

Isoprene synthesis protects transgenic tobacco plants from oxidative stress

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ABSTRACT

Isoprene emission represents a significant loss of carbon to those plant species that synthesize this highly volatile and reactive compound. As a tool for studying the role of isoprene in plant physiology and biochemistry, we developed transgenic tobacco plants capable of emitting isoprene in a similar manner to and at rates comparable to a naturally emitting species. Thermotolerance of photosynthesis against transient high-temperature episodes could only be observed in lines emitting high levels of isoprene; the effect was very mild and could only be identified over repetitive stress events. However, isoprene-emitting plants were highly resistant to ozone-induced oxidative damage compared with their non-emitting azygous controls. In ozone-treated plants, accumulation of toxic reactive oxygen species (ROS) was inhibited, and antioxidant levels were higher. Isoprene-emitting plants showed remarkably decreased foliar damage and higher rates of photosynthesis compared to non-emitting plants immediately following oxidative stress events. An inhibition of hydrogen peroxide accumulation in isoprene-emitting plants may stall the programmed cell death response which would otherwise lead to foliar necrosis. These results demonstrate that endogenously produced isoprene provides protection from oxidative damage.

Key-words: isoprene synthase; oxidative damage; ozone; reactive oxygen species; thermotolerance.

INTRODUCTION

Isoprene (2-methyl-1,3-butadiene) is synthesized by many, but not all, plant species (Hewitt, Street & Scholefield 1997). Because of its high volatility, it is rapidly emitted to the atmosphere. Because of its high reactivity and large flux rate (~500 Tg C year⁻¹) (Guenther *et al.* 2006), isoprene has major impacts on atmospheric chemistry (Fehsenfeld *et al.* 1992; Shallcross & Monks 2000), mediating in the oxidant

budget of the lower atmosphere (Pierce *et al.* 1998), potentially controlling the lifetime of the greenhouse gas methane (Shallcross & Monks 2000) and forming secondary organic aerosol particles (Claeys *et al.* 2004). Isoprene, therefore, influences the radiation balance of the atmosphere.

Isoprene production in plants is catalysed by the enzyme isoprene synthase, which converts dimethylallyl diphosphate (DMADP) to isoprene and pyrophosphate (Silver & Fall 1991, 1995). Under normal conditions, DMADP for isoprene synthesis is produced primarily via the chloroplastic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Lichtenthaler 2000). Isoprene emission represents a very significant loss of energy and carbon from emitting plants (Sharkey & Yeh 2001); under stress conditions, losses of 10–20% of recently fixed carbon can occur (Harley, Guenther & Zimmerman 1996). Global carbon loss is equivalent to about 1.3% of net primary productivity (NPP), while in particular ecosystems under certain conditions, losses may account for as much as 10% of NPP (Guenther *et al.* 1995), figures that may in fact be an underestimate (Harley *et al.* 1996). It is assumed that plants which lose carbon and energy through isoprene production must gain some benefits from its synthesis (Niinemets *et al.* 1999; Sharkey & Yeh 2001; Lerda 2007).

The role of isoprene in plant physiology and biochemistry is still under investigation. Early experiments relied on depleting the DMADP substrate by chemical inhibition of the MEP pathway and/or fumigating non-emitting or inhibited species with exogenous isoprene. These experiments suggested that isoprene may act as a thermoprotectant of the photosynthetic apparatus and/or as an antioxidant (Sharkey & Singsaas 1995; Loreto & Velikova 2001; Loreto *et al.* 2001; Sharkey, Chen & Yeh 2001; Affek & Yakir 2002; Peñuelas *et al.* 2005). However, inhibition of the MEP pathway (achieved by feeding fosmidomycin) affects products other than isoprene; for example, abscisic acid (ABA) biosynthesis is altered (Barta & Loreto 2006).

Concerns about the specificity of the inhibitor treatment stimulated transgenic approaches to examine the thermotolerance hypothesis. This hypothesis suggests that photosynthetic thermotolerance is caused by the ability of isoprene to stabilize membranes involved in photosynthetic

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processes, in particular, during short, intense heat spikes (e.g. as in sun flecking) (Singsaas *et al.* 1997; Singsaas & Sharkey 1998; Sharkey & Yeh 2001). In this scenario, high light and increased temperature are coincident. Results in transgenic systems were conflicting. Transgenic *Arabidopsis* (*Arabidopsis thaliana*) emitting isoprene displayed an enhanced growth phenotype in early stages of growth when grown under moderate heat stress (29 °C) (Loivamäki *et al.* 2007) and when subjected to 2.5 h of severe heat stress (60 or 70 °C, but not at 50 °C) (Sasaki *et al.* 2007). However, wild-type *Arabidopsis* plants did not display reduced photosynthesis under repetitive heat and light stress, so the hypothesis could not be tested in this system (Loivamäki *et al.* 2007). The thermotolerance hypothesis was also investigated in grey poplar using RNAi to inhibit isoprene synthase expression and eliminate isoprene emission (Behnke *et al.* 2007). In this case, photosynthesis in transgenic plants was more sensitive to heat spikes than that of non-transgenic plants. However, because of the long generation time of poplar trees, these experiments were performed in primary transgenic plants, using non-transgenic plants as controls. Altered stress responses often occur in primary transgenics because of transformation and tissue culture processes. Such effects are particularly problematic for stress-related experiments, and can be carried through several transgenic generations (Molinier *et al.* 2006).

To date, isoprene-emitting transgenic plants have not been tested for resistance to oxidative damage. Moreover, *Arabidopsis* plants transformed with isoprene synthase have not produced isoprene at levels comparable to a naturally high-emitting species (Loivamäki *et al.* 2007; Sasaki *et al.* 2007), and concerns have been raised as to the legitimacy of using *Arabidopsis* as a model for studying isoprene emission (Loivamäki *et al.* 2007). To address these issues, we transformed tobacco (*Nicotiana tabacum* L.) with an isoprene synthase gene extracted from poplar (*Populus alba*) and obtained plants emitting isoprene at levels comparable to a naturally emitting species. These plants were challenged with abiotic stresses, including heat and combined heat/light to investigate thermotolerance, and ozone fumigation to investigate oxidative stress tolerance.

MATERIALS AND METHODS

Plant material

Populus alba trees were obtained from Weasdale Nurseries (Cumbria, UK), grown in 10 L pots in John Innes No. 2 potting mix and fertilized with Miracle-Gro (Miracle-Gro, Marysville, OH, USA). Tobacco plants (*N. tabacum* cv. Samson NN and transgenic lines derived from this genotype) were potted in 1.5 L pots with Levington M3 potting mix (Scotts Company UK Ltd, Suffolk, UK) and maintained in a controlled environment at 25/20 ± 2 °C and 40/50 ± 10% relative humidity (RH) day/night at 14 h photoperiod of 500 µmol m⁻² s⁻¹.

Plasmid construction

Genomic DNA was extracted from *P. alba* leaves using a DNeasy Plant kit (Qiagen, West Sussex, UK), according to the manufacturer's directions. PCR was performed using the Expand Long High Fidelity polymerase (Roche Diagnostics, East Sussex, UK) and primers based on the *P. × canescens* IS cDNA (Miller, Oschinski & Zimmer 2001) (IS1F and IS5R; Supporting Information Table S1). PCR product (GenBank EF638224) was cloned into the Klenow-blunted *Not* I and *Xba* I sites of p35SsGFP (Vickers *et al.* 2007) to make p35S:ISA15. p35SsGFP contains an expression cassette consisting of the cauliflower mosaic virus 35S promoter fused to a green fluorescent protein (GFP) coding sequence containing the S65T mutation, followed by the nopaline synthase (nos) terminator region. In p35S:ISA15, the GFP coding sequence is replaced by IS gene. To generate a binary plasmid, p35S:ISA15 was digested with *Pvu* II and *Sda* I followed by a partial digestion with *Eco* RI. The 35S:IS:NOS fragment was cloned into the *Pst* I and *Eco* RI sites of pGFPGUS*Plus* (Vickers *et al.* 2007) to generate pB:35S:ISA15. pGFPGUS*Plus* (GenBank EF546437) is a binary vector which bears expression cassettes for both GFP and GUS*Plus*. In the pB:35S:ISA15 construct, the 35S:GFP expression cassette is essentially replaced by the 35S:ISA15 cassette; the 35S:GUS*Plus* cassette is retained for use as a transformation and segregation marker. The binary thus consisted of adjacent 35S:GUS*Plus* and 35S:ISA15 cassettes, cloned in opposing orientations with the CaMV35S promoters adjoining (Supporting Information Fig. S1).

In order to isolate isoprene synthase cDNA, RNA was extracted from *P. alba* using an RNeasy Plant kit (Qiagen). cDNA was generated by using (dT)17-adaptor primer (Frohman, Dush & Martin 1988) and RevertAid reverse transcriptase (Fermentas, York, UK) according to the manufacturer's directions. Full-length and truncated isoprene synthase cDNAs were amplified using IS5R coupled with IS1F and IS2F, respectively (Supporting Information Table S1). PCR products were cloned into pCR2.1-TOPO (Invitrogen, Paisley, UK) according to the manufacturer's instructions, to create pISc (full length) and pISΔc (truncated). Transcription of the resulting genes in the correct orientation is controlled by the *lac* operon promoter. All PCR products were sequenced after cloning.

Tobacco transformation, line selection and generations

Tobacco transformation was performed essentially as described previously (Guerineau *et al.* 1990), with the following changes: MES was omitted from all media; leaf sections were cut while suspended in the *Agrobacterium* culture; cocultivation was carried out on solidified medium (0.8% agar); following cocultivation, leaf sections were washed in liquid selection medium; augmentin (200 µg mL⁻¹) was used to remove agrobacteria, and hygromycin (20 µg mL⁻¹) was used as a selective agent in the selective medium; and rooting was performed on a medium

identical to selection medium, but omitting plant hormones. Primary transgenic plants (T0) were screened for GUS*Plus* activity, and GUS-positive plants were screened for isoprene emission by mass spectrometry using a membrane inlet mass spectrometer (MIMS). Emitting lines were selected for segregation analysis. β -Glucuronidase activity was used as a marker for transgene locus segregation in first (T1) and second (T2) transgenic generations as described previously (Vickers *et al.* 2007) using the histochemical assay (Jefferson 1987). Azygous plants were selected in the T1 generation, and homozygous T1 plants were identified by segregation analysis in the T2 generation. Transgenesis and segregation analysis were confirmed, and copy number was estimated, in the T1 generation by Southern blot. For each of five isoprene-emitting single-locus lines, one homozygous and one null segregant plant were selected for analysis and production of further generations. Seed was collected and stored (4 °C) for every generation; lines were carried through to T3 (fourth transgenic) generation and beyond.

Southern analysis

DNA was extracted from tobacco plants using the CTAB method (Doyle & Doyle 1990). Extracted DNA was digested with *Xba* I, and 60 μ g was used for Southern analysis using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics) according to the manufacturer's directions. A DIG-labelled probe targeted towards the 3' region of the IS gene was generated using the IS3F and IS5R primers (Supporting Information Table S1).

Transcript analysis

RNA was extracted from tobacco plants using an RNEasy Plant kit (Qiagen). cDNA was generated by using (dT)17-adaptor primer (Frohman *et al.* 1988) and RevertAid reverse transcriptase (Fermentas) according to the manufacturer's directions. Isoprene synthase cDNA was amplified by PCR using primers IS5F and IS6R (Supporting Information Table S1), cloned into pCR2.1-TOPO (Invitrogen) according to the manufacturer's directions and sequenced to confirm identification.

Western analysis

Leaf tissues were powdered under liquid nitrogen. Total protein was extracted by adding 10 volumes of 1 × Laemmli buffer (Laemmli 1970) without bromophenol blue, mixing thoroughly and incubating 5 min on ice. Insoluble material was removed by centrifugation at 20 000 *g* for 10 min. Supernatant samples (20 μ L) were electrophoresed through 10% polyacrylamide gels. Proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Watford, England) and probed according to the manufacturer's instructions, using 60 min incubations with 1/5000 dilutions of both primary and secondary antibodies. Blocking solution was 5% low-fat milk powder in phosphate-buffered saline (PBS). Primary antibody was generated in

rabbit commercially (Sigma-Aldrich, Dorset, UK) against a synthetic peptide (SSDTDESIEVYKDK) located near the N-terminus of the IS protein. The secondary antibody was an anti-rabbit IgG conjugated to horseradish peroxidase (Perbio Science, Northumberland, UK). Between antibodies, membranes were washed 4 × 10 min (primary) and 3 × 10 min (secondary) with PBS-T. Immunoblots were visualized using chemiluminescent ECL substrate (Perbio Science) according to the manufacturer's instructions.

Isoprene detection in T0 and T1 plants

Leaf discs (1.5 cm diameter) were placed in 2 mL clear glass vials with 100 μ L water. Vials were sealed and incubated for 60 min at 30 °C and 1000 μ mol m⁻² s⁻¹ light, and then 1 mL headspace samples were injected into a quadrupole mass spectrometer with Faraday collection (QMG 422, Pfeiffer Vacuum, Asslar, Germany). Isoprene was identified at masses 67 and 68.11 (protonated).

Gas exchange and isoprene emission measurements in T2 and subsequent generations

Leaf gas exchange measurements from tobacco and *P. alba* were performed using an open gas exchange system (LI-6400; Li-Cor Inc, Lincoln, NE, USA) with an integrated fluorescence chamber head (LI-6400-40 leaf chamber fluorometer). Gas exchange measurements in the ozone experiments were made using fully expanded leaves (third and fourth nodes from the apical meristem) at a CO₂ mixing ratio of 370 μ mol mol⁻¹ and growth temperature and light conditions [25 °C and 500 μ mol m⁻² s⁻¹ photosynthetically active radiation (PAR)]. Measurements for the thermotolerance experiments were made using fully expanded leaves (fourth node from the apical meristem) at a CO₂ concentration of 380 μ mol mol⁻¹, a leaf temperature of 30 °C and light intensities of either 400 or 1000 μ mol photons m⁻² s⁻¹ PAR. Prior to measurements, leaves were clamped and allowed to adapt for 5–10 min to attain steady-state CO₂ and H₂O fluxes.

Isoprene concentration was determined in the air exiting the Li-Cor cuvette using a proton transfer reaction-mass spectrometer (PTR-MS; Ionicon GmbH, Innsbruck, Austria). The operational characteristics of this instrument have been well described (Hansel *et al.* 1995; Lindinger, Hansel & Jordan 1998; Hayward, Tani & Hewitt 2002), and it has been used extensively for the determination of isoprene emissions from plants (e.g. Hayward *et al.* 2002; Hewitt, Hayward & Tani 2003; Tani, Hayward & Hewitt 2003; Hayward *et al.* 2004). The cuvette exhaust was connected by a PTFE tubing (0.25 inch OD) to the PTR-MS via a T-junction. The air leaving the cuvette head-space was sampled for isoprene at a flow rate of 100 mL min⁻¹. The PTR-MS instrument featured two turbomolecular pumps, a heated silica steel inlet system and a 9.6-cm-long stainless steel drift tube. According to the manufacturer, the

response time was about 1 s for these instruments. The operating parameters of the PTR-MS were held constant during measurements, except for the secondary electron multiplier voltage, which was optimized before every calibration. The drift tube pressure, temperature and voltage were 2.2 hPa, 50 °C and 600 V, respectively. The parameter E/N was approximately 125 Td, and the reaction time was about 97 μ s. The count rate of $\text{H}_3\text{O}^+\text{H}_2\text{O}$ ions was 1–2% of the count rate of H_3O^+ ions, which was $(2.9\text{--}3.5) \times 10^6$ counts s^{-1} . Normalized sensitivities and isoprene volume mixing ratios (VMRs) were calculated as described previously (Taipale *et al.* 2008) using 700 ppb isoprene in nitrogen (Linde, Tunstall, Staffordshire, UK) diluted in zero air from a Zero Air Generator (model 75-83; Parker Hannifin Ltd, Hemel Hempstead, UK). Protonated isoprene was detected by the PTR-MS as its molecular mass plus one (i.e. $M + \text{H}^+ = 69$) using a dwell time of 5 s (ozone experiment) or 15 s (thermotolerance experiment).

Emission of isoprene from the tobacco leaves was confirmed using thermal desorption GC-MS. At least 1 L of air exiting the cuvette was trapped onto sampling tubes containing the adsorbent resins Tenax TA and Carbotrap (Supelco Inc, Bellefonte, PA, USA). Samples were desorbed using automated thermal desorption (Turbomatrix ATD; Perkin Elmer, Norwalk, CT, USA) by heating the tubes at 280 °C and focusing the desorbed isoprene on a Tenax TA cold trap at –30 °C for 6 min. The cold trap was flash heated to 300 °C, and the sample was injected onto an Ultra-2 capillary column (50 m $\times$ 0.22 mm i.d. $\times$ 1.05 μ m film thickness, 5% phenylmethylsilica, Hewlett Packard; Varian Inc, Palo Alto, CA, USA) for compound separation. The GC oven was initially held at 35 °C for 2 min, heated to 160 °C at 4 °C min^{-1} , then heated at 45 min^{-1} to 300 °C, which was held for 10 min. Identification and quantification of the individual compounds were by comparison of retention time and fragmentation pattern of the compounds with those of an external commercially available standard (700 ppb isoprene; Linde, Tunstall, Staffordshire, UK) and using the Wiley (John Wiley & Sons, Hoboken, NJ, USA) and NIST Mass Spectral Libraries in conjunction with the TurboMass software (Turbomass version 4.4.0.014; Perkin Elmer Instruments).

Leaf gas exchange parameters (net carbon assimilation, transpiration and isoprene emission) were calculated considering the flow rates through the cuvette and the leaf area.

Light/heat stress protocol

Gas exchange measurements were taken at standard conditions (30 °C and either 400 or 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) before any heating. Leaves were heated from the standard conditions to 45 °C at a rate of ~ 3 °C min^{-1} , maintained at 45 °C for 2 min, then returned to 30 °C. Heating of the leaves was controlled jointly by thermoelectric modules that are part of the LI-COR 6400 system and a heat gun blowing hot air onto the leaf cuvette. Gas exchange was measured 20 and 60 min after heating. The heat stress was applied three times. Biological replications consisted of

leaves from individual plants. Data points are reported as means of biological replications.

Ozone fumigation

Five-week-old plants from the T3 generation (a different set from those used for thermotolerance experiments) were transferred to duplicate controlled environmental chambers and acclimated for 24 h before ozone treatment. The chamber design for growth and ozone fumigation has been described previously (Stokes, Terry & Hewitt 1998). Environmental conditions in the chambers were $25/20 \pm 2$ °C day/night, 35–40% RH, with a 14 h photoperiod (500 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Ozone was generated by flowing oxygen through a UV O_3 generator (OPSIS, Teledyne Instruments, City of Industry, CA, USA); this ozone was mixed with the ambient air supplied to the treatment chambers. The ozone generator was adjusted until the desired ozone concentration in the treatment chambers (120 or 200 ppb) was achieved. The ozone concentration in the chambers was measured using a 400E photometric O_3 analyser (Teledyne Instruments) connected to the chamber outlet. Previous experiments using these chambers indicate that ozone loss by deposition on the chamber walls was 15–20%, and ozone loss by deposition on the tobacco leaf surfaces was $\sim 10\%$ (data not shown). The ozone concentration in the control chambers did not exceed 20 ppb. Plants of each transgenic line were exposed to either 120 or 200 ppb for 6 h d^{-1} for two consecutive days. For each transgenic line, 1 leaf per plant from three plants for both heterozygous and azygous lines was analysed (biological replications).

Biochemical analyses

Leaf samples were taken before ozone fumigation, immediately after ozone fumigation and at 1 and 5 d post-fumigation. Samples from the fourth node (from the apical meristem) were frozen immediately on liquid nitrogen and ground to a fine powder. For each biological replicate (described in 'Ozone fumigation'), three technical replications were performed. Technical replications were averaged to give the value for the biological replication. Results are reported as the mean of the biological replications.

H_2O_2 was determined using Amplex Red (Invitrogen), essentially according to the manufacturer's instructions. Leaf tissue (0.1 g) was ground in ice-cold 0.1 M HCl. After centrifugation (13 000 rpm, 10 min, 4 °C), the supernatant was purified by spinning through a small charcoal column. Reaction buffer contained 50 mM HEPES, 20 U mL^{-1} horseradish peroxidase and 10 mM Amplex Red. In control tubes, catalase (Sigma-Aldrich) was added to the reaction buffer to remove H_2O_2 .

Lipid peroxidation was assessed using the corrected thiobarbituric acid (TBA)-reactive substances (TBARS) test (Hodges *et al.* 1999) with minor changes. Fresh leaf tissue (100 mg) was homogenized in 1 mL of 5% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged for 15 min at 12 000 g, and the supernatant was

mixed with an equal volume of TBA solution (0.5% w/v TBA in 20% TCA). The solution was boiled for 30 min at 100 °C then placed on ice. Samples were centrifuged at 10 000 g for 5 min, then assayed.

Total ascorbate (Asc.) and reduced Asc. were determined by using a variation of a previous method (Law, Charles & Halliwell 1983). Frozen leaf material (0.1 g) was homogenized in an ice bath with 1.4 mL 6% (w/v) TCA. The homogenate was centrifuged for 5 min at 15 000 g and 4 °C. The supernatant was placed on ice and assayed immediately. For total Asc. analysis, 0.4 mL of 0.2 mM potassium phosphate buffer and 0.2 mL of 10 mM dithiothreitol were added to 0.2 mL supernatant, the solution was mixed and incubated at 42 °C for 15 min and then 0.2 mL of 0.5% (w/v) *N*-ethylmaleimide was added. For Asc. analysis, 0.6 mL of 0.2 mM K-phosphate buffer (pH 7.4) and 0.2 mL double distilled water were added. To both samples, 1.0 mL of 10% (w/v) TCA, 0.8 mL of 42% (v/v) H₃PO₄, 0.8 mL of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 0.4 mL of 3% (w/v) FeCl₃ were added. After vortex mixing, the samples were incubated at 42 °C for 40 min, and the absorbance was read at 525 nm. Dehydroascorbate content was determined by subtracting Asc. values from total Asc. values.

Chlorophylls *a* and *b*, and total carotenoids were extracted in acetone. About 40 mg of frozen leaf tissue was homogenized in 10 mL of 80% acetone and centrifuged for 13 min at 4600 rpm. The supernatant was removed and the pellet was again macerated with another 10 mL of 100% acetone and centrifuged for 13 min as before. The combined supernatants were used for chlorophyll and carotenoid determination. The pigment content was calculated by absorbance at 646, 663 and 470 nm using an Ultrospec 2100 Pro (Amersham Bio-Sciences, GE Healthcare, Buckinghamshire, UK) as described previously (Lichtenthaler & Wellburn 1983).

Statistical analyses

Statistical analysis was performed with SPSS for Windows (release 14.0; SPSS, Chicago, IL, USA). For thermotolerance experiments, the means ($\pm 1\sigma$) of three or more independent experiments per treatment were analysed. Mean values of gas exchange, at preheat and after each stress event, were analysed for significant differences between genotypes (homozygous and azygous) using Student's *t*-test at the $P \leq 0.05$ level. For analysis of data normalized to preheat values, data were transformed using an arcsine function to meet statistical assumption requirements. Statistically significant differences between genotypes over the duration of the repeated heat stress treatments were calculated using a hierarchical linear model with each independent experiment treated as a random effect. This model allows for non-constant variability and correlation within the data; the assumptions of a *t*-test or analysis of variance (ANOVA) are violated when comparing time-points after heating as both models require homogeneity of variance, which is not observed in these data.

For physiological analyses of ozone-treated plants, three independent experiments were performed, each with 4

plants per treatment (non-emitting/isoprene emitting, +ozone/–ozone). One sample was taken from each plant. The data points presented are means from the pooled replications ($n = 12$) with standard deviations. Means of emitting and non-emitting plants were separated using Tukey's test. For biochemical analyses of ozone-treated plants, three independent experiments were performed, each with 1 plant per treatment (non-emitting/isoprene emitting, +ozone/–ozone), except for Asc. samples, for which there were 2 plants per treatment. One sample was taken from each plant. The data points presented are means ($n = 6$ for Asc. data; $n = 3$ for all other data sets) with standard deviations. Significant differences and mean separation were performed as described earlier.

RESULTS

Generation of transgenic tobacco plants

Nicotiana tabacum cv. Samsun NN was transformed with a poplar (*P. alba*) isoprene synthase gene (GenBank EF638224) placed under the control of the constitutive CaMV35S promoter. The isoprene synthase expression cassette was linked to a CaMV35S:: β -glucuronidase (*GUS*) reporter gene in a binary Ti plasmid vector T-DNA to facilitate identification of putative transgenic plants and segregation analysis (Vickers *et al.* 2007). Putative transgenic lines (*GUS*-positive) were screened for isoprene emission, and transgenesis was confirmed in primary transgenic (T0) plants using Southern analysis (Supporting Information Table S1). Expression of the isoprene synthase RNA and protein was confirmed in subsequent generations by sequencing isoprene synthase cDNAs isolated from transgenic lines and by immunoblot analyses (e.g. Fig. 1a), respectively. Homozygous and azygous plants of each line were cultivated through to the fourth (T3) generation before stress experiments were performed. Physiological measurements showed that there were no major morphological differences between homozygous and azygous plants of the same line, except for line 22, where the homozygous plants were smaller and had fewer smaller leaves than the azygous plants (Supporting Information Fig. S2). We assume this to be caused by a random genetic effect in the homozygous plants of this line, because it was not observed in any of the other lines. No difference was observed in carotenoid or chlorophyll levels between homozygous and azygous plants (Supporting Information Fig. S3).

Emission patterns in transgenic tobacco plants mimic those of normal isoprene-emitting plants

For phenotypic analysis of lines bearing the full-length isoprene synthase gene, five independent, isoprene emission-positive, single-locus, homozygous transgenic lines were generated, along with azygous (transgene null) sibling lines as controls (see Materials and methods for details). Transgenic lines harbouring the isoprene synthase gene displayed a range of emission levels (0.5–18 nmol

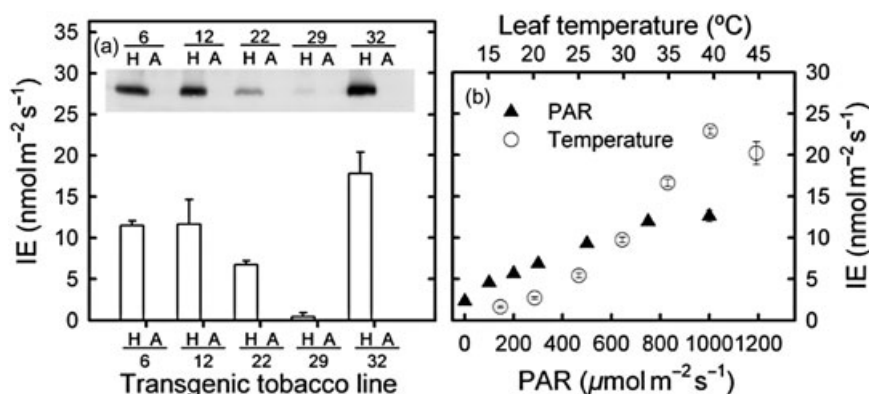


Figure 1. Isoprene emission rates (IE) of homozygous T3 (fourth generation) transgenic plants. Emission rates from different transgenic lines are shown in (a). Numbers on the *x*-axis are transgenic line designations. Antigenic identification of isoprene synthase protein by Western blot analysis of crude protein extracts is shown for homozygous (H) and azygous (A) plants each line [insert, (a)]. Light and temperature responses for line 32 are shown in (b); other lines are shown in Supporting Information Fig. S4. All measurements were taken from the fourth node (from the apical meristem) of 5-week-old tobacco plants or from 8-week-old poplar leaves, and at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) and 30°C except where noted in (b). $n \geq 3$ for each data point; error bars are standard deviations.

isoprene $\text{m}^{-2} \text{s}^{-1}$; e.g. Fig. 1a). These emission levels are comparable with emissions from *P. alba* leaves under the same conditions of temperature and light ($2\text{--}14 \text{ nmol isoprene m}^{-2} \text{s}^{-1}$). No isoprene emission, and no isoprene synthase protein, were detected in azygous control lines (Fig. 1a). A quantitative relationship was observed between isoprene synthase protein and emission levels on homozygous plants (Fig. 1a). The correlation between emission levels and isoprene synthase protein levels is consistent both with previous observations in isoprene-emitting species (Sharkey, Wiberley & Donohue 2008) and with transgenic plants exhibiting a range of emission levels (Behnke *et al.* 2007; Loivamäki *et al.* 2007).

Light and temperature responses were typical of normally emitting plants; responses for line 32 are shown in Fig. 1b, and are representative of patterns observed in other lines (see Supporting Information Fig. S4): isoprene emission rate increased as photosynthetically active photon flux density (PPFD) increased, and emission rates peaked around 40°C .

Responses of photosynthesis to temperature and light stress in isoprene-emitting tobacco

Transgenic plants and azygous controls from four transgenic lines (6, 12, 22 and 32) were placed under a repetitive heat/light stress regime, where three stress events ($45^\circ\text{C}/1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) were applied. This experiment was repeated with heat stress only (ambient light maintained at $400 \mu\text{mol m}^{-2} \text{s}^{-1}$). The recovery of net photosynthetic rate (P_n) was measured following each stress event (Fig. 2). Post-stress changes in P_n were normalized to pre-stress readings to compare relative changes in P_n (inserts, Fig. 2). Two statistical analyses were applied to each data set. The first analysis compared P_n and stomatal conductance (g_s) after each stress event. This type of analysis has been used previously to compare chemically inhibited leaves with

non-inhibited leaves (e.g. Sharkey *et al.* 2001; Velikova, Pinelli & Loreto 2005; Velikova *et al.* 2006). No significant differences were observed between isoprene-emitting and non-emitting plants after the stress events were applied (Fig. 2). We also used a second statistical test (a hierarchical mixed-model analysis) to identify differences in the overall performance of the plants over the duration of the entire experiment (across all stress events) rather than at individual time points. In this case, statistically significant differences were observed between emitting and non-emitting plants in lines 6 and 32 under both thermal stress and combined thermal/light stress (Table 1), with emitting plants showing greater P_n than non-emitting plants. The observed differences were maintained when data were standardized for pre-stress variations, except in line 32 under thermal stress, where the significance was lost (Table 1). In all cases for the relative data, and in many cases for the absolute data, an increased P_n in emitting plants was coincident with a significant increase in stomatal conductance.

Isoprene-emitting plants are resistant to ozone-induced damage

Azygous plants displayed typical ozone damage by 5 d after fumigation with 200 ppb ozone for 6 h, whereas isoprene-emitting plants were substantially protected from damage (Fig. 3). To investigate the physiological basis of this response, plants from two transgenic lines (6 and 32) were fumigated with ozone at 120 ppb for 6 h d^{-1} over 2 d. Isoprene emissions in fumigated and non-fumigated plants were similar, except for a transient decrease in fumigated plants relative to non-fumigated plants after the second fumigation event (Fig. 4a,b). This indicates that at that time there was either a change in the synthesis of isoprene (caused by a decrease in either substrate availability or enzyme production/activity) or that isoprene was being

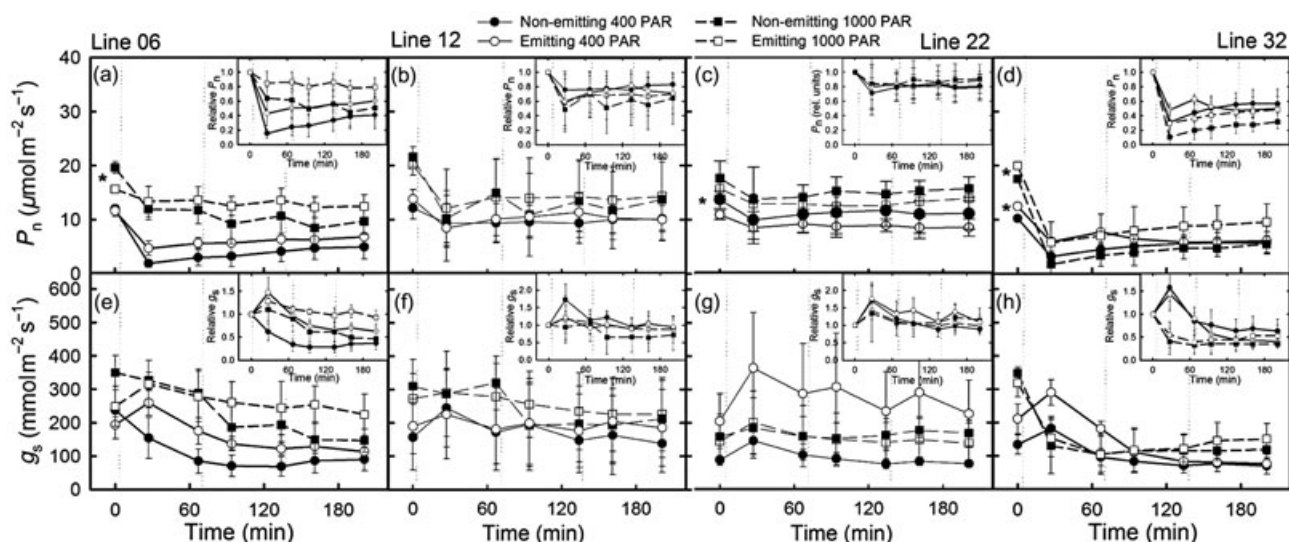


Figure 2. Thermotolerance experiments on transgenic tobacco plants. Gas exchange measurements were taken at standard conditions [30 °C and either 400 or 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR)] prior to heating. Leaves were then subjected to three heat stress events, and gas exchange was measured 20 and 60 min after each stress event. Net photosynthesis (P_n ; a–d) and stomatal conductance (g_s ; e–h) are shown for lines 6, 12, 22 and 32. Vertical broken lines represent stress events. Insets show data normalized to pre-stress measurements. Significant differences (at $P < 0.05$ level) between azygous and homozygous plants under the same treatments are represented by asterisks. $n \geq 3$; error bars are standard deviations.

Table 1. Statistical data for relative leaf gas exchange using hierarchical linear model analysis to measure differences in data sets for thermotolerance experiments at two light levels [400 or 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR)]

	400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR								1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR							
	Line 6		Line 12		Line 22		Line 32		Line 6		Line 12		Line 22		Line 32	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
P_n	4.79	0.04	0.04	0.84	1.91	0.21	12.3	≤ 0.01	4.93	0.03	0.10	0.77	2.21	0.18	24.1	≤ 0.01
g_s	0.60	0.44	0.07	0.81	9.19	0.02	19.1	≤ 0.01	0.12	0.73	0.05	0.84	0.08	0.78	2.84	0.10
Relative P_n	7.25	0.01	0.23	0.65	≤ 0.01	0.94	1.07	0.33	159	≤ 0.01	0.21	0.66	0.06	0.79	44.7	≤ 0.01
Relative g_s	11.3	≤ 0.01	0.77	0.40	1.805	0.22	1.13	0.31	153	≤ 0.01	0.89	0.37	0.08	0.78	7.30	0.01

Statistically significant differences are identified between homozygous emitting and azygous non-emitting plants of the same line. The assumptions of normality and homogeneity of variance were met through arcsine transformations. The P value indicates significant differences over the course of the whole experiment; P values in bold indicate statistical significance at the 0.05 level or higher.

consumed *in planta*. Ozone treatment resulted in a decrease in P_n in both emitting and non-emitting plants (Fig. 4c,d). The magnitude of this decrease was significantly greater in non-emitting plants relative to emitting plants after the second fumigation. The difference in response between non-emitting and emitting plants was not caused by differences in CO_2 availability or ozone uptake, because stomatal conductance was unchanged between the emitting and non-emitting plants (Fig. 4e,f).

Isoprene-emitting plants accumulate less H_2O_2 and exhibit less membrane damage through lipid peroxidation after ozone fumigation

In isoprene-emitting plants of line 6, H_2O_2 levels increased after ozone fumigation, but over subsequent days levels

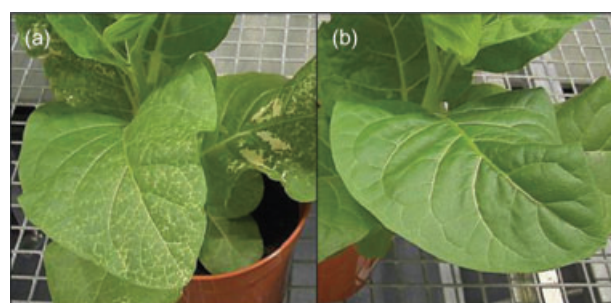


Figure 3. Phenotype of ozone-fumigated plants. Plants (line 6) were fumigated with ozone (200 ppb) for 6 h d^{-1} over 2 d, and then allowed to recover for 5 d. Azygous, non-emitting plants showed a classical ozone-induced cell death response, evidenced by necrotic lesions (a), whereas homozygous, isoprene-emitting plants were protected from ozone-induced damage (b).

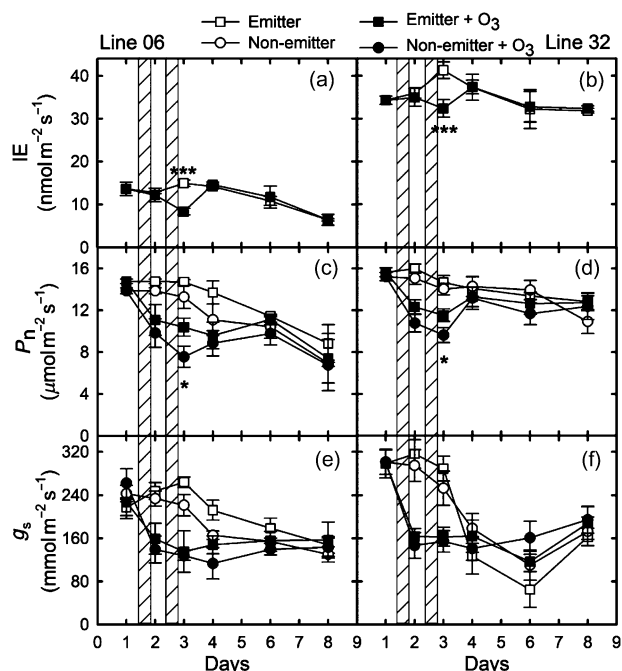


Figure 4. Physiological data collected from tobacco plants (lines 6 and 32) fumigated with 120 ppb ozone, 6 h d⁻¹ for 2 d. Measurements were taken before fumigation; between fumigation events; immediately after fumigation; and at 1, 3 and 5 d post-fumigation. Isoprene emission rates (IE) are shown in (a,b); net photosynthetic rates (P_n) are shown in (c,d) and stomatal conductances (g_s) are shown in (e,f). Fumigation events are represented by cross-hatched bars. $n = 3$; error bars are standard deviations. Means of emitting and non-emitting plants were separated using Tukey's test. Asterisks (*, ** or ***) indicate differences significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

decreased to normal (Fig. 5a). In isoprene-emitting plants of line 32 (which emitted about 1.5 times as much isoprene as line 6 plants under the same conditions), H_2O_2 levels remained low (Fig. 5b). In contrast, non-emitting plants significantly and continually accumulated H_2O_2 throughout the experiment, resulting in an increase of ~400% compared to emitting plants after 5 d. Lipid peroxidation levels were also lower in isoprene-emitting plants following ozone fumigation (Fig. 5c,d). Notably, in line 32, lipid peroxidation levels were ~2.5 times higher in non-emitting plants than in emitting plants, and this was maintained 5 d after fumigation. Interestingly, in line 6, lipid peroxidation levels recovered to levels observed in non-fumigated plants by 5 d post-fumigation; the reason for the discrepancy between the two lines is unclear and requires further investigation.

Antioxidant capacity is higher in isoprene-emitting plants

In line 6, Asc. levels were also examined. In ozone-fumigated plants, total Asc. levels increased following fumigation (Fig. 5e). This increase occurred more rapidly in

isoprene-emitting plants than in non-emitting plants, although by 24 h post-fumigation, total Asc. levels were similar in both emitting and non-emitting plants. However, the proportion of reduced Asc. was consistently much lower in non-emitting plants after ozone fumigation (Fig. 5f).

DISCUSSION

Transgenic tobacco plants which emit isoprene at levels comparable to a naturally emitting species have been engineered and thoroughly characterized (Fig. 1; Supporting Information Figs S2–S4; Supporting Information Table S2). They display stable phenotypes over generations (Supporting Information Fig. S5), due in part to the stable zygosity and single locus characteristic of each transgenic line. Isoprene emission from these plants is only observed when

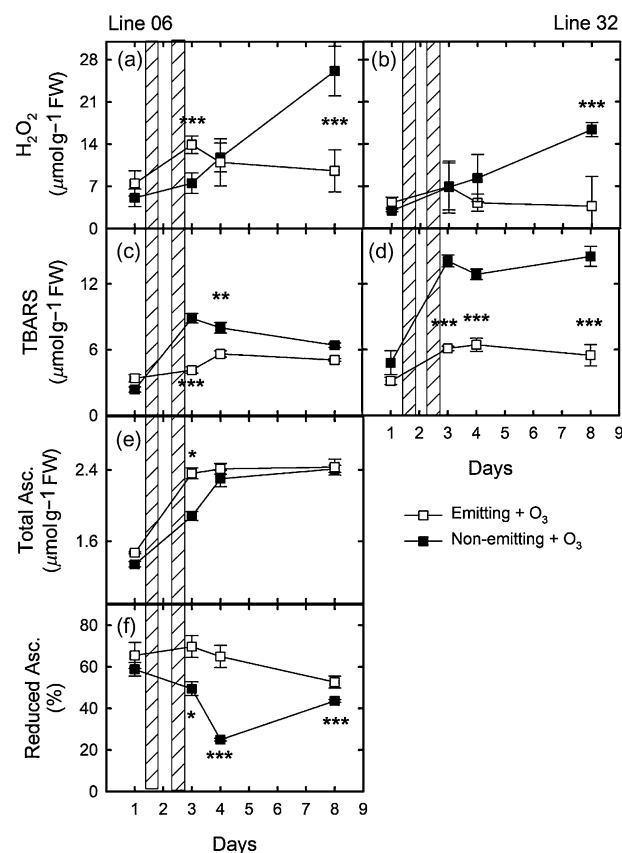


Figure 5. Biochemical data collected from tobacco plants (lines 6 and 32) fumigated with 120 ppb ozone, 6 h d⁻¹ for 2 d. Measurements were taken before fumigation, immediately after fumigation and at 1 and 5 d post-fumigation. H_2O_2 levels are shown in (a,b). Lipid peroxidation products were measured using the thiobarbituric acid-reactive substances (TBARS) test (c,d). In line 6, ascorbate levels were also analysed; total ascorbate (Asc.) is shown in (e) and reduced Asc. is shown in (f). Ozone fumigation events are represented by cross-hatched bars. $n = 3$; error bars are standard deviations. Means of emitting and non-emitting plants were separated using Tukey's test. Asterisks (*, ** or ***) indicate differences significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

light is present, and plants also display characteristic emission responses to light–dark transition (Laothawornkitkul *et al.* 2008). Light and temperature responses of isoprene emission (Fig. 1b; Supporting Information Fig. S4) displayed patterns typically observed in isoprene-emitting species (Fehsenfeld *et al.* 1992; Zeidler *et al.* 1997; Sharkey *et al.* 2001). The introduction of isoprene biosynthesis did not seem to interfere with plant growth under normal conditions, because no major morphological differences were observed between plants from transgenic lines and azygous controls (Supporting Information Fig. S2). This suggests that isoprene raiding of the DMADP pool in the chloroplast did not unduly perturb downstream functioning of the MEP pathway; this supposition is supported by the observation that there were no variations in carotenoid or chlorophyll levels between homozygous and azygous plants (Supporting Information Fig. S3). In short, these plants mimic normal isoprene-emitting species as far as possible in a transgenic system, and, under normal conditions, azygous plants and homozygous plants appear to be very similar apart from isoprene emission. These transgenic lines are therefore ideal tools for examining the biology and physiology of isoprene emission.

We investigated the thermotolerance hypothesis using fourth generation (T3) transgenic tobacco plants. Azygous non-emitting and homozygous isoprene-emitting plants were exposed to transient heat and light stress or to heat only stress, interspaced with recovery periods during which photosynthesis was assessed by gas exchange. No statistically significant differences in net photosynthesis (P_n) or stomatal conductance (g_s) were observed at individual time-points between isoprene-emitting and non-emitting plants after stress events (Fig. 2). However, when analysed over the duration of the experiment (rather than at individual post-stress time-points), plants from two high-emitting lines (6 and 32) showed better overall P_n compared to non-emitting plants (Table 1). This suggested that the transgenic plants emitting isoprene may show some thermotolerance under thermal and combined thermal/light stress conditions when high levels of isoprene are present. The effect is subtle and not as pronounced as has been previously observed in fosmidomycin-poisoned or isoprene-fumigated plants (e.g. Sharkey & Yeh 2001; Sharkey *et al.* 2001, 2008; Peñuelas *et al.* 2005; Velikova & Loreto 2005). The present study does not examine the biological significance of this effect, although it might be expected that very small changes in photosynthetic efficiency may have a significant effect on the growth of an individual when integrated over the lifetime of a plant.

We also examined the effect of oxidative stress on transgenic tobacco plants by subjecting them to ozone fumigation. Non-emitting plants displayed typical foliar damage when fumigated with ozone, whereas isoprene-emitting plants were substantially protected from damage, showing a remarkable reduction in visible foliar lesions (Fig. 3). Non-emitting plants showed greater damage to photosynthesis (Fig. 4c,d). There was no difference in stomatal conductance between emitting and non-emitting plants (Fig. 4e,f),

indicating that stomatal aperture was not a source of variation in ozone access to leaf tissue.

When ozone enters the leaf, it triggers an oxidative burst resulting in the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). Apart from inducing direct oxidative damage, increased ROS levels trigger programmed cell death, often leading to the formation of visible foliar lesions (Wohlgemuth *et al.* 2002; Pasqualini *et al.* 2003). Isoprene-emitting plants accumulated far less H_2O_2 than non-emitting plants (Fig. 5a,b), suggesting that isoprene-synthesizing plants are either more efficient at removing H_2O_2 , or produce less in the first instance. One explanation for the difference in phenotype observed between emitting and non-emitting plants (Fig. 3) is that, in transgenic isoprene-emitting plants, levels of H_2O_2 did not pass the threshold required to initiate cell death processes.

Isoprene is highly hydrophobic, and is likely to partition into the lipid phase in membranes (Siwko *et al.* 2007). It has been proposed that isoprene may physically stabilize membranes and/or behave as an antioxidant (Sharkey & Singsaas 1995; Loreto & Velikova 2001; Loreto *et al.* 2001; Sharkey *et al.* 2001; Affek & Yakir 2002), presumably preventing lipid peroxidation and minimizing oxidative damage to membranes. In support of this, isoprene-emitting plants had substantially lower lipid peroxidation levels following ozone fumigation (Fig. 5c,d). Isoprene is unlikely to be effective as an antioxidant in the aqueous phase, and is therefore unlikely to have directly scavenged H_2O_2 . However, aqueous and lipid-phase antioxidant capacities are linked (e.g. through Asc.-mediated regeneration of tocopherol moieties) (Foyer, Trebst & Noctor 2006), so an increase in lipid-phase antioxidant capacity may be expected to impact on aqueous-phase antioxidant capacity. Ascorbate is a major water-soluble antioxidant; it directly scavenges ozone (Baier *et al.* 2005) and is involved in scavenging H_2O_2 (Asada 1992). Although the total Asc. pools displayed relatively little variation in transgenic tobacco (Fig. 5e), the amount of reduced Asc. in isoprene-emitting plants was greater than in non-emitting plants after ozone fumigation (Fig. 5f). This shows that isoprene-emitting plants have a reduced requirement for antioxidant capacity in the aqueous phase, most likely as an indirect consequence of lipid-phase protection by isoprene.

Together, our data show that endogenous isoprene emission plays a protective role against three abiotic stresses (heat, combined heat/light and ozone-induced oxidative stress). We also show that, in the case of ozone-induced stress, this effect is exerted through mediation of the oxidative status of the plant. This allows plants to respond better to oxidative stress caused by ozone, minimizing potential damage and preventing the over-accumulation of ROS, which would otherwise result in cell death. As has been noted previously (Sharkey *et al.* 2008), it is unlikely that isoprene emission evolved as a mechanism to deal specifically with ozone toxicity, because high ground-level ozone concentrations are a recent phenomenon. Rather, it seems probable that isoprene assists plants in coping with myriad oxidative stresses to which they are exposed throughout the

course of their growth and development. It should be noted that isoprene emission is one of a collection of defences available to plants to deal with oxidative stress. In isoprene-emitting plants, emission is maintained under many stress conditions (e.g. drought, salinity, high light, high temperature) that promote oxidative stress, despite the large increase in relative carbon loss (Sharkey & Loreto 1993; Loreto & Delfine 2000). The ability to synthesize isoprene may confer a competitive advantage as ground-level ozone episodes become more frequent in the future (Fiscus, Booker & Burkey 2005; UNECE 2006), and ultimately may have implications for biodiversity (Lerdau 2007), biomass accumulation and carbon sequestration in specific plant communities.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic diagram of the T-DNA region of pB:35S:ISA15.

Figure S2. Physiological measurements of T3 generation transgenic plants.

Figure S3. Carotenoid and chlorophyll analysis.

Figure S4. Light and temperature responses of other transgenic lines.

Figure S5. Isoprene emissions from homozygous T2 and T3 generation transgenic lines.

Table S1. Primers used for plasmid and probe construction.

Table S2. Details of T0 transgenic lines generated and molecular analysis.

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