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e-Xtra*

Elevated Ozone Alters Soybean-Virus Interaction

Damla D. Bilgin,[1] Mihai Aldea,[2] Bridget F. O'Neill,[3] Marisol Benitez,[4] Min Li,[4] Steven J. Clough,[4,5] and Evan H. DeLucia[1,2]

[1]Institute for Genomic Biology, University of Illinois at Urbana-Champaign (UIUC), 1206 W. Gregory Drive; [2]Department of Plant Biology, UIUC, 265 Morrill Hall, 505 South Goodwin Avenue; [3]Department of Entomology, UIUC, 320 Morrill Hall, 505 South Goodwin; [4]Department of Crop Sciences, UIUC, 1101 W. Peabody Drive; and [5]United States Department of Agriculture–Agricultural Research Service, 1101 W. Peabody Drive, Urbana, IL 61801, U.S.A.

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Increasing concentrations of ozone (O_3) in the troposphere affect many organisms and their interactions with each other. To analyze the changes in a plant–pathogen interaction, soybean plants were infected with *Soybean mosaic virus* (SMV) while they were fumigated with O_3. In otherwise natural field conditions, elevated O_3 treatment slowed systemic infection and disease development by inducing a nonspecific resistance against SMV for a period of 3 weeks. During this period, the negative effect of virus infection on light-saturated carbon assimilation rate was prevented by elevated O_3 exposure. To identify the molecular basis of a soybean nonspecific defense response, high-throughput gene expression analysis was performed in a controlled environment. Transcripts of fungal, bacterial, and viral defense-related genes, including *PR-1*, *PR-5*, *PR-10*, and *EDS1*, as well as genes of the flavonoid biosynthesis pathways (and concentrations of their end products, quercetin and kaempherol derivatives) increased in response to elevated O_3. The drastic changes in soybean basal defense response under altered atmospheric conditions suggest that one of the elements of global change may alter the ecological consequences and, eventually, coevolutionary relationship of plant–pathogen interactions in the future.

Additional keywords: microarray, photosynthesis, ROS, SoyFACE.

Changes in atmospheric chemistry caused by human activities are potentially reshaping the coevolutionary relationships between plants and viruses. The combustion of fossil fuels is increasing the concentration of ozone (O_3) in the atmosphere (Byrne 2001), and these increases affect myriad aspects of plant physiology and chemistry (Ainsworth and Rogers 2007; Bernacchi et al. 2006). In the past 20 years, tropospheric O_3 concentration increased 0.5 to 2.5% per year and is expected to reach a global mean of >60 nmol mol^{-1} by the year 2050; even today, it can episodically reach levels as high as 100 nmol mol^{-1} (Gard et al. 1998; Houghton et al. 2001).

Highly phytotoxic O_3 enters the plant through stomata and is converted into reactive oxygen species (ROS), triggering a series of metabolic reactions (Kanofsky and Sima 2000; Langebartels et al. 2000; Sandermann 1996; Schraudner et al. 1996). Excess ROS can disrupt plant metabolism by causing irreversible damage to cell membranes, proteins, carbohydrates, and DNA (Apel and Hirt 2004). Even in the absence of visible symptoms, O_3 can inhibit the growth and development of a variety of forest and grassland plants (Ashmore et al. 2007; Campbell 2007; Krupa 2003; Sandermann 1996).

Long-term exposure to elevated O_3 decreased the yield of soybean and caused early senescence (Morgan et al. 2004). Oxidative bursts and rapid accumulation of ROS caused by elevated O_3 not only disrupts plant metabolism but also alters gene expression (Gadjev et al. 2006; Mahalingam et al. 2006; Sharma et al. 1996). Exposure of *Arabidopsis* to elevated O_3 modulated the abundance of 1,390 stress-related genes, including key components of salicylic acid (SA) and ethylene (ET) signaling pathways. Redox-sensitive transcription factors (TFs) and *WRKY* TFs also were upregulated by acute O_3 fumigation (Li et al. 2006; Mahalingam et al. 2006; Tosti et al. 2006).

Plant responses to elevated O_3 resemble defense responses to pathogens, involving common genes and nodes (Mittler 2006; Singh et al. 2002). By mimicking the oxidative burst generated at the early stages of hypersensitive response (HR), acute O_3 (150 to 300 nmol mol^{-1} for 4 to 6 h) induces HR-like localized cell death in plants, similar to the resistance (*R*) gene-mediated host defense response (Overmyer et al. 2005; Rao and Davis 1999). Oxidative bursts caused by acute O_3 treatments also affect antagonistic and synergistic interaction between the plant growth regulators jasmonic acid (JA), SA, ET, and abscisic acid (ABA). The biosynthesis of JA triggered by excess linoleic acid may attenuate SA-dependent HR and cell death (Rao et al. 2000a and b). Ozone reacts primarily with apoplastic fluid and cell membrane, which causes changes in the lipid composition and increases the production of linoleic acid (Mudd 1998). On the other hand, functional SA-signaling pathways are required for O_3-induced ET biosynthesis and induction of HR-like cell death in *Arabidopsis* (Rao et al. 2002). An increase in the O_3-induced biosynthesis of ET production boosts the biosynthesis of ABA, which regulates processes such as glucose signaling and stomatal conductance in plants (Ahlfors et al. 2004; Leon and Sheen 2003).

Ozone-induced changes in host defense response alter plant–pathogen interactions, making plants potentially resistant to obligate pathogens such as viruses (Manning and Tiedemann 1995). Viruses are intimately associated with and dependent on host cellular processes for invasion. In case of a systemic infection, virus can suppress multiple layers of innate host defenses that result in disease (Bilgin et al. 2003; Whitham et al. 2003). Any distinct change in plant gene expression directly influences its relationship with the infecting virus. There is a delicate coevolutionary relationship between

Corresponding author: Evan H. DeLucia; E-mail: delucia@life.uiuc.edu

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viruses and their hosts. Their coexistence depends on the defense strategies developed by both sides, in which host gene expression plays an important role. During this antagonistic coevolution, both sides acquired new traits. It is proposed that plant viruses might have acquired their movement protein (MP) from their hosts (Roossinck 2003). Plants express proteins that function like viral MPs (*Knotted1*), associating with plasmadesmatal structures and facilitating the movement of RNA between plant cells (Lucas and Lee 2004; Lucas et al. 1995). On the plant side, development of posttranslational gene silencing is a unique defense strategy against viruses (Baulcombe 2004).

Plants adjust to environmental challenges by tightly and differentially regulating their transcriptome (Baker et al. 1997; Chen et al. 2002; Cushman and Bohnert 2000; Yamaguchi-Shinozaki and Shinozaki 2006). Ozone induces oxidative bursts, which resemble the biotic elicitors that disrupt plant gene expression and metabolism (Mahalingam et al. 2006; Sharma et al. 1996). Thus, plant–pathogen interactions may be altered by rapid changes in atmospheric composition. To test the hypothesis that elevated O_3 alters the relationship between plants and viruses, we used soybean and *Soybean mosaic virus* (SMV) as a model system. Prior to SMV infection, young soybean plants were exposed to elevated levels of O_3 in the field, and the changes in SMV systemic infection, photosynthesis, antioxidant capacity, and biomass were examined. The effect of O_3 on soybean gene expression, its role as an elicitor, and the changes it caused in soybean–SMV interaction were explored further in an experiment conducted in controlled-environment chambers. Analysis of the soybean gene-expression profile allowed us to identify soybean basal defense response components and to investigate the complexity of O_3-induced nonspecific resistance.

RESULTS

Field experiment.

SMV-susceptible soybean plants were infected with virus under elevated O_3 and ambient atmospheric conditions. Disease symptoms, including mottling, leaf curling, and stunted growth, were observed, and the systemic infection of the virus was determined by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), expressed as relative fluorescence units (RFU). The effects of elevated O_3 and SMV on photosynthesis and antioxidant capacities were determined by measuring A_{sat} and total oxygen-radical absorbance capacity (ORAC), respectively.

Elevated O_3 slowed systemic infection and the rate of disease development by SMV (Table 1). Inoculated plants had classic SMV disease symptoms of a yellow-green mosaic and leaf curling by 7 to 10 days. Samples from inoculated leaves were collected and analyzed quantitatively at 8 h postinfection (hpi) when no significant differences were observed between plants of different treatment (data not shown). On the following days, the magnitude of SMV infection was determined by measuring the accumulation of a viral coat protein at four timepoints for 28 days following the initial infection. Under elevated O_3, the relative SMV titer in soybean was significantly lower for a period of 3 weeks after infection (analysis of variance [ANOVA] tables can be found in Supplementary Table 3).

SMV infection reduced the light-saturated rate of photosynthesis by approximately 10% in ambient atmospheric conditions (Table 1). There was no main effect of elevated O_3. There was, however, a significant statistical interaction between SMV infection and atmospheric composition; the inhibition of photosynthesis by SMV observed under ambient conditions was eliminated in plants exposed to elevated O_3 (Table 1). No effects of elevated O_3 or SMV on stomatal conductance were detected (Supplementary Table 5).

Activation of the ROS scavenging network is part of oxidative stress response in plants. We used the total ORAC assay to quantify hydrophilic chain-breaking antioxidant capacity (Table 1). ROS scavenging capacity increased with time and viral infection (Table 1). The treatment modulated the effect of SMV on ROS scavenging capacity but was unaffected by exposure to elevated O_3.

Across all treatments, SMV infection caused a 42% reduction in aboveground biomass and yield of soybean (Table 1). The decrease in vegetative biomass caused by SMV infection did not significantly differ in O_3 treatments. The pod yield decreased by approximately 55% for SMV-infected plants (Table 1). There was no indication of an interactive effect of SMV infection with changes in the composition of the atmosphere.

Growth chamber experiments.

A controlled-environment experiment was conducted to more closely examine the interactive effects of SMV infection and elevated O_3 on soybean gene transcription, flavonoid content, and ROS formation. The Affymetrix oligonucleotide-based GeneChip Soybean Genome Array that represents 35,611 soy-

Table 1. Summary of significance analysis of SoyFACE experiment[z]

			Ambient		O_3	
Measurements	**Significance**	**Day**	**–V**	**+V**	**–V**	**+V**
Virus Infection	T, V, T × V	7	0.07 (0.05)	0.45 (0.05)	0.07 (0.05)	0.24 (0.05)
DAS-ELISA (RFU)	D, D × V	14	0.05 (0.13)	1.45 (0.13)	0.05 (0.13)	0.97 (0.13)
		21	0.06 (0.12)	1.45 (0.12)	0.06 (0.12)	1.04 (0.12)
		28	0.04 (0.47)	1.91 (0.47)	0.05 (0.47)	1.07 (0.47)
Antioxidant Capacity	D, V	7	5.6 (1.2)	5.7 (0.5)	5.8 (0.4)	6.1 (0.2)
ORAC (Trolox units)	T × V	14	6.9 (0.3)	6.4 (1.0)	7.0 (0.2)	7.4 (0.6)
		21	6.6 (0.7)	6.9 (0.5)	7.1 (0.8)	7.6 (0.9)
Photosynthesis	D, T, D × T	4	19.1 (2.8)	18.8 (2.7)	21.9 (2.8)	25.7 (2.8)
A_{sat} (μmol m^{-2} s^{-1})	T × V	8	23.8 (2.8)	25.1 (2.8)	25.1 (2.8)	25.7 (2.8)
		16	23.9 (2.8)	19.0 (2.8)	22.9 (2.8)	21.6 (2.7)
		22	23.8 (2.9)	17.3 (2.9)	18.6 (2.9)	20.6 (2.9)
		41	16.0 (2.9)	15.4 (2.9)	16.3 (3.0)	14.2 (3.0)
Vegetative mass (g)	V	82	87.2 (8.0)	45.8 (8.0)	87.6 (8.0)	50.1 (8.0)
Pod mass (g)	V, T	82	28.6 (1.9)	10.3 (1.9)	24.1 (1.9)	13.6 (1.9)

[z] The measurements were done weekly after *Soybean mosaic virus* (SMV) infection. Plants were assayed for SMV by double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) in relative fluorescence units (RFU). Total antioxidant capacity was measured by oxygen-radical absorbance capacity (ORAC) in Trolox units, up to 21 days after inoculation. Net photosynthesis (A_{sat}) was measured at saturating light intensity. The dry weight of leaves and stems (vegetative mass) and pods were measured at the end of the season. Each value is a mean ($n = 4$) followed by the one standard error. Only statistically significant values or interactions ($P < 0.05$) for overall measurements were designated as D = day, T = treatment, and V = virus.

bean transcripts was used to determine the changes in transcript levels. Significant gene lists were obtained after applying a cutoff false discovery rate (FDR) of <0.01.

Viral infection and elevated O_3, singly and in combination, caused a significant change in the transcript levels of 2,280 genes at 8 hpi and 1,191 genes at 72 hpi (Fig. 1). Exposure to O_3 elicited substantially more genes at 8 hpi than viral infection or the O_3-plus-SMV treatment. The genes regulated by exposure to O_3 alone decreased by 72 hpi. The number of genes regulated by all the treatments increased at the later timepoint (Fig. 1).

SMV infection alone did not affect the transcription of as many genes as elevated O_3; 219 transcripts were solely regulated by SMV infection (Fig. 1). A comparison with the National Center for Biotechnology Information (NCBI) nonredundant database revealed that 82 of these genes coded for proteins with unknown functions. Multiple Kunitz-type trypsin inhibitors (*KTI*) were downregulated 8 h after SMV infection. In contrast, elevated O_3 increased the levels of *KTI* transcripts. Levels of transcripts related to genes regulating circadian rhythm, such as late elongated hypocotyl (*LHY*) and *Arabidopsis* pseudoresponse regulator 5 (*APRR5*) soybean homologs, also were increased by virus infection (Supplementary Table 1).

Soybean plants responded to elevated O_3 and SMV infection by enhancing the transcript levels of several genes coding antioxidant enzymes. Ascorbate peroxidase was increased at 8 hpi in response to elevated O_3 and the O_3-plus-SMV treatment. Multiple glutathione S-transferase (GST) transcripts increased in response to SMV and elevated O_3 (Table 2). *GST7*, *GST15*, and *GST18* transcripts increased only in the presence of elevated O_3 at 72 hpi. In contrast, *GST12* and

Table 2. Selection of soybean transcripts that were differentially expressed by *Soybean mosaic virus* (SMV) infection, elevated O_3, and combination of O_3 plus SMV infection at 8 and 72 hpi; ambient mock treatment was used as a control

			Transcript fold change (log_2), FDR *P*-value < 0.01					
			8 hpi			72 hpi		
Gene description	**Affymetrix ID**	**TIGR TC[y]**	**SMV**	**O_3**	**SMV+O_3**	**SMV**	**O_3**	**SMV+O_3**
Oxidative stress related genes								
Alternative oxidase	Gma.1439.1.S1_at	TC217347	–	–	1.96	–	–	–
Ascorbate peroxidase	GmaAffx.44716.1.A1_at	BI969342	–	1.13	–	–	–	–
Ascorbate oxidase precursor	GmaAffx.47157.1.A1_at	TC204959	–	1.26	2.26	–	–	–
GSTX_SOYBN	GmaAffx.19480.1.S1_at	TC214400	2.11	–	2.23	–	1.11	1.13
GSTX6_SOYBN	GmaAffx.88762.1.S1_at	TC214058	1.43	–	2.31	–	2.50	1.91
GST 15	Gma.2593.1.S1_s_at	TC215837	–	–	3.00	–	3.40	2.80
Peroxidase precursor	GmaAffx.92917.1.S1_s_at	TC215016	–	2.04	1.58	–	–	–
SRG1 (senescence related gene1) oxidoreductase	Gma.8240.1.S1_at	TC231312	–	–	–	–	1.52	1.20
Defense–related genes								
PR1a precursor	GmaAffx.42847.2.S1_at	TC227432	–	2.41	2.92	–	3.98	4.43
PR5 thaumatin–like protein	Gma.2821.2.S1_a_at	TC226940	–	1.13	1.56	–	3.02	2.39
PR10-like protein	Gma.6999.1.S1_x_at	TC227429	2.04	1.11	2.99	–	4.08	3.50
PR10-like protein (stress–induced protein SAM22)	Gma.6999.3.S1_s_at	TC224834	2.79	1.38	3.33	–	3.44	3.18
EDS1 putative	GmaAffx.80842.1.S1_at	BE440732	–	1.00	1.64	–	1.43	1.63
COI1	GmaAffx.21596.1.S1_at	BE329784	–	1.37	–	–	–	–
Disease-resistance gene (*RPP13*–like)	GmaAffx.57686.1.S1_at	AW597616	–	–	1.54	–	–	–
Disease-resistance gene (*RPM1*)	GmaAffx.67437.1.S1_at	TC224491	–	–	1.75	–	–	–
TIR–NBS[z] disease resistance–like protein	GmaAffx.92922.1.S1_at	TC208227	–	–	1.73	–	–	–
NDR1/HIN1–like protein 3 (NHL3)	Gma.9397.1.S1_at	CA802074	–	–	1.62	–	–	–
Disease resistance–responsive family protein	Gma.4332.1.S1_at	TC215635	–	–	–	–	1.79	1.98
DRR4_PEA disease-resistance response	GmaAffx.93611.1.S1_s_at	TC208219	–	–	–	–	1.15	1.34
Disease resistance–like gene (*Cf-4/9*)	Gma.3351.1.S1_at	BQ454138	–	–	–	–	1.16	1.13
Endo–1,3–beta–glucanase	GmaAffx.86629.1.S1_at	TC228694	–	–	1.14	–	2.12	1.75
Chitinase	Gma.3702.1.S1_at	TC228060	–	1.63	2.7	–	2.79	2.51
CHIT1_tulip bulb chitinase–1 (TBC–1)	GmaAffx.89972.1.S1_s_at	TC228060	–	1.46	2.42	–	2.51	2.05
Acidic chitinase	GmaAffx.494.1.S1_at	TC216469	–	–	1.46	–	2.17	1.86
Ethylene signaling related genes								
Transcription factor EIL1	GmaAffx.13724.1.S1_s_at	TC214361	–	–	–	1.98	1.84	1.68
Transcription factor EIL2	GmaAffx.86555.1.S1_s_at	TC214228	–	–	–	2.64	2.53	2.48
EBF1 (EIN3–BINDING F BOX PROTEIN 1)	GmaAffx.54144.1.S1_at	TC212384	–	–	–	2.79	2.67	2.64
Jasmonic acid signaling related genes								
Phospholipase A2	Gma.7515.1.S1_at	TC207273	–	1.746	–	–	–	–
Lipoxygenase LOX1	GmaAffx.45573.1.S1_at	TC233575	–	–	–	2.27	2.09	1.78
Lipoxygenase (AtLox2)	Gma.2382.1.S1_s_at	TC207342	–	–	1.72	–	1.26	1.78
Allene oxide synthase	Gma.6281.3.S1_at	CA800304	–	–	–	1.94	1.75	1.82
12–oxophytodienoate reductase	GmaAffx.6142.1.S1_at	TC228335	1.16	–	–	–	–	–
NAC transcription factors								
NAC domain protein NAC1	GmaAffx.32328.1.A1_at	TC216429	–	–1.90	–1.44	–	–	–
NAC domain protein NAC1	Gma.4643.1.S1_at	TC216430	–	–1.69	–1.55	–	–	–
NAC domain protein NAC2	Gma.4774.2.S1_at	TC215443	–1.53	–1.47	–1.33	–	–	1.04
NAC domain protein NAC3	GmaAffx.57970.1.S1_at	TC215245	–1.61	–2.27	–2.16	–	–	–
NAC domain protein NAC4	Gma.5331.1.S1_at	TC215244	–1.23	–2.84	–1.79	–	–	–
NAC family protein	GmaAffx.90028.1.S1_s_at	TC205356	–1.64	–1.59	–1.22	–	–	–
NAC family protein	Gma.5519.1.S1_at	TC205356	–1.60	–1.63	–1.33	–	–	1.32
ANAC062; transcription factor	GmaAffx.32877.1.S1_at	TC234989	–	–	–	1.13	1.37	1.15
ANAC017; transcription factor	GmaAffx.75215.1.S1_at	TC234607	–	–	–	–	1.08	–

[y] TIGR TC = The Institute for Genomic Research tentative consensus.
[z] TIR–NBS = toll interleukin 1 receptor-nucleotide binding site.

GST14 transcripts accumulated in response to SMV infection with or without elevated O_3 at the early timepoint. Several peroxidase genes responded to both stresses. In particular, class III peroxidases were increased by SMV infection at the early timepoint (Table 2).

The accumulation of transcripts for several antioxidant enzymes suggested that ROS was formed in response to elevated O_3, SMV infection, and the O_3-plus-SMV treatment. The spatial production of H_2O_2 and $O_2^{\bullet-}$ in the foliar tissue following exposure to SMV alone or in combination with elevated O_3 was determined in a subsequent growth-chamber experiment. The presence of a brown precipitate in leaves infiltrated with diaminobenzidine (DAB) indicated that virus infection caused H_2O_2 to accumulate in dense patches throughout the leaf mesophyll (Fig. 2B). Exposure to elevated O_3 alone caused localized bursts of H_2O_2 production (Fig. 2C). The pattern of accumulation of H_2O_2 in the combination treatments (O_3 plus SMV) was similar to leaves exposed only to SMV infection, but the intensity of the response was lower (Fig. 2D). Exposure to elevated O_3, singly or in combination with SMV infection, caused localized accumulation of $O_2^{\bullet-}$, evident as a blue precipitate (Fig. 2F, G, and H). The number of spots and the intensity of nitroblue tetrazolium (NBT) accumulation were strongest in leaves exposed simultaneously to O_3 and virus infection (Fig. 2H).

Flavonoid biosynthesis gene transcripts accumulated at both timepoints with a maximum up to 10.6-fold in the presence of O_3; however, by 72 hpi, these particular flavonoid biosynthesis genes were not affected by SMV infection (Fig. 3). Elevated O_3 and the O_3-plus-SMV treatments increased the transcript levels of phenylalanine ammonia-lyase (*PAL*), 4-coumarate:CoA ligase (*4CL*), chalcone synthase (*CHS*), chalcone reductase (*CHR*), chalcone isomerase (*CHI*), isoflavone synthase (*IFS*), isoflavone reductase (*IFR1*), and 2-hydroxyisoflavanone dehydratase (*HID*) (Fig. 3 and Supplementary Table 2).

The analysis of flavonoids by liquid chromatography mass spectrometry (LC/MS) revealed that O_3 treatment and virus infection caused significant changes in flavonoid concentrations in soybean leaves (Table 3 and Supplementary Fig. 1). Quercetin triglycoside was significantly greater in O_3-plus-virus samples than virus treatment alone. The quercetin triglycoside amount did not change with time. Kaempherol triglycoside increased over time in ambient conditions and its amount was significantly higher in virus treatment than O_3 treatment at early timepoint. This difference disappeared by 72 h. Virus infection decreased the production of methyl quercetin triglycoside; however, when the plants were pre-exposed to elevated O_3, the decrease was not observed. Quercetin diglycoside concentration was significantly higher in O_3-plus-SMV-infected plants than the virus-infected plants under ambient conditions at 8 hpi. Methyl quercetin diglycoside increased only when plants were infected with virus in the presence of elevated O_3 (Table 3). The kaempherol diglycoside amount was significantly less than mock-inoculated plants in ambient conditions when both O_3 and SMV infection were applied. Genistein malonylhexose and kaempherol aglycone increased by time in O_3-treated samples. They were significantly higher in O_3-treated plants than virus-infected plants in ambient conditions at 72 hpi.

In addition to genes in flavonoid biosynthesis, defense response genes induced by SA, JA, and ET were differentially regulated by elevated O_3, SMV, and the O_3-plus-SMV treatment. Isochorismate synthase (*ICS*) transcript levels increased at an early timepoint and PAL gene transcripts increased at a later timepoint. *ICS* and *PAL* are required to synthesize SA for defense. Pathogenesis-related 1 and 10 (*PR-1* and *PR-10*), thaumatin-like *PR-5*, and enhanced disease susceptibility (*EDS1*) transcripts were increased up to fourfold (log_2) by elevated O_3 treatment (Table 2). The transcript levels of these genes were verified by real-time reverse-transcribed polymerase chain reaction (RT-PCR) (Supplementary Fig. 2 and Supplementary Table 4). The direction of the response of genes measured by real-time RT-PCR and microarray were similar but the magnitude of the response was greater when meas-

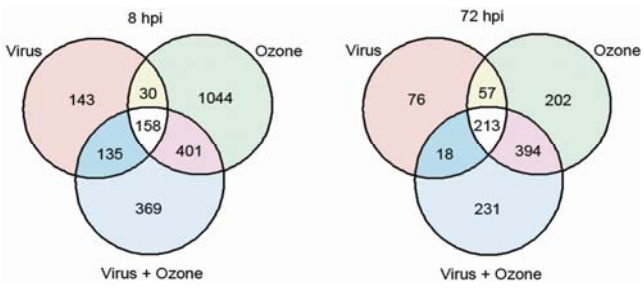

Fig. 1. Venn diagram depicting the number of soybean transcripts showing common or differential expression in response to elevated O_3, *Soybean mosaic virus* (SMV), and the combination of elevated O_3 plus SMV at 8 and 72 h postinfection (hpi).

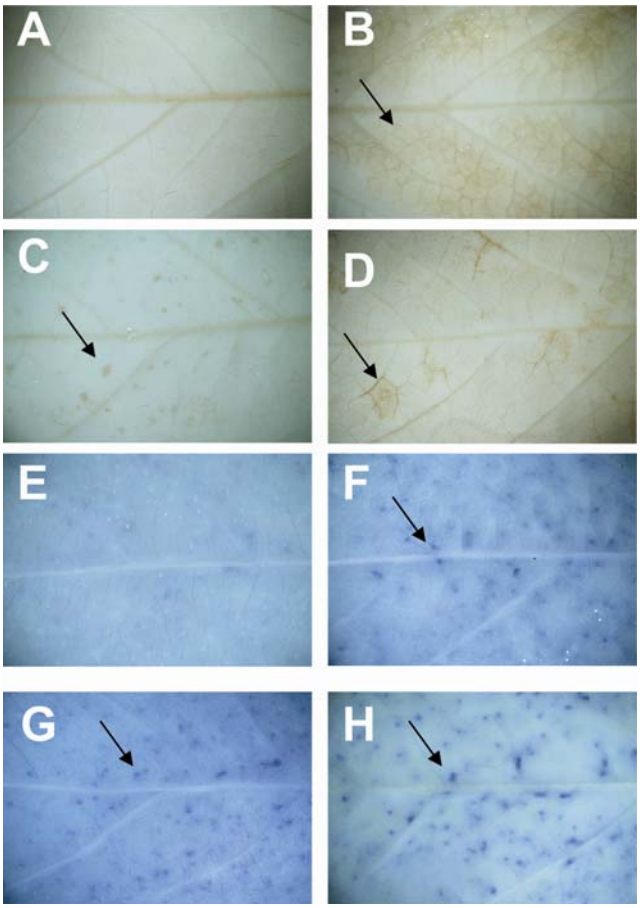

Fig. 2. Accumulation of H_2O_2 and superoxide in soybean plants grown in temperature-controlled growth chambers. To observe the effect of *Soybean mosaic virus* (SMV) systemic infection and elevated O_3, the top soybean leaves were infiltrated with diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) at 7 days postinoculation. **A** through **D,** Staining for H_2O_2 accumulation with DAB. **A,** Mock-inoculated plants in ambient conditions; **B,** SMV-infected plants in ambient condition; **C,** elevated O_3-treated plants; **D,** elevated O_3-treated and SMV-infected plants. **E** through **H,** Staining for superoxide with NBT. **E,** Mock-inoculated plants in ambient condition; **F,** SMV-infected plants in ambient condition; **G,** elevated O_3-treated plants; **H,** elevated O_3-treated and SMV-infected plants.

ured by real-time RT-PCR. Transcripts related to a large number of 1,3-β-glucanase, endo-1,4-β-glucanase, and chitinase genes were also increased in elevated O3 and the O3-plus-SMV treatment (Table 2). The transcript levels of 10 different glucanase genes and 7 chitinase genes were increased by elevated O_3 (Table 2).

Transcript levels for genes related to ET signaling were greater in response to all treatments. ET is perceived by the membrane-bound histidine kinase receptor ETR1 and triggers defense and development-related responses (Luthen et al 2007). At 72 hpi, six transcripts representing four ET-regulated TFs such as ethylene-insensitive 3 (*EIN3*) and *EIN3*-like 1 and 2 homologs (*EIL1* and *EIL2*) increased in unison (Table 2). Also, elevated O_3 increased the transcription of several brassinosteroid (BR) and auxin-signaling-related genes.

Transcript levels of genes in the octadecanoid pathway leading to the production of JA were increased by O_3 and the O_3-plus-SMV treatment, especially at 72 hpi. Phospholipase A2 transcripts increased by elevated O_3 at an early timepoint. The expression of lipoxygenase 1 (*LOX1*) increased in response to all treatments whereas soybean *LOX2* homolog responded only to elevated O_3 and the O_3-plus-SMV treatment (Table 2). Lipoxygenases also function in the hydroperoxide lyase pathway; however, there were no significant changes in hydroperoxide lyase (*HPL*) transcript levels. Transcripts of allene oxide synthase (*AOS*) that leads to JA biosynthesis were increased in response to all the treatments at 72 hpi.

TFs play a central role in orchestrating the genomic responses to external stimuli. Exposure to elevated O_3 decreased transcript levels of *NAC* TFs. In all, 32 transcripts that represent 15 different *NAC* TFs decreased more than twofold (log_2) by elevated O_3 and the O_3-plus-SMV treatment (Table 2). Five of these genes also were decreased by SMV infection at an early timepoint. Alternatively, two *NAC* TFs that showed homology to *Arabidopsis ANAC062* and *ANAC017* had higher transcript levels at 72 hpi.

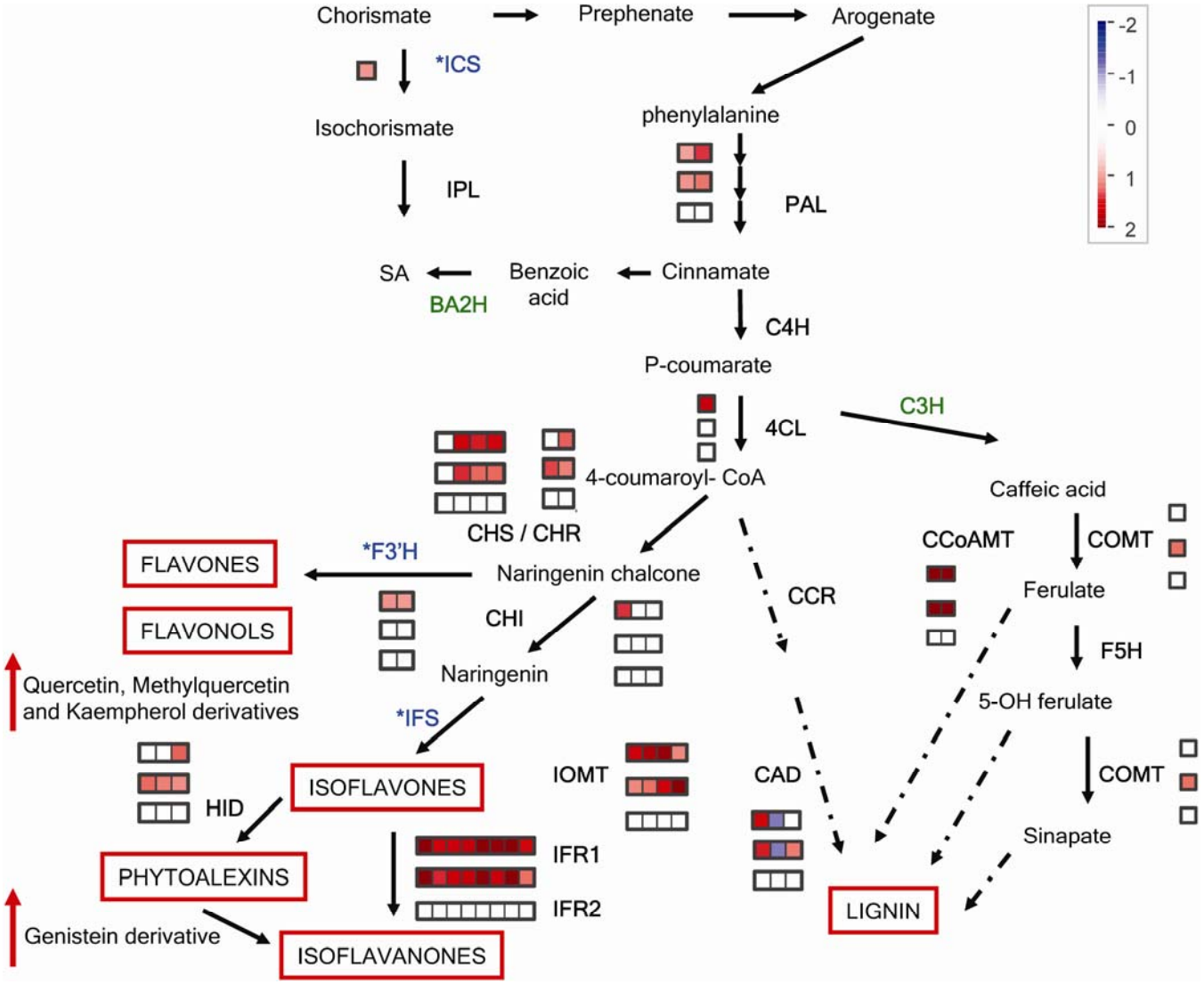

Fig. 3. Graphical representation of significantly differentiated gene transcripts in phenylpropanoid metabolism in response to elevated O_3, elevated O_3 plus *Soybean mosaic virus* (SMV), and SMV treatments at 72 h postinfection (hpi). Each box corresponds to one soybean transcript. For each gene, the upper block of squares represents genes regulated by elevated O_3 treatment, the middle block represents genes regulated by O_3 plus SMV, and the lower block represents genes regulated by SMV infection. The genes marked with an asterisk were detected only at 8 hpi. No annotated expressed sequence tags exist in soybean public databases for the genes in green. Values for gene regulation are expressed by color intensity, where upregulation is indicated by shades of red and downregulation by shades of blue. Abbreviations: benzoic acid 2-hydroxylase (BA2H), caffeic acid *O*-methyltransferase (COMT), caffeoyl-CoA 3-*O*-methyltransferase (CCoAMT), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), cinnamyl alcohol dehydrogenase (CAD), 4-coumarate:CoA ligase (4CL), 2-hydroxyisoflavanone dehydratase (HID), isoflavone synthase (IFS), isoflavone reductase (IFR1), isochorismate synthase (ICS), p-coumarate 3 hydroxylase (C3H), and phenylalanine ammonia-lyase (PAL).

DISCUSSION

The response of a plant to a given stress is governed by its genetic background. To study the changes in the soybean–SMV interaction, we used an SMV-susceptible but moderately O_3-tolerant soybean line (*Glycine max* cv. Pioneer 93B15). As a result of long-term elevated O_3 treatment, the soybean–SMV interaction was altered and a nonspecific defense response was induced. The induced response slowed systemic infection by SMV and reduced its negative effect on photosynthesis. However, the nonspecific defense response induced by O_3 did not provide life-time protection, and plants infected with SMV had typical disease symptoms and lower end-of-season biomass and yield (Table 1). Analysis of transcript levels by high-throughput gene expression profiling provided possible explanations for the mechanisms of nonspecific defense response.

Ozone enters the plant through stomata and dissociates in the apoplast as a function of endogenous hydroxyl ion concentration (Rao et al. 2000a and b). It generates ROS such as H_2O_2 and $O_2^{\bullet-}$ and alters redox balance (Mano 2002). Whether the O_3 treatment is acute (150 to 300 nmol mol^{-1} for 4 to 6 h) or chronic (<100 nmol mol^{-1} for months), the apoplast soon becomes saturated with ROS and causes activation or accumulation of signaling intermediates, such as SA, JA, and ET, and eventually induces a defense response (Apel and Hirt 2004; Heath 2007; Kangasjarvi et al. 2005). The production of ROS is an important initial determinant of plant–pathogen interactions, and its intensity and duration differ in host-incompatible and host-compatible interactions (Bolwell et al. 2002; Scheel 2002). In plants, it has been shown that various biotic and abiotic stress response pathways share common nodes (Lee et al. 2006; Mazarei et al. 2007; Sohn et al. 2006). In our experiments, exposure to elevated levels of O_3 mimicked the effect of pathogen infection and the defense response induced by elevated O_3 improved resistance against SMV for a period of 3 weeks (Table 1). Even though the soybean line was moderately tolerant to O_3, plants were sensitive enough that the O_3 treatment transiently enhanced SMV resistance, possibly by modulating defense-related gene expression levels and the concentration of individual flavonoids.

Individual viral, bacterial, and fungal infection strategies are diverse; therefore, each plant–pathogen interaction under elevated O_3 may show different characteristics (Manning and Tiedemann 1995). Similarly, acute ozone treatment (150 to 300 nmol mol^{-1}) protected tobacco from *Tobacco mosaic virus* (TMV) and increased tolerance of *Arabidopsis thaliana* to *Pseudomonas syringae* (Sandermann 2000). However, *Peanut stunt virus* (PSV) infection and sustained levels of O_3 treatment had additive effects on O_3-sensitive *Trifolium repens* (Heagle et al. 1992).

There are strong similarities between defenses induced by elevated O_3 and systemic acquired resistance (SAR) (Durrant and Dong 2004; Kotchoni and Gachomo 2006). During the response to SAR and O_3, microbursts of ROS may activate defense responses throughout the plant. The transcription of many defense genes involved in SAR is activated by the long-term exposure to elevated O_3. For example, SA plays a major role in HR resistance and SAR and may be synthesized by two pathways that involve *PAL* and *ICS* (Kachroo and Kachroo 2007; Wildermuth et al. 2001). In *Arabidopsis*, SA biosynthesis and *ICS* transcript level started to increase within 3 h in response to acute O_3 treatment (Ogawa et al. 2007). There was a slight increase in *PAL2* transcript but not in *PAL1*. In our study, elevated O_3 increased the transcript of *ICS* and *PAL* genes in soybean at early and later timepoints, respectively. The increase in transcript levels of these genes may cause increases in SA production. The increase in *PR-1* gene transcript (TC227431; greater than fourfold; $\log_2$) may have played a role in defense against SMV together with several SA-regulated defense genes such as *PR5* and *EDS1* homologs. Among PR genes, the transcript level of a soybean *PR-10*-like gene (TC227429) increased approximately fourfold ($\log_2$). The *PR-10* gene from *Capsicum annuum* was induced after TMV infection and was shown to have ribonuclease activity against TMV RNA (Park et al. 2004).

Another defense pathway induced by elevated O_3 with or without SMV infection is phenylpropanoid biosynthesis (Fig. 3 and Supplementary Fig. 4). Flavonoids have antioxidant capacity and may function by inhibiting ROS-producing enzymes such as NADPH oxidase, cyclooxygenase, lipoxygenase, and chelating trace elements involved in free radical production (Brown et al. 1998; Korkina 1997, 2007; Pietta 2000; Ursini et al. 1994). Oxidative bursts caused by elevated O_3 lead to ROS formation that may exceed the apoplastic antioxidative capacity. Ozone-induced excess ROS formation shows similarity to HR and activates defense response and cell death via SA, ET, and JA signaling pathways (Kangasjarvi et al. 2005; Rao and Davis 2001; Tuominen et al. 2004). In susceptible plants, the activation of a phenylpropanoid pathway may affect SA levels and basal defense response against pathogens.

Many secondary metabolites that are part of the defense response against pathogens are derived from multiple branches of the phenylpropanoid pathway, including flavonols, isoflavonoid phytoalexins, and lignins. Protein engineering approaches were successfully used to construct multienzyme biosynthesis pathways in nonlegume plants to increase their pathogen resistance (Shadle et al. 2003; Tian et al. in press). Acute O_3 exposure increased the transcript of stilbene synthase (*STS*) and *CHS* genes in pine, grapevine, and *Arabidopsis* (Chiron et al. 2000; Schubert et al. 1997; Tosti et al. 2006).

Table 3. Flavonoids of noninfected and infected soybean plants in ambient and elevated O_3 conditions[a]

Flavonoids	Quercetin triglycoside	Kaempferoltri glycoside	Methylquercetin triglycoside	Quercetin diglycoside	Methylquercetin diglycoside	Kaempherol diglycoside	Genistein malonyl hexose	Kaempherol aglycone
8 hours postinoculation (hpi)								
A	17.99 ± 4.1(ab)	33.95 ± 5.6(bc)	23.5 ± 4.09(ab)	63.25 ± 12.8(ab)	42.89 ± 6.82(ab)	52.47 ± 7.5(ab)	9.04 ± 8.8(c)	0 ± 3.9(b)
V	9.18 ± 4.1(b)	43.38 ± 5.6(ab)	31.62 ± 4.09(ab)	29.7 ± 12.8(b)	46.42 ± 6.82(ab)	53.78 ± 7.5(ab)	7.9 ± 8.8(c)	0 ± 3.9(b)
O_3	17.09 ± 4.1(ab)	25.9 ± 5.6(c)	23.45 ± 4.09(ab)	52.18 ± 12.8(ab)	32.19 ± 6.82(b)	33.02 ± 7.5(b)	15.51 ± 8.8(bc)	2.64 ± 3.9(b)
O_3+V	22.75 ± 4.1(a)	39.91 ± 5.6(abc)	34.49 ± 4.09(a)	70.78 ± 12.8(a)	49.48 ± 6.82(ab)	53.84 ± 7.5(ab)	19.04 ± 8.8(bc)	2.33 ± 3.9(b)
72 hpi								
A	16.45 ± 4.1(ab)	51.07 ± 5.6(a)	33.33 ± 4.09(a)	49.09 ± 12.8(ab)	52.9 ± 6.82(a)	63.44 ± 7.5(a)	23.6 ± 8.8(bc)	9.22 ± 3.9(ab)
V	8.35 ± 4.1(b)	35.72 ± 5.6(abc)	19.38 ± 4.09(b)	27.15 ± 12.8(b)	33.41 ± 6.82(ab)	43.73 ± 7.5(ab)	19.61 ± 8.8(bc)	4.14 ± 3.9(b)
O_3	18.67 ± 4.1(ab)	36 ± 5.6(abc)	25.53 ± 4.09(ab)	55.16 ± 12.8(ab)	40.65 ± 6.82(ab)	43.30 ± 7.5(ab)	50.31 ± 8.8(a)	19.61 ± 3.9(a)
O_3+V	16.12 ± 4.1(ab)	36.11 ± 5.6(abc)	23.32 ± 4.09(ab)	43.11 ± 12.8(ab)	33.10 ± 6.82(ab)	37.62 ± 7.5(b)	35.56 ± 8.8(ab)	10.53 ± 3.9(ab)

[a] Student *t*-test analysis of flavonoid accumulation in soybean leaves in response to elevated O_3, *Soybean mosaic virus* (SMV) and O_3 plus SMV treatments at 8 and 72 hpi. The values represent least square means ± standard error. Within the column, the values with the same letter do not differ significantly ($P < 0.05$).

Several soybean *CHS* transcripts (*CHS1*, *CHS2*, *CHS3*, *CHS5*, *CHS6*, and *CHS7/8*) accumulate during the basal defense and HR to pathogens (Lozovaya et al. 2007; Zou et al. 2005). Although avirulent strains elicit a faster gene expression response than virulent strains, induction of *PAL* and *CHS* genes are observed in both interactions. Zabala and associates (2006) concluded that the synthesis of lignin or suberin is one of the early responses to the bacterial infection because of high accumulation of *CAD* transcripts 2 h after *P. syringae* inoculation.

Elevated O_3 or elevated O_3-and-SMV combination treatments induced genes coding for key regulatory enzymes in the biosynthesis of flavonoids, causing changes in the concentrations of genistein, kaempherol, quercetin, and methyl quercetin derivatives in soybean leaves. Genistein and its more complex isoflavonoid derivatives exhibit broad spectrum antimicrobial activity. They can function as preformed "phytoanticipins" and inducible "phytoalexins" against pathogens (Dixon and Ferreira 2002). The increase in genistein malonyl hexose concentration may have contributed to defense against SMV. Moreover, preexposure to O_3 prevented the decrease in methylquercetin triglycoside caused by SMV infection and induced the biosynthesis of kaempherol diglycoside (Table 3). External application of quercetin and derivatives effectively inhibited systemic infection of *Potato virus X* (PVX) and *Tomato ringspot virus* (TomRSV) up to 70% (French and Towers 1992; Malhotra et al. 1996). Methylated quercetin compounds reduced infectivity of TMV, exposing the viral RNA to attack by host RNase by weakening virion subunit binding (French 1991). Increase in the kaempherol and quercetin derivatives by long-term elevated O_3 treatment was part of a nonspecific host defense response but the defense was transient. As a response to elevated O_3, soybean flavonoid concentrations increased at the beginning of the field season; however no, change was detected after July 14, 2005 (B. F. O'Neill, *unpublished*). Long-term O_3 treatment may cause plants to acclimate to environmental conditions.

Several nucleotide binding site leucine-rich repeat family genes were upregulated by elevated O_3; however, the overall defense response did not resemble a pathogen-specific response because it was transient. The main driving force for the induction of defense-related genes could have been excess ROS production under elevated O_3. The events triggered were similar to SAR following HR response. The defense genes that showed an increase in transcript levels functioned downstream in an incompatible interaction and SAR, such as *EDS1* and *SGT1*.

Transcripts homologous to a large number of *NAC* TFs (negative regulators of PR genes) were decreased by elevated O_3 and the combination of O_3 plus SMV. Overexpression of *Arabidopsis ATAF2*, a member of the NAC-domain TF family, increased the susceptibility of transgenic plants to fungal infection (Delessert et al. 2005). A short period of acute O_3 treatment induced the transcription of an *NAM-like* gene (At5g63790) in *Arabidopsis* (Mahalingam et al. 2006) but sustained levels of exposure to O_3 downregulated the transcription of 15 *NAC* TFs in mature soybean plants (Table 2). By contributing to greater expression of *PR1*, *PR5*, and *PR10*, the inhibition of *NAC*s by O_3 would enhance resistance to SMV. Also, *NAC* TFs regulate various basic cellular processes, including cell division, secondary cell wall synthesis, and auxin-dependent formation of lateral roots (Kim et al. 2006; Xie et al. 2000; Zhong et al. 2006). Transcription of *NAC* TFs is regulated by microRNA (Mallory et al. 2004), and two of the soybean *NAC* genes that are downregulated had a potential miR164 complementary site with one mismatch (Table 2).

Vigorous ROS production by SMV was detected in ORAC assay with field samples and confirmed by histochemical staining in growth chambers. Susceptible soybean plants produced H_2O_2 in response to SMV infection, and the location and intensity of production were dependent on the strength of the infection (Fig. 2B). Although total ORAC increased with time and viral infection, it was not affected by elevated O_3 under field conditions. Plants in natural field conditions are subjected to other stresses, including herbivory, drought, and heat, that cause sustained production of ROS. The production of ROS in response to other stresses may have masked its response to elevated O_3 in the field. The production of ROS by elevated O_3 was milder than ROS production following SMV infection (Fig. 2B through D). The spatial distribution of H_2O_2 accumulation was very different between treatments. H_2O_2 levels increased as SMV infection spread throughout the plant; on the other hand, superoxide production was the greatest when more than one stress was applied. This suggests different roles for ROS species.

Although elevated O_3 induced temporary defense against SMV infection, it also affects photosynthesis and yield (Morgan et al. 2004, 2006). Elevated O3 reduces soybean photosynthesis during the vegetative stage and affects yield in some cultivars. Production of ROS may contribute to reduced photosynthesis and growth in elevated O_3. Excess ROS improves resistance against various pathogens but causes damage as well. Acute doses of O_3 exposure induce HR and cell death (Pasqualini et al. 2003). The major difference between ROS induced by plant–pathogen interactions and the effect of elevated O_3 is that, during plant–pathogen interactions, ROS is generated initially under controlled conditions as a defense to restrict the pathogen, whereas exposure to elevated O_3 induces ROS in an uncontrolled and widespread manner (Fig. 2).

In nature, plants rapidly lose their resistance as pathogens evolve to overcome their defenses (Roossinck 2003). Once inside the host cell, viral populations explode and vast numbers greatly increase the opportunity to develop new avenues of attack. A rapidly growing population of wild-type bacteriophage in a chemostat acquired adaptive traits in 180 days (Wichman et al. 2005). Growth in elevated O_3 induced a nonspecific defense response against SMV; however, this defense was active for a limited time only. Although the increase in SMV population was retarded by elevated O_3 (Table 1), the virus ultimately overcame nonspecific defenses mounted by soybean and caused a substantial reduction in final biomass. The strong positive selection promotes the rapid evolution of defense strategies on both sides. Thus, soybean has gone through several genetic bottlenecks because of domestication (Hyten et al. 2006) and may be limited in developing counterstrategies against viruses in the long term.

To our knowledge, this study is the first global gene expression analysis of soybean–SMV interaction. SMV infection caused differential regulation of 830 transcripts, of these 219 were unique to SMV treatment. The majority of these genes coded for proteins with unknown functions. SMV infection suppressed expression of several *KTI* gene expressions, possibly, as part of its infection strategy. *KTI*s function in plant defense response and their expression increases in response to herbivory, drought, and exogenous application of ABA, ET, and methyl jasmonate (Kang et al. 2002). Similarly, TF *MYB6* (TC204916) was downregulated at early hours of the SMV infection. *Cucumber mosaic virus* (CMV) infection downregulates *MYB* TFs in *Arabidopsis* (Marathe et al. 2004). The downregulation of TF *MYB6* could be because of viral dominance on the host transcription. This small cluster of genes was uniquely regulated by SMV infection and the genes were different from the nonspecific resistance genes initiated by elevated O_3 and O_3-and-SMV combination treatments.

Concluding remarks.

Elevated O_3 altered gene expression in soybean and induced a nonspecific defense response. Although the host defense re-

sponse was not induced specifically to stop SMV infection but to cope with the environmental changes, it retarded the replication and spread of the host-dependent pathogen temporarily. The induced nonspecific defense has multiple components. Transcriptional increases of PR genes and ET, SA, and JA signaling genes contributed to the defense response to a certain degree. The increase in flavonoid biosynthesis genes and the increase in several flavonoids indicated that flavonoid biosynthesis was one of the major players in the nonspecific defense response.

These stress-induced plant responses may change predisposition and enhance tolerance for a second stress factor. Many preformed and induced defense systems against pathogens involve activation of the same or overlapping signaling pathways with abiotic stresses. At the early stages of the stress, the induction of genes involved in a signal transduction pathway is often specific to the particular stimulus. However, downstream events involving stress adaptation are orchestrated by the same genes across different types of stress factors. The number of differentially regulated soybean genes that were common in response to all stresses increased from 30 to 50% over time. Therefore, biotic and abiotic signaling pathways may share multiple nodes and their outputs may have significant functional overlap.

The dynamics of plant–pathogen interactions are shaped by a complex set of ecological and evolutionary influences. The altered molecular and physiological state of the plant influenced its interaction with the virus. The changes in the preformed defenses and the strength of induced defenses will affect the arms race between plants and pathogens, and future changes in the composition of the atmosphere may alter the coevolution of plants and pathogens.

MATERIALS AND METHODS

Field experiment.

Soybean plants (*G. max* cv. 93B15; Pioneer Hi-Bred, Des Moines, IA, U.S.A.) were exposed to elevated O_3 and infected with SMV under natural field conditions. Plants were grown in large plots (314 m^2/plot) at the University of Illinois Soybean Free-air Concentration Enrichment facility (SoyFACE), where atmospheric levels of O_3 were precisely controlled. Each experimental plot ($n = 4$) was surrounded by pipes that injected O_3 at supersonic velocity from 300-μm pores above the canopy. The rate and position of gas release was automatically altered with wind speed and direction to maintain the desired gas concentrations. Plots were fumigated during daylight hours from planting until harvest. The target concentration for O_3 (1.2× ambient; 8 h O_3, 59.8 nmol mol^{-1}) represents the predicted atmospheric levels by 2050. Cross-contamination between treatments was prevented by separating the experimental plots by at least 100 m. Dermody and associates (2006) provide a more detailed description of the SoyFACE facility.

The SoyFACE experiment included fully replicated plots exposing plants to elevated CO_2 (550 μmol mol^{-1}) during daylight hours from planting until harvest. Measurements of SMV infection, antioxidant capacity, and photosynthesis for plants exposed to elevated CO_2 were included in the statistical analysis applied to the data in Table 1 (full factorial analysis of variance).

The crop was planted on day of the year (DOY) 145 in 2005; on DOY 189, 16 plants in each experimental plot were randomly selected and the mature fourth trifoliates of 8 plants were rub-infected with SMV-G2 as in Chen and associates (2004). Another set of eight randomly selected plants was mock inoculated. The inoculums were prepared by grinding previously SMV-infected (for SMV inoculation) and noninfected (for mock treatment) *G. max* cv. Williams leaves in chilled 0.025 M potassium phosphate buffer (pH 7.1), 0.01 M sodium sulfite, and Carborundum (320 grit).

Each experiment was conducted as four biological replicates (plots), and each replicate consisted of a pooled sample from four plants. In addition to measurements of gas exchange, final biomass, and yield, samples were collected from inoculated leaves 8 and 72 hpi for RNA isolation.

DAS-ELISA.

For quantitative analysis of virus accumulation, samples from top leaves were collected at 7, 14, 21, and 28 days postinfection (dpi). Detection of SMV coat protein was carried out following the standard procedures for DAS-ELISA assay as described in the manufacturer's protocol (PathoScreen Kit; Agdia, Elkhart, IN, U.S.A.). The results were expressed as RFU measured at an optical density of 405 nm on a plate reader (EL 340; Bio-Teck Services Inc., Richmond, VA, U.S.A.). Four biological replicates and two technical replicates were processed for each treatment. Separate samples from inoculated leaves were collected at 8 hpi and were analyzed quantitatively to determine the initial amount of infection.

Antioxidant capacity assay.

To assess total ORAC (Cao et al. 1993) of soybean leaves, disks (1.5 cm in diameter) were collected at 7, 14, and 21 dpi, flash frozen in liquid nitrogen, and stored at –80°C until analysis. Four disks collected from top leaves of four inoculated (SMV-G2 or mock) plants were pooled for each biological replicate and there were four replicates per treatment. Samples (1 mg) were ground in a bead beater at room temperature, extracted with acidic acetonitrile, and resuspended in methanol (1 ml) before analysis as described by Lee and associates (2004). The final mixture for assay contained a 1:1,000 dilution of the sample in 75 mM K_2HPO_4 buffer, 12 mM 2,2′-Azobis(2-amidino-propane)dihydrochloride), and 70.3 nM fluorescein (Huang et al. 2002). Fluorescence was measured at 530 nm with excitation at 485 nm using a 96-well plate fluorimeter (Synergy HT; Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.).

Gas-exchange and chlorophyll fluorescence measurements.

Light-saturated rates of carbon assimilation (A_{sat}) were measured on infected and noninfected plants with an open-path gas-exchange system (LI-6400, LI-COR, Inc., Lincoln, NE, U.S.A.) at 4, 8, 16, 22, and 41 dpi. Four infected and four control plants were measured on each day in each experimental plot. The leaf immediately above the one inoculated with the virus and the corresponding leaf on control plants were measured. The portion of the leaflet inside the gas-exchange cuvette was maintained at 29 to 30°C (50% relative humidity) and CO_2 at 400 μmol mol^{-1} in the reference air stream for the ambient and elevated O_3 treatments. Photon flux density at the leaf surface was the maximum PFD determined in the field above the canopy (LI-190; LI-COR, Inc.) on the date of each measurement (2000 μmol m^{-2} s^{-1} for days 4, 8, 22, and 41; 800 μmol m^{-2} s^{-1} for day 16). Carbon assimilation rates were light saturated with PFD above 700 μmol m^{-2} s^{-1} (data not shown).

Biomass and leaf-area measurements.

The aboveground portions of a subset of infected and control plants ($n = 5$ each) in each plot were harvested on DOY 227 and separated into leaves, stems, and pods. Total leaf area for the harvested plants in each experimental plot was measured with a leaf-area meter (LI-3100C; LI-COR, Inc.). The tissue was then dried to constant mass (60°C) and weighed.

Statistical analysis of field experiment.

Variables measured (DAS-ELISA, ORAC, gas exchange, and biomass) in the field experiment were evaluated by full-factorial ANOVA (Proc Mixed, SAS 9.1; SAS Institute Inc.,

Cary, NC, U.S.A.). To control for topographic and soil variations among the FACE plots, a priori blocks were included as random effects in the gas exchange statistical analyses. Measurements performed on multiple dates were analyzed in a repeated design (with day as the repeated factor) in an autoregressive covariance structure. Statistical significance was considered for $P \leq 0.05$. For gas exchange and biomass results, post-hoc multiple comparisons were conducted using the Tukey-Kramer adjustment.

Growth chamber experiment.

To further explore the interactions between SMV and O_3, the same soybean cultivar as the field experiment was maintained in two growth chambers at 27 and 22°C and a photoperiod of 16 and 8 h of light and darkness, respectively, with an irradiance of 500 μmol m^{-2} s^{-1} (PFD). In one chamber, plants were exposed from germination to O_3 at 90 ± 5 nmol mol^{-1} for 8 h/day using an O_3 generator (Model HTU-500AC; Azco Industries Ltd., Surrey, British Columbia, Canada) and analyzer (Model 1008-RS; Dasibi Environmental Corp., Glendale, CA, U.S.A.). Another cohort of plants was grown in a nearby growth chamber under the same environmental conditions, except air was charcoal filtered to remove ambient O_3. Plants and treatments were rotated between chambers weekly to minimize potential "chamber effects". When plants developed one mature third trifoliate (approximately 3 weeks after germination), half of the plants in each chamber were randomly selected and were rub infected with SMV-G2 strain as described previously. The remaining half of the plants were mock inoculated as controls. For each treatment and sampling timepoint, four biological replicates were created. For each biological replicate, four plants were pooled.

Samples were collected from SMV- and mock-inoculated leaves at 8 and 72 hpi for measurement of global gene expression by microarray. The 8-hpi sampling timepoint was selected to detect the early response of genes involved in signaling and cellular communication and the 72-hpi timepoint was selected to monitor the genes involved in defense responses, metabolic adaptation, and protein translation and modifications.

RNA isolation and microarray hybridization.

The total RNA was isolated with an isolation procedure optimized for high-polysaccharide-containing soybean leaves. For each biological replicate, the trifoliate collected from four plants was pooled and flash frozen in liquid nitrogen. The frozen leaves were ground in precooled mortars with liquid nitrogen until they became fine powder. For 1 g of tissue, 4 ml of homogenization buffer (38% acidic phenol, 0.4 M ammonium thiocyanate, 0.8 M guanidine thiocyanate, 0.1 M of 3 M sodium acetate [pH 5.5], and 5% glycerol) was added. The mixture was transferred to Phase Lock Gel-Heavy (PLG) (Brinkmann Instruments, Inc., Westbury, NY, U.S.A.) and one-fifth volume of chloroform was added. The tubes were centrifuged for 10 min at 2,000 × *g*. The aqueous phase was transferred to a new PLG tube and equal volumes of phenol/chloroform/isoamylalcohol (5:4:1) and cold, 3 M potassium acetate, pH 5.5 (1 ml of potassium acetate per 3 ml of aqueous phase) were added. The mixture was left on ice for 1 h and centrifuged for 10 min at 2,000 × *g*. The aqueous phase was transferred to a clean centrifuge tube. Sodium citrate (0.8 M)/sodium chloride (1.2 M) salt mixture was added (0.25 volume) and the RNA was precipitated with 0.25 volume of ethanol for 30 min at –20°C. The concentration of samples was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and was verified with a BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA, U.S.A.).

Target preparation was performed according to the manufacturer's protocol (Affymetrix, Inc., Santa Clara, CA, U.S.A.). RNA from four biological replications for each treatment and mock-inoculated control was hybridized to oligonucleotide microarrays (Affymetrix GeneChip; Affymetrix) for measurement of global gene transcription. Hybridization and scanning were performed at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.

Statistical analysis of microarray data and clustering.

The microarray data from four biological replicates for each timepoint from the growth chamber experiment were analyzed. Raw data were background corrected with Robust Multi-chip Average in the BioConductor *affy* module for R. The background-corrected and log-transformed perfect match intensities were normalized using the quantile normalization method developed by Bolstad and associates (2003). The normalized data were analyzed statistically using SAS (v9.1.2; SAS Institute Inc.) by the following ANOVA model:

$$y_{ijklm} = \mu + P_i + O_j + V_k + T_l + (PO)_{ij} + (Pv)_{ik} + (PT)_{il} + (OV)_{jk} + (OT)_{jl} + (VT)_{kl} + (OVT)_{jkl} + \varepsilon_{ijklm}$$

where μ is the overall mean, y_{ijklm} is the PM-logarithm of the *m*th replicate model-based-expression value at the *i*th probe gene ($i = 1,\ldots,11$), *j*th treatment ($j = 1,2$), *k*th virus ($k = 1,2$), and *l*th time ($l = 1,2$); *P*, probe set; *O*, elevated O_3; *V*, virus; and *T*, time. Significant gene lists were obtained after applying a cutoff FDR of <0.01, which was calculated based on the *P* value from the ANOVA as in Benjamini and Hochberg (1995) using SAS.

Gene annotation.

The Affymetrix oligonucleotide-based GeneChip Soybean Genome Array is prepared from the available expressed sequence tag sequences consisting of 37,593 probe sets representing 35,611 soybean transcripts. This chip does not fully represent the soybean genome because it is not yet sequenced. To understand the possible function of individual transcripts, we compared the sequence used by Affymetrix to The Institute for Genomic Research soybean gene indices tentative consensus (TC) and NCBI nonredundant sequences and *Arabidopsis* sequences.

Quantitative RT-PCR analysis.

The expression level of two PR genes (*PR-10* and *PR-5*) and a small subunit of ribulose bisphosphate carboxylase gene (*Rubisco SSII*) were measured by quantitative RT-PCR for infected plants grown under elevated O_3 in growth chambers and elevated O_3 and CO_2 in the field experiment. Primers were designed using Primer 3 software (Rozen and Skaletsky 2000). Total RNA from leaf samples was extracted as described above and was enriched for mRNA using affinity columns (QIATEX; Qiagen, CA, U.S.A.). cDNA was synthesized from 20 ng of mRNA using Superscript III (Invitrogen, Carlsbad, CA, U.S.A.). Serial dilutions up to 1×10^4 of the cDNA were used in a standard real-time RT-PCR reaction containing Sybergreen dye to determine reaction efficiencies for each primer set and sample. Based on calculated PCR efficiencies, 0.2 to 2.0 ng of mRNA equivalent cDNA was used to monitor changes in transcript level. A dissociation curve analysis was performed and confirmed the absence of multiple amplification products prior to relative gene expression analysis. Data were normalized to the expression level of *actin 3 isoform B* (TC204824), an endogenous, constitutively expressed reference gene, and relative gene expression was calculated according to Pfaffl and associates (2002).

Analysis of flavonoids with LC/MS.

For each timepoint and treatment, there were three biological replicates. For each biological replicate, the infected or mock-treated leaves from four plants were pooled and freeze dried for 2 days. Lyophilized tissue (50 mg) was weighed and placed in a 2-ml microcentrifuge tube. A glass bead (6 mm) was placed into the same tube and the tissue was shaken in a Qiagen bead beater for 2 min until it became a fine powder. To the powder sample, 500 µl of extraction buffer (9 parts methanol and 10 parts 0.5% formic acid, with 0.001% xanthotoxol as an internal standard) was added. The samples were placed on a shaker for 30 min at room temperature and thoroughly mixed; then, the samples were spun for 30 s at maximum speed (14,000 rpm) in a microcentrifuge. The supernatant was transferred to a 1.5 ml-microcentrifuge tube containing 50 mg of C-18 silica, which removed highly nonpolar compounds. The sample was vortexed briefly and spun for 30 s at maximum speed (14,000 rpm) in a microcentrifuge. The supernatant (approximately 200 µl) was transferred to a microinsert tube inside a 2-ml glass autosampler vial. Sample (10 µl) was injected into the Shimadzu 2010 spectrometer for LC/MS and was analyzed by Shimadzu Labsolutions software. The LC/MS separation was performed using a Luna C18 column (250-by-2-mm column, 5-µm particle size) at 25°C. The LC/MS eluents were aqueous 0.05% formic acid (A) and acetonitrile (B). The initial gradient condition was 90% A and 10% B, changing linearly to 70% B in 48 min. After analysis, the column was equilibrated for 17 min at initial conditions, giving a total analysis time of 65 min. The eluent flow rate was 0.2 ml/min. To identify some of the flavonoids with the same base mass of charge (*m/z*), acid hydrolysis was performed. In all, 20 µl of 2 N HCl was added to 150 µl of extract and boiled for 60 min in an airtight vial. As a control instead of HCl, 20 µl of high-performance liquid chromatography-grade water was added to the extract. Both extracts were analyzed by LC/MS with the conditions previously described. To identify compounds, several standards were used as references. The standard solutions were prepared by dissolving 564 µg of daidzein, 81 µg of daidzin, 72 µg of genistin, 232 µg of glycitin, 292 µg of kaempherol, 266 µg of kaempherol-3-*O*-glucoside, 224 µg of luteolin-7-*O*-glucoside, 140 µg of quercitrin, 281 µg of quercetin, and 427 µg of rutin in 3 ml of methanol. The standards (10 µl) were injected into the spectrometer for LC/MS analysis.

The variables measured were evaluated by full-factorial ANOVA (Proc Mixed, SAS 9.1; SAS Institute Inc.). Statistical significance ($P < 0.05$) of paired least square means was compared by using Student's *t* test.

DAB and NBT staining.

Elevated O_3 induces oxidative stress and mimics the effect of biotic elicitors. To determine whether O_3 and SMV elicited similar responses in soybean leaves, the spatial and temporal distribution of ROS was examined by histochemical staining.

In a separate controlled-environment experiment, plants were exposed to elevated O_3 with and without SMV infection. One group of plants was grown from germination in a chamber with O_3 at 90 ± 5 nmol mol^{-1} for 8 h/day. Another group of plants was grown in ambient conditions and half of the plants in each chamber were challenged with SMV. DAB was used to determine hydrogen peroxide (H_2O_2) and NBT for superoxide ($O_2^{\bullet-}$) accumulation as described by Fryer and associates (2002). DAB forms a dark brown precipitate upon reacting with H_2O_2 in the presence of peroxidase. When NBT reacts with $O_2^{\bullet-}$, a dark blue insoluble formazan is produced. To avoid the wound-induced ROS production from rubbing inoculated leaves with Carborundum and to observe the differences in the systemic infection of SMV, the fourth fully developed trifoliate (above the infected leaf) was stained 1 and 2 weeks postinfection. Tissues were boiled in destaining solution that contained glycerol, lactic acid, acidic phenol, ethanol, and water (1:1:1:2:1 parts) to remove chlorophyll. Leaves were imaged under a stereo microscope (SZX12; Olympus, Center Valley, PA, U.S.A.) and digital camera (DP70; Olympus). Although these dyes have low specificity, they are widely used to verify oxidative stress in plants (Shulaev and Oliver 2006).

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