaves

The duration of the whitefly developmental period was negatively correlated with the concentrations of soluble sugars ($P = 0.022$, $r = -0.877$) and free amino acids ($P = 0.019$, $r = -0.884$) in the tomato plants, and whitefly fecundity was positively correlated with these two measures (soluble sugars: $P = 0.017$, $r = 0.893$; free amino acids: $P = 0.022$, $r = 0.875$). Whitefly developmental period was positively correlated with the total

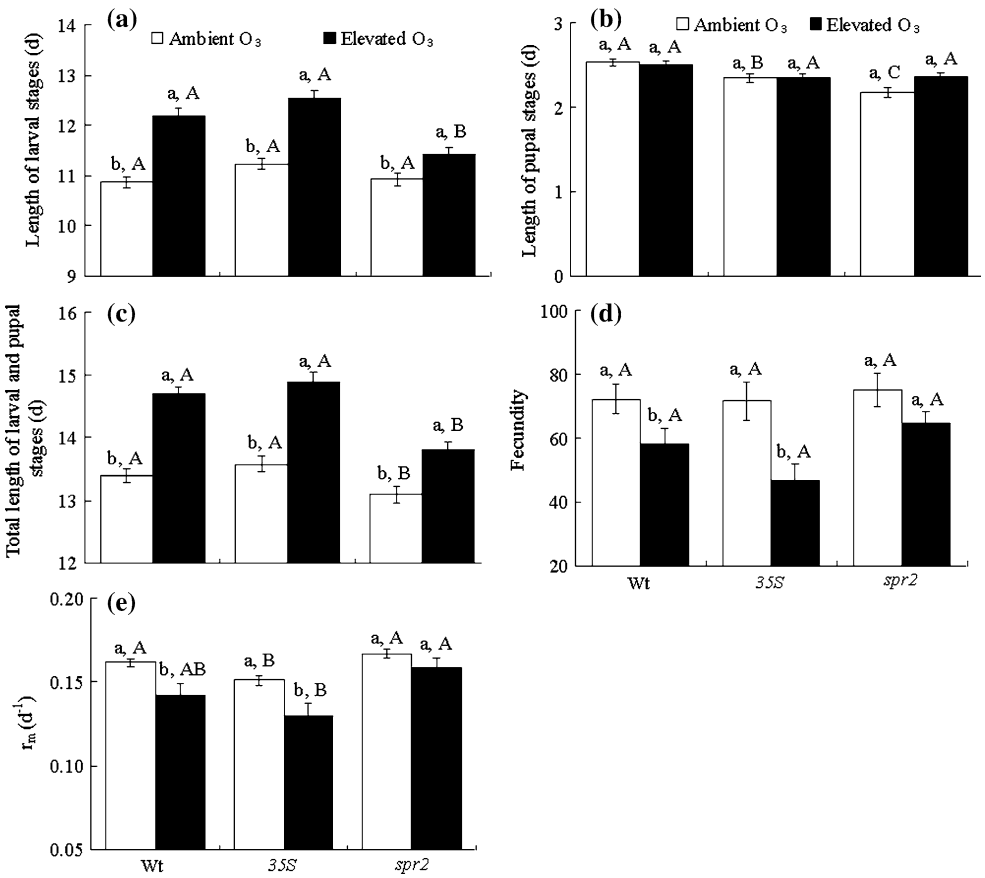

Fig. 6 Developmental times (larval stage (**A**), pupal stage (**B**) and both of these combined (**C**)), fecundity (**D**) and intrinsic rate of increase (r_m, **E**) of whiteflies reared on three tomato genotypes (Wt, *35S* and *spr2*) grown in ambient and elevated O_3 levels. Each value represents the average (±SE) across all individuals within a treatment. Different *lowercase letters* indicate significant differences between O_3 treatments within a tomato cultivar, and different *uppercase letters* indicate significant differences between tomato cultivars within a given O_3 level (Tukey's test: $P < 0.05$)

phenolics ($P = 0.003$, $r = 0.955$) and the condensed tannin content ($P = 0.022$, $r = 0.878$) of the tomato plants, and whitefly fecundity was negatively correlated with these two measures (total phenolics: $P = 0.001$, $r = -0.975$; condensed tannins: $P = 0.031$, $r = -0.851$). The r_m of the whiteflies was also negatively correlated with the total phenolics ($P = 0.002$, $r = -0.963$) and the condensed tannin content ($P = 0.011$, $r = -0.912$) of the tomato plants. Whitefly developmental period was positively correlated with the free SA content ($P = 0.011$, $r = 0.915$) and the relative *PR* mRNA expression ($P = 0.005$, $r = 0.942$) of the tomato plants, and whitefly fecundity was negatively correlated with both of these measures (free SA: $P < 0.001$, $r = -0.985$; relative *PR* mRNA expression: $P < 0.001$, $r = -0.995$). The r_m of the whiteflies was also negatively correlated with the free SA content ($P = 0.028$, $r = -0.859$) and relative *PR* mRNA expression ($P = 0.013$, $r = -0.905$) of the tomato plants (Table 2).

Discussion

Elevated O_3 can affect herbivores by altering the nutritional quality, the secondary metabolites and the resistance of plants (Percy et al. 2002; Agrell et al. 2005). However, the observed effects of elevated O_3 on herbivorous insects are often inconsistent across species (Jondrup et al. 2002). Generally, the developmental rates of chewing herbivorous insects are enhanced when these insects feed on O_3-exposed plants (Jackson et al. 2000; Mondor et al. 2004). Phloem-feeding insects such as aphids exhibit variable responses, including increased, decreased, or unchanged growth or oviposition rates, when fed on plants grown in conditions of increased O_3 (Holopainen 2002; Menendez et al. 2010). In our study, the exposure of host tomato of three genotypes to elevated O_3 prolonged the duration of the developmental period in whiteflies. This was true for both the duration of the larval stage alone and the duration of the combined larval and pupal stages. Furthermore, elevated O_3 reduced the fecundity and the r_m of whiteflies fed on tomato plants of the *35S* and Wt genotypes. These results suggest that, in terms of r_m, elevated O_3 negatively affects the fitness of whiteflies.

Changes in growth-limiting resources, such as the physical and chemical qualities of plant tissues, are expected to affect herbivorous insects (Percy et al. 2002; Agrell et al. 2005). Plant quality, particularly with respect to amino acid and carbohydrate levels, can influence the performance of phloem-sap-feeding insects (Awmack et al. 2004). Nitrogen, one of the basic nutrients required for

Table 2 Pearson correlations between population parameters of *B. tabaci* and biochemical indices of tomato leaves

Tomato constituents	Developmental time		Fecundity		r_m^a	
	r	*P*	*r*	*P*	*r*	*P*
Soluble sugars	−0.877*	0.022	0.893*	0.017	0.779	0.068
Free amino acids	−0.884*	0.019	0.875*	0.022	0.789	0.062
Total phenolics	0.955*	0.003	−0.975**	0.001	−0.963**	0.002
Condensed tannins	0.878*	0.022	−0.851*	0.031	−0.912*	0.011
Free SA[b]	0.915*	0.011	−0.985**	0.000	−0.859*	0.028
*PR*1[c]	0.942**	0.005	−0.995**	0.000	−0.905*	0.013

[a] The intrinsic rate of increase

[b] Free salicylic acid

[c] Relative pathogenesis-related mRNA expression

* $P < 0.05$

** $P < 0.01$

growth, is frequently limiting for phytophagous animals (Mattson 1980). Free amino acids in plants represent the major source of nitrogen for herbivorous insects (Helden et al. 1994). Low levels of available amino acids in host plants adversely affect life-history traits of whiteflies and aphids (Blackmer and Byrne 1999). In several species of aphids as well as in *B. tabaci*, fecundity has been shown to exhibit a close positive relationship with the amino acid and soluble sugar levels of host plants (Bentz et al. 1995; Douglas 2003). In our study, we similarly found that the developmental rate and fecundity of *B. tabaci* is positively correlated with the levels of soluble sugars and free amino acids in tomato plants. Moreover, the levels of free amino acids and soluble sugars in these plants were reduced under conditions of elevated O_3, resulting in longer developmental time of the whiteflies fed on three plant genotypes and lower fecundity of the whiteflies fed on the *35S* and wild-type plants. Thus, we conclude that elevated O_3 prolongs whitefly development and reduces fecundity in this species by reducing the nutritional content of the tomato plants that serve as its host.

The natural defenses of plants are also important to insect performance (Pegadaraju et al. 2005). JA and SA have been recognized as important endogenous regulatory signals that mediate the chemical defenses of plants against numerous stressors such as O_3, herbivores and physical injury (Meur et al. 2008). Previous studies have found that plant JA content increases significantly after 12 h under 200 ppb O_3 or 6 h under 300 ppb O_3 (Rao et al. 2000; Tamaoki et al. 2003). However, our study found no effect of elevated O_3 on JA content in different JA-dependent pathway plant mutants. One possible explanation is that the fumigation time and the O_3 level used in this study may not be sufficient to elicit an increase in JA. In any case, other biochemical components are also important in plant defense. The PAL enzyme plays a key role in SA biosynthesis and in the regulation of synthesis in secondary metabolism (Wen et al. 2005; Liu et al. 2006). *PR* proteins have been shown to increase the levels of plant resistance to aphids (Chaman et al. 2003; Martinez de Ilarduya et al. 2003). The products of secondary metabolism, such as phenolics and tannins, have been found to decrease the r_m values and the population densities of piercing-sucking insects (Leszczynski et al. 1989; Mansour et al. 1997). In the present study, elevated O_3 levels increased PAL activity, SA content, relative *PR* mRNA expression, total phenolics and the levels of condensed tannins in tomato plants. Additionally, the SA content, the relative *PR* mRNA expression, the total phenolics and the condensed tannin content of these plants were all negatively correlated with the rate of developmental time, the fecundity and the r_m of *B. tabaci*. These findings suggest that elevated O_3 reduces the population fitness (in terms of r_m) of *B. tabaci* by increasing the SA content, relative *PR* mRNA expression and the products of secondary metabolism in tomato plants.

Previous research has found that plants with different JA pathway mutations respond differently to elevated O_3 levels (Kanna et al. 2003). JA-induced defense plays an important role in protecting plants from pests (Abe et al. 2008; Browse 2009). Several JA-overexpression mutants have been found to exhibit greater resistance against insects than wild-type plants, whereas JA-deficient mutants exhibit weaker resistance (Ellis et al. 2002; Li et al. 2003; Kempema et al. 2007). Similarly, our results indicated that the JA-overexpression tomato mutant *35S* has the highest resistance to *B. tabaci* of three tomato genotypes. Moreover, elevated O_3 levels reduce the fitness of *B. tabaci* fed on *35S* and wild-type plants, but not that of insects fed on the *spr2* plants. Although the SA defense pathway was triggered in *spr2* plants, the inhibited JA-dependent pathway in *spr2* plants may explain the lack of change in

whitefly fitness under elevated O_3 conditions in this tomato mutant.

The SA-dependent defense pathway has been shown to be important in the induced resistance of tomato cultivars to piercing-sucking insects such as potato aphids (Cooper et al. 2004; Li et al. 2006). Similarly, JA-regulated defenses have been found to delay the development of phloem-feeding whiteflies on *Arabidopsis* (Zarate et al. 2007). In the present study, JA levels exhibited a hierarchy in plants with different JA pathway mutations, and this was negatively related to the fitness of whiteflies. At the same time, in tomato plants cultivated under elevated O_3 conditions, increased SA levels resulted in the reduction of the fitness of the associated whiteflies. Together, these results indicate that increasing the endogenous levels of either JA or SA in plants may reduce the fitness of whiteflies, suggesting that elevated O_3 not only affects plant phenotypes but also modifies plant resistance to herbivorous insects by altering the levels of SA phytohormones. It will be interesting to further explore the mechanisms of plant-induced defenses against insects under elevated O_3 conditions using multi-pathway plant defense signaling mutants.

To our knowledge, this is the first systematic study of the responses to elevated O_3 of tomato plants with different JA defense pathway mutations and of the herbivorous insects associated with them. Our results indicate that elevated O_3 reduces the nutrition of tomato plants and increases the SA content, the relative *PR* mRNA expression and the products of secondary metabolism in these plants, which together result in a decrease in the fitness of whiteflies on these hosts. Such changes suggest that the carrying capacity of the environment with respect to whiteflies will decrease if the atmospheric concentration of O_3 continues to increase.

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