Rapid report

Differential responses of G-protein *Arabidopsis thaliana* mutants to ozone

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Summary

• Ground-level ozone (O_3) curtails agricultural production in many regions worldwide. However, the etiology of O_3 toxicity remains unclear. Activated oxygen species appear to inflict biochemical lesions and propagate defense responses that compound plant injury. Because some plant defense responses involve membrane-delimited GTPases (G proteins), we evaluated the O_3 sensitivity of *Arabidopsis* mutants altered in the heterotrimeric G-protein pathway.

• Eight genotypes were treated with a range of O_3 concentrations (0, 100, 175 and 250 nmol mol^{-1}) for 13 d in controlled environment chambers.

• After treatment with O_3, the epinasty typically observed for wild type leaves did not occur in mutant plants lacking the alpha subunit of the G-protein complex (*gpa1*). O_3-induced suppression of leaf chlorophyll levels and leaf mass per unit leaf area were less for *gpa1* mutants and were not due to differences in O_3 flux.

• There was a positive correlation between the lack of a G-protein alpha subunit and decreased O_3 sensitivity. Our results suggest that a heterotrimeric G-protein is critically involved in the expression of O_3 effects in plants.

Introduction

Ground-level O_3 concentrations in most agricultural regions near urbanized areas worldwide currently suppress the growth and yield of susceptible crops by 5–15% (Heck *et al.*, 1983; US Environmental Protection Agency, 1996). Furthermore, tropospheric O_3 concentration is expected to increase in the 21st century as emissions of its precursors continue to grow (Prather *et al.*, 2001). Regional O_3 episodes from local smog production build upon background tropospheric levels to produce highly phytotoxic atmospheric conditions periodically during the spring and summer. However, the etiology of O_3 phytotoxicity remains unclear.

Ozone toxicity appears to consist of biochemical lesions inflicted by a strong chemical oxidant (O_3 and its metabolites) that promotes production of plant-originated activated oxygen species (AOS) and other metabolites that cause injury (Kangasjärvi *et al.*, 1994; Sharma & Davis, 1997; Overmyer *et al.*, 2000; Rao & Davis, 2001). Injury arises from protein oxidation, impaired ion regulation, and toxic byproduct production (Sharma & Davis, 1997; Kanoun *et al.*, 2002). In addition to suppressing plant growth, chronic O_3 exposure lowers net photosynthesis, ribulose bisphosphate carboxylase (Rubisco) activity and mRNA transcript levels for the small subunit of Rubisco (Pell *et al.*, 1997). It also causes foliar injury and accelerates senescence in many plants. Evidence is

accumulating that responses to O_3 are modulated by ethylene, jasmonic and salicylic acid levels, and the interactions among their signaling pathways (Kangasjärvi *et al.*, 1994; Sharma *et al.*, 1996; Pell *et al.*, 1997; Koch *et al.*, 2000; Overmyer *et al.*, 2000; Rao *et al.*, 2000; Rao & Davis, 2001; Rao *et al.*, 2002; Overmyer *et al.*, 2003; Tamaoki *et al.*, 2003; Vahala *et al.*, 2003). For example, *Arabidopsis* mutants that overproduce ethylene (*eto1*) or are insensitive to jasmonic acid (*jar1*) are more extensively injured by O_3 than Columbia wild-type plants (Overmyer *et al.*, 2000; Rao *et al.*, 2000; Tamaoki *et al.*, 2003). Ethylene acts as a promoting factor for O_3 injury, whereas jasmonates might have a role in minimizing injury (Overmyer *et al.*, 2000; Overmyer *et al.*, 2003).

Ozone is believed to be perceived by the plant in the extracellular space (Kangasjärvi *et al.*, 1994). Questions remain, however, regarding the signal transduction mechanisms involved in putative O_3-elicited responses. Especially, early events transmitting information of O_3 perception into the cell remain unknown. We hypothesize that membrane-bound GTPases (G-proteins) that transduce extracellular signals to intracellular receptors are involved. G-proteins are thought to be involved in interactions with plant hormones such as auxin and with plant defense responses to wounding or pathogen infection (Assmann, 2002; Jones, 2002). The binding of O_3, or more likely an O_3-derived reaction product, to a G-protein receptor could activate the protein and initiate a signal cascade process that activates target proteins in this signal transduction. The objective of this study was to test directly whether the *Arabidopsis* G-protein complex is involved in O_3 signal transduction.

Methods and Materials

Plant culture and O_3 treatment conditions

Five G-protein null mutants (Jones, 2002; Chen *et al.*, 2003; Jones *et al.*, 2003; Ullah *et al.*, 2003), two O_3-sensitive mutants (*rcd1* and *eto1*) (Overmyer *et al.*, 2000; Rao *et al.*, 2002; Tamaoki *et al.*, 2003), all in ecotype Columbia (Col-O) background, and Col-O were used in this study. *gpa1–3* and *gpa1–4* are transcript null mutants in the single canonical G alpha subunit gene (Jones *et al.*, 2003), *rgs1–1* and *rgs1–2* are null mutants of the gene encoding the single Regulator of G Signaling (Chen *et al.*, 2003), and *gcr1–1* and *gcr1–2* are null mutants of the gene encoding a candidate G protein coupled receptor (J.-G. Chen *et al.*, unpublished). A transcript null mutant in the G beta subunit gene (*agb1–2*) and a double-null mutant (*gpa1–4*, *agb1–2*) (Jones *et al.*, 2003) were also tested.

Plants were grown individually in 100-ml pots containing a 3 : 2 : 1 mixture of Pro-Mix BX (Premier Horticulture, Inc., Quakertown, PA, USA), perlite and sand in a growth chamber at 23°C, 300 μmol m^{-2} s^{-1} photosynthetic photon fluence rate (PPFR), 8/16 h light/dark cycle and 50% relative humidity (RH) for 21 d. Before planting, the growth medium was fertilized with an aqueous solution containing 0.7 g l^{-1} of soluble fertilizer (20-20-20, N-P-K) (Peters Professional, Scotts-Sierra Horticultural Products Co., Marysville, OH, USA). Plants were then transferred to four 0.8 m^3 continuous-stirred tank reactor chambers (CSTRs) (Rogers *et al.*, 1977) located in a controlled environment chamber in the NC State University Phytotron. CSTRs are cylindrical, Teflon-covered chambers designed for the rapid mixing of treatment gases with charcoal-filtered air. After placement in CSTRs, plants were acclimated at 23°C, 300 μmol m^{-2} s^{-1} PPFR, 12/12 h light/dark cycle, and 50% RH for 4–7 d. Plants were then exposed to 0, 100, 175 and 250 nmol O_3 mol^{-1} 7 h daily for 13 d. Ozone was produced by electrostatic discharge in dry O_2 (model GTC-1 A, Ozonia North America, Elmwood Park, NJ, USA) and monitored using a UV photometric O_3 analyzer (model 49, Thermo Environmental Instruments Co., Franklin, MA, USA). The experiment was conducted three times.

Gas exchange, chlorophyll and biomass assays

Net photosynthesis and stomatal conductance (g_s) of Col-O and G-protein mutants were measured in the CSTRs on three leaves on each of three plants from the 0 and 100 nmol O_3 mol^{-1} treatments after exposure for 11 d. Gas exchange was measured using a portable photosynthesis system (model 6400, Li-Cor, Inc., Lincoln, NB, USA) and a sampling chamber with a 0.79 cm^2 aperture (Model 6400–15, Li-Cor, Inc.). Net photosynthesis and g_s were computed from measurements of CO_2 and H_2O vapor concentrations in the leaf sample and reference chambers using infrared gas analyzers and other environmental measurements. During the measurements, average leaf-to-air vapor pressure deficit, leaf temperature, RH and PPFR in the leaf chamber were 1.5 kPa, 23°C, 46% and 287 μmol m^{-2} s^{-1}, respectively. After O_3 treatment for 13 d, leaves were sampled for chlorophyll analysis and shoots were harvested for dry mass measurements. For the chlorophyll assay, five leaf disks (0.85 cm diameter) from each plant were extracted twice with 3 ml of 95% ethanol overnight at 4°C. Extracts were pooled by sample, and chlorophyll concentrations were determined as previously described (Lichtenthaler & Wellburn, 1983). Ten leaf disks from leaves sampled for chlorophyll concentration were taken for dry mass measurements. Leaf disks and remaining shoots were frozen in liquid nitrogen, freeze-dried and weighed.

Leaf curvature assay

In two additional replications of the experiment, four randomly selected leaves ≥ 2 cm in length were harvested from each of three plants in the 0 and 100 nmol O_3 mol^{-1} treatments after exposure for 6–12 d. The distal two-third of each leaf was transversely sectioned into three segments along the longitudinal axis. A 0.8-mm wide strip of tissue obtained by transverse section of each segment was placed on a glass slide under a dissecting microscope where its curvature was assessed.

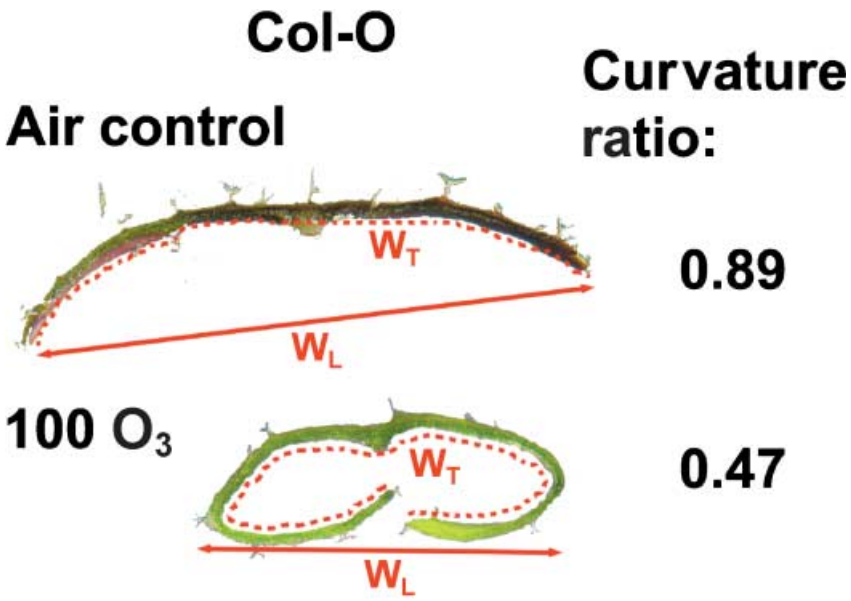

Fig. 1 Leaf curvature was determined as the ratio of leaf width (W_L) (length of red line) to total leaf width (W_T) (length of hand-traced, dashed red line). Leaf widths were measured on enlarged digital images of transverse leaf sections. Leaf sections from Col-O plants treated with either 0 (air control) or 100 nmol O_3 mol^{-1} (100 O_3) 7 h daily for 12 d are shown. Leaf curvature ratios (W_L : W_T) for air control = 0.89 and for 100 O_3 = 0.47.

Curvature was determined by photographing each fresh section from the side followed by measurements of enlarged images using a digital take-off tool. The width of the leaf in cross-section (W_L) was divided by the total width (W_T) of the leaf to obtain a ratio that varied from 1 for a flat leaf to 0.5 for a highly curled leaf (Fig. 1). W_T in Fig. 1 is indicated by the red hand tracings of the two leaves shown. Leaf section curvature was not observed to change following sectioning, careful placement on the slide or during image acquisition on the illuminated microscope stage. While there are many methods to quantify leaf curvature, this method was chosen because it is sensitive in the low curvature range and is rapid.

Statistical analysis

The experiment was conducted three times in a randomized complete block design. Each time, three to four plants of each line were exposed to the various O_3 treatments. A Log_e transformation was applied to the chlorophyll and aerial dry mass data before statistical analysis. Treatment effects and means were estimated using a split-plot model in which chambers constituted the whole-plot and genotype was the within-plot factor (SAS Proc Mixed) (Littell *et al.*, 1996). Means were considered significantly different if $P \leq 0.05$, and marginally significantly different if $P \leq 0.1$.

Results

Leaf curvature

After treatment with 100 or 175 nmol O_3 mol^{-1} for 5–6 d, leaf morphology differed among genotypes. As shown in Fig. 2(a), wild type leaves respond to O_3 by displaying epinastic growth, that is severe lateral down curling and infolding of leaf margins along the longitudinal axis. As shown in Fig. 1, the typical curvature ratio for control Col-O leaves is 0.89 and after O_3 treatment the curvature ratio is dramatically reduced. However, *gpa1* single and the *gpa1–4*, *agb1–2* double mutant leaves did not display leaf curvature (Fig. 2b, Table 1). Leaf curling in *agb1–2* plants was variable. Visible foliar injury was absent in all genotypes in the 100 nmol O_3 mol^{-1} treatment except for the O_3-hypersensitive mutants, *rcd1* and *eto1* plants (Fig. 3). The *rcd1* and *eto1* plants exhibited leaf curling (Table 1), chlorosis and necrotic lesions in the 100 nmol O_3 mol^{-1} treatment that increased in severity with increasing O_3 concentrations. All genotypes, however, exhibited chlorotic lesions and necrosis after treatment with 250 nmol O_3 mol^{-1} (Fig. 3). Plants in the control treatment showed only minor leaf curling and no chlorosis or necrosis.

Gas exchange, chlorophyll, leaf mass per unit area and shoot biomass assays

Net photosynthesis in Col-O was suppressed by 60% by 100 nmol O_3 mol^{-1} after exposure for 11 d (Table 2). However, there was no statistically significant O_3 effect on net photosynthesis among the G-protein mutants. Stomatal conductance among the controls averaged 315 ± 131 (SD) mmol H_2O m^{-2} s^{-1} after treatment for 11 d. There was no indication of significant inherent differences in g_s among genotypes under control conditions ($P > 0.1$). Stomatal conductance was lower in all genotypes following treatment with 100 nmol O_3 mol^{-1} for 11 d although the differences were statistically significant only for the *gpa1–4*, *agb1–2* and the double mutant lines (Table 3).

Leaf chlorophyll concentrations were not strongly altered by the 100 and 175 nmol mol^{-1} O_3 treatments (Table 3). Chlorophyll concentration in the highest O_3 treatment was

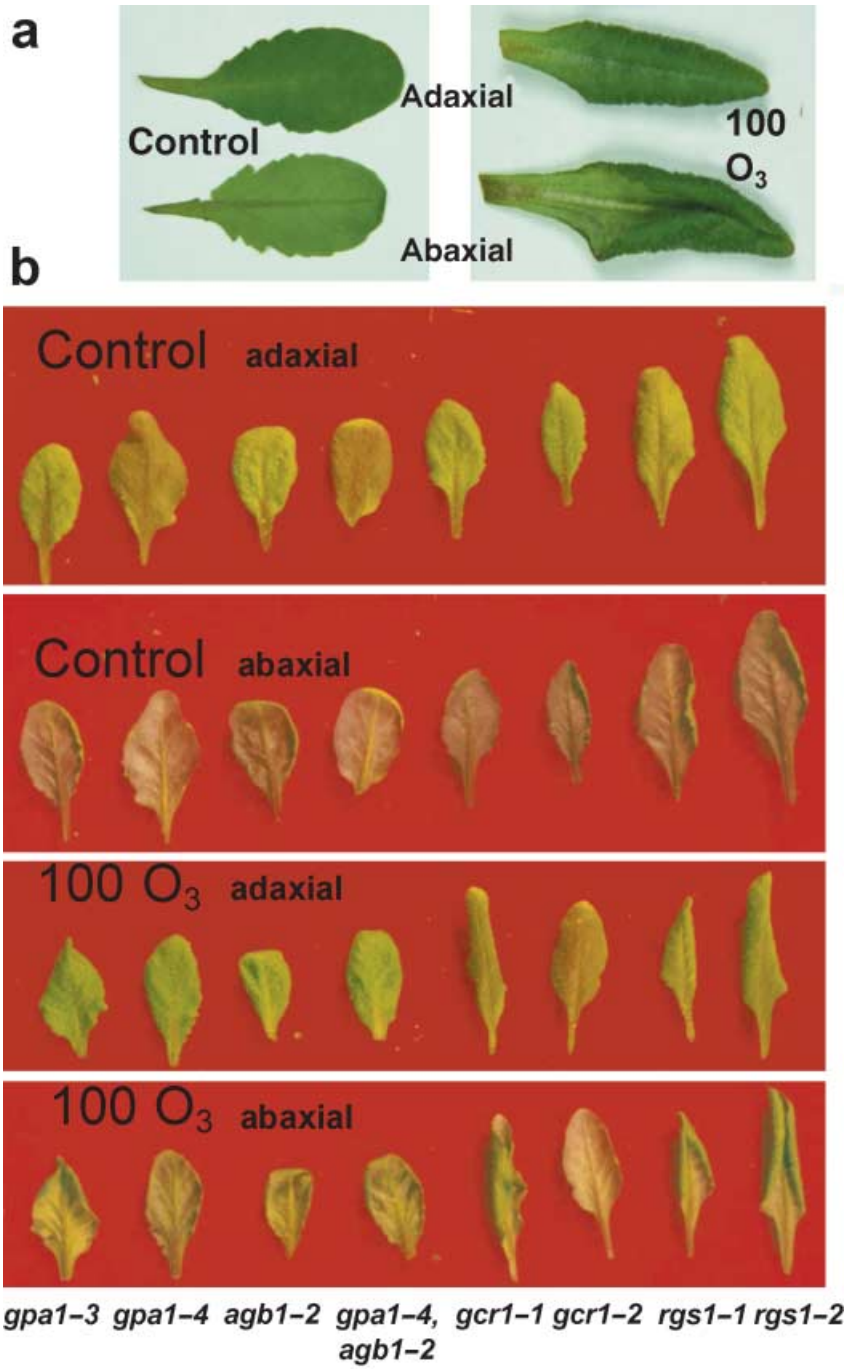

Fig. 2 The *Arabidopsis* heterotrimeric G protein complex is required for O_3-induced leaf curling. (a) Example of leaf curling after treatment of Col-O plants with 100 nmol O_3 mol^{-1} 7 h daily for 6 d. (b) Leaves from the indicated *Arabidopsis* mutants after treatment with 0 or 100 nmol O_3 mol^{-1} (100 O_3) 7 h daily for 12 d. Adaxial and abaxial views are indicated.

significantly lower in the *agb1–2*, *gcr1–2* and *rgs1–1* genotypes. The two O_3-hypersensitive mutants, *rcd1* and *eto1*, displayed the greatest O_3-induced reductions in chlorophyll concentration compared with wild type plants. In *rcd1*, there was insufficient tissue available for sampling in the 175 and 250 nmol mol^{-1} treatments.

Leaf mass per unit leaf area decreased in Col-O, *gcr1*, and *rgs1* lines with increasing O_3 treatment concentrations (Table 3). However, it did not decrease in response to O_3 in the *gpa1* lines. In *agb1–2* and the double mutant, leaf mass per unit area was lower in the highest O_3 treatment, although the effect was only statistically significant for *agb1–2*.

Average aerial tissue dry mass values were all lower in the elevated O_3 treatments after 13 d compared with the air controls, although the magnitude of the treatment effects varied among genotypes (Table 3). This parameter did not prove to be a robust indicator for distinguishing differences in O_3 sensitivity.

Fig. 3 Ozone induces vegetative damage that is independent of a heterotrimeric G protein complex. Representative *Arabidopsis* Col-O, and indicated mutants after treatment with 0, 100, 175 or 250 nmol O_3 mol^{-1} 7 h daily for 12 d.

Discussion

In this study, we found that two G-protein α null mutants (*gpa1–3* and *gpa1–4*) and the double null mutant (*gpa1–4*, *agb1–2*) responded differently to O_3 compared with Col-O and *gcr1*, *rgs1*, *rcd1* and *eto1* mutant plants. The major difference among genotypes was the lack of leaf curling in the *gpa1* lines in response to O_3 compared with the other genotypes.

The *gcr1* mutants used in this study did not result in attenuated O_3-induced leaf curling responses (Table 1). This clearly demonstrates that GCR1 is not involved in O_3 responses and implies that another pathway leading to G-protein activation

Table 1 Leaf curvature ratio of Col-O, G-protein mutants, *rcd1* and *eto1* plants following treatment with 0 and 100 nmol O_3 mol^{-1} 7 h daily for 12 d. Leaf curvature was evaluated by the ratio of curled leaf width to total leaf width (see Fig. 1)

Genotype	O_3 treatment (nmol mol^{-1})	Curvature ratio
Col-O	0	0.81 ± 0.06
	100	0.67 ± 0.06†
gpa1–3	0	0.85 ± 0.06
	100	0.77 ± 0.06
gpa1–4	0	0.88 ± 0.06
	100	0.82 ± 0.06
agb1–2	0	0.75 ± 0.03
	100	0.66 ± 0.03*
gpa1–4, *agb1–2*	0	0.76 ± 0.03
	100	0.82 ± 0.03
gcr1–1	0	0.80 ± 0.03
	100	0.58 ± 0.03***
gcr1–2	0	0.82 ± 0.03
	100	0.70 ± 0.03**
rgs1–1	0	0.78 ± 0.03
	100	0.68 ± 0.03*
rgs1–2	0	0.76 ± 0.03
	100	0.54 ± 0.03***
rcd1–1	0	0.87 ± 0.03
	100	0.59 ± 0.04***
eto1	0	0.84 ± 0.04
	100	0.65 ± 0.04***

Values shown are means ± SE Significant O_3 effects within genotype are indicated as $P \leq 0.1$ (†), ≤ 0.05 (*), ≤ 0.01 (**), ≤ (***). A value of 1 indicates a flat leaf while a value of 0.5 represents a severely epinastic leaf.

is at work in O_3-exposed plants. Currently the number of G-protein coupled receptors is unknown (Jones, 2002). Thus one of the unknown receptors may be involved in O_3 responses. Alternatively, a receptor independent pathway for G-protein activation has been suggested in plants (Jones, 2002).

Leaf curling in response to O_3 was noted in a previous study with Col-O (Sharma & Davis, 1994). Sharma & Davis (1994) suggested that leaf curling was related to enhanced ethylene production. Increased ethylene production has been associated with O_3 sensitivity in plants in previous studies (Tingey *et al.*, 1976; Tamaoki *et al.*, 2003). The *eto1* plants, which exhibit enhanced ethylene production, showed leaf curling, foliar injury and suppressed biomass that increased in severity with the O_3 concentrations. Based on a variety of experiments with different *Arabidopsis* lines including *eto1*, Tamaoki *et al.* (2003) concluded that ethylene-induced responses were primarily responsible for enhanced O_3 sensitivity. Ethylene responses may be modulated by salicylic acid concentrations (Rao *et al.*, 2002).

Chlorophyll, leaf mass per unit area and aerial dry mass measurements also indicated that G-protein α mutants were affected less by O_3 compared with the other genotypes. Chlorophyll concentration and leaf mass per area typically decrease in response to O_3 in cotton (*Gossypium hirsutum* L.) and soybean [*Glycine max* (L.) Merr.] leaves chronically exposed to O_3 (Booker, 2000; Morgan *et al.*, 2003). Decreased chlorophyll concentration is an often-used measure of O_3 injury (Knudson *et al.*, 1977). Decreased leaf mass per unit area is associated with O_3-related decreases in net photosynthesis, C assimilation and starch accumulation (Miller, 1988; Morgan *et al.*, 2003). The types of injury responses observed here for

Table 2 Net photosynthesis and stomatal conductance (g_s) of Col-O and G-protein null mutants following treatment with 0 and 100 nmol O_3 mol^{-1} 7 h daily for 11 d

Genotype	O_3 treatment (nmol mol^{-1})	Net photosynthesis (μmol CO_2 m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})
Col-O	0	3.5 ± 0.6	311 ± 48
	100	1.4 ± 0.6**	238 ± 50
gpa1–3	0	3.1 ± 0.7	238 ± 56
	100	2.2 ± 0.7	209 ± 63
gpa1–4	0	2.9 ± 0.6	303 ± 45
	100	1.8 ± 0.6	191 ± 50†
agb1–2	0	2.7 ± 0.6	431 ± 48
	100	2.8 ± 0.6	233 ± 50**
gpa1–4, *agb1–2*	0	1.8 ± 0.9	494 ± 79
	100	2.3 ± 0.9	287 ± 75†
gcr1–1	0	3.5 ± 0.9	245 ± 79
	100	2.9 ± 1.0	144 ± 86
gcr1–2	0	2.8 ± 0.9	237 ± 79
	100	nd	nd
rgs1–1	0	2.9 ± 0.7	286 ± 58
	100	2.5 ± 0.7	250 ± 79
rgs1–2	0	2.9 ± 0.9	255 ± 80
	100	1.7 ± 1.0	156 ± 86

Values shown are means ± SE. Significant O_3 effects within genotype are indicated as $P \leq 0.1$ (†), ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***); nd – not determined.

Table 3 Leaf chlorophyll concentration, leaf mass per unit area and shoot dry mass of Col-O, G-protein, *rcd1* and *eto1* mutants following treatment with 0–250 nmol O_3 mol^{-1} 7 h daily for 13 d

Genotype	O_3 treatment (nmol mol^{-1})	Chlorophyll (μg cm^{-2})	Leaf mass per area (mg cm^{-2})	Dry mass (mg per plant)	Dry mass relative to air control (%)
Col-O	0	20.6 ± 4.4	2.5 ± 0.4	238 ± 35	100
	100	21.6 ± 4.3	2.3 ± 0.3	274 ± 37	115
	175	17.7 ± 4.2	1.7 ± 0.3	189 ± 32	79
	250	16.6 ± 3.8	1.7 ± 0.3†	200 ± 33	84
gpa1–3	0	22.2 ± 5.0	2.6 ± 0.5	399 ± 65	100
	100	19.7 ± 4.5	2.4 ± 0.4	379 ± 62	95
	175	29.5 ± 9.2	2.6 ± 0.8	385 ± 94	96
	250	25.8 ± 8.0	2.8 ± 0.8	294 ± 72	74
gpa1–4	0	20.0 ± 3.9	2.2 ± 0.3	372 ± 49	100
	100	26.4 ± 5.2	2.5 ± 0.4	347 ± 46	93
	175	31.3 ± 7.2*	2.6 ± 0.5	314 ± 52	84
	250	21.4 ± 4.9	2.2 ± 0.4	276 ± 45	74
agb1–2	0	21.3 ± 4.2	2.3 ± 0.4	343 ± 46	100
	100	23.8 ± 4.7	2.4 ± 0.4	269 ± 36	78
	175	27.4 ± 6.3	2.4 ± 0.4	269 ± 44	78
	250	13.7 ± 3.1*	1.4 ± 0.3*	242 ± 40†	70
gpa1–4, *agb1–2*	0	17.8 ± 3.9	2.2 ± 0.4	324 ± 51	100
	100	26.8 ± 5.9†	2.5 ± 0.4	280 ± 44	86
	175	23.5 ± 6.7	1.8 ± 0.5	233 ± 52	72
	250	14.8 ± 4.2	1.5 ± 0.4	224 ± 50	69
gcr1–1	0	18.6 ± 3.7	2.6 ± 0.4	367 ± 49	100
	100	26.2 ± 5.2†	2.6 ± 0.4	297 ± 40	81
	175	18.7 ± 4.3	2.0 ± 0.4	288 ± 47	78
	250	15.3 ± 3.5	1.8 ± 0.3†	290 ± 48	79
gcr1–2	0	24.5 ± 7.6	3.4 ± 1.0	413 ± 101	100
	100	29.5 ± 9.1	2.8 ± 0.8	300 ± 73	73
	175	22.8 ± 7.1	2.8 ± 0.8	369 ± 90	89
	250	9.1 ± 2.8**	1.1 ± 0.3**	162 ± 39**	39
rgs1–1	0	23.3 ± 4.6	2.8 ± 0.4	329 ± 44	100
	100	27.8 ± 5.5	2.6 ± 0.4	342 ± 46	104
	175	18.2 ± 4.2	1.8 ± 0.3*	279 ± 46	85
	250	12.2 ± 2.8**	1.3 ± 0.2**	240 ± 40†	73
rgs1–2	0	23.5 ± 4.6	3.1 ± 0.4	357 ± 48	100
	100	20.4 ± 4.0	2.0 ± 0.3*	247 ± 33*	69
	175	16.1 ± 3.7†	2.1 ± 0.4†	228 ± 37*	64
	250	18.1 ± 4.1	1.8 ± 0.3*	216 ± 36**	60
rcd1–1	0	15.7 ± 3.6	1.3 ± 0.2	148 ± 24	100
	100	7.3 ± 1.7***	0.7 ± 0.1**	141 ± 23	95
	175	nd	nd	88 ± 14*	59
	250	nd	nd	82 ± 14**	55
eto1	0	18.3 ± 5.2	1.8 ± 0.5	146 ± 32	100
	100	7.9 ± 2.2**	0.9 ± 0.2 *	98 ± 22	67
	175	6.9 ± 2.2**	1.1 ± 0.3 †	56 ± 13**	38
	250	5.8 ± 1.8***	1.0 ± 0.3 †	50 ± 11**	34

Values shown are means ± SE. Significant O_3 effects within genotype are indicated as $P \leq 0.1$ (†), ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***); nd – not determined. Dry mass data for the elevated O_3 treatments were also normalized to the 0 nmol O_3 mol^{-1} treatment (air control) for each genotype.

Arabidopsis were similar to those caused by ambient O_3 pollution, and were distinct from the widespread loss of cellular integrity, wilting and massive necrosis that can occur with acute O_3 treatments.

Measurements of g_s indicated that O_3 uptake was not substantially different among genotypes under control conditions. Stomatal conductances were lower after treatment with 100 nmol O_3 mol^{-1} for 11 d, but g_s rates were still similar among O_3-treated genotypes. Also, the relative decreases in g_s in O_3-treated *gpa1–4*, *agb1–2* and *gpa1–4*, *agb1–2* lines were similar to *gcr1–1* and *rgs1–2* lines. However, leaf curling did not occur in the *gpa1* lines while it did in the *gcr1–1* and *rgs1–2* lines. Leaf curling also occurred in Col-O plants that had conductances similar to the *gpa1* lines. Therefore, differential

responses among genotypes to the O_3 treatments were likely not due to differences in O_3 flux.

It is possible that pleotropic effects of the mutation on cell structure might be involved in the lack of O_3 responses. Specifically, *gpa1* mutants have reduced cell divisions and increased cell expansion. These or other related changes might physically limit the ability of the leaves to curl. However, when exposed to short, very high episodes of O_3 (> 300 nmol mol^{-1}), which cause lesions on wild type plants, *gpa1–1* remained O_3 tolerant (K. Overmyer, unpublished). The induction of cell death by O_3 is not subject to the same restrictions that changes in cell structure may impose on the leaf curling phenotype. This suggests the results seen in this paper are due to altered perception of O_3 or one of its downstream signals and cannot be attributed simply to general pleotropic effects.

Results from this study suggest, for the first time, that G-proteins are involved in the transduction of O_3-derived signals. This departs from the general view that O_3 action is mainly through its toxicity. While O_3 toxicity does occur, evidence for O_3-related signal transduction can now be genetically dissected from its toxicity. Furthermore, these results evoke parallels between O_3-induced and pathogen-induced responses, a topic that has received much attention (Kangasjärvi *et al.*, 1994; Pell *et al.*, 1997; Sharma & Davis, 1997; Sandermann, 1998; Overmyer *et al.*, 2000; Rao & Davis, 2001). Both pathogens and O_3 induce plant AOS production, which is preceded and regulated by plasma membrane ion fluxes and membrane-bound oxidases such as NAD(P)H oxidase (Rao & Davis, 2001). In response to pathogens, G-proteins have a demonstrated role regulating Ca^{++} influx, H^+-influx (Xing *et al.*, 1997; Aharon *et al.*, 1998).Results presented in this study are consistent with a role for G-proteins as an early signaling link relaying information into the cell from the extracellular site of O_3 perception. However, further studies will be required to show if G-proteins have such a direct role in O_3 signal transduction. Alternate explanations can also be found. G-proteins have been shown to modulate, via both direct and indirect mechanisms, the sensitivities of plants to multiple hormones (Jones, 2002). Given the demonstrated role of interactions between hormones such as ethylene, jasmonate, and ABA in the expression of O_3 responses (Overmyer *et al.*, 2003), it is possible that G-proteins modulate O_3 responses via their roles in hormone signal transduction. These facts notwithstanding, there was a positive correlation between the lack of a G-protein alpha subunit and decreased O_3 sensitivity. Our results suggest that a heterotrimeric G protein is critically involved in the expression of O_3 effects in plants.

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