

Transgenic, non-isoprene emitting poplars don't like it hot

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This paper is dedicated to the memory of Wolfgang Zimmer † 14 August 2002.

Summary

The physiological role of isoprene emission in plants is a matter of much debate. One of the most widely propagated hypotheses suggests a function of isoprene in the protection of leaf physiological processes against thermal and oxidative stress. To test this hypothesis, we developed transgenic Grey poplar (*Populus x canescens*) plants in which gene expression of isoprene synthase (ISPS) was either silenced by RNA interference (RNAi) or upregulated by over-expression of the *ISPS* gene. Despite increased *ISPS* mRNA levels, we did not observe consistent increases in isoprene emission in the over-expressing lines, indicating post-transcriptional control of *ISPS* by co-suppression. In the RNAi lines, levels of isoprene emission were effectively suppressed to virtually zero. Transgenic plants were subjected to temperature stress with three transient heat phases of 38–40°C, each followed by phases of recovery at 30°C. Parallel measurements of gas exchange, chlorophyll fluorescence and isoprene emission provided new insights into the physiological link between isoprene and enhanced temperature tolerance. Transgenic non-isoprene-emitting poplars showed reduced rates of net assimilation and photosynthetic electron transport during heat stress, but not in the absence of stress. The decrease in the efficiency of photochemistry was inversely correlated with the increase in heat dissipation of absorbed light energy, measured as NPQ (non-photochemical quenching). Isoprene-repressed poplars also displayed an increased formation of the xanthophyll cycle pigment zeaxanthin in the absence of stress, which can cause increased NPQ or may indicate an increased requirement for antioxidants. In conclusion, using a molecular genetic approach, we show that down-regulation of isoprene emission affects thermotolerance of photosynthesis and induces increased energy dissipation by NPQ pathways.

Keywords: isoprene emission, poplar, thermotolerance, photosynthesis, non-photochemical quenching.

Introduction

Isoprene (2-methyl-1,3-butadiene) is one of the most widely studied biogenic volatile organic compounds. The global annual isoprene flux from vegetation is of similar magnitude as that of methane (Guenther *et al.*, 1995, 2006). Isoprene is emitted from many but not all plant species. In general, most isoprene emitters are tree species, with the highest emission rates found in the genera *Quercus* and *Populus* (Harley *et al.*, 1999; Kesselmeier and Staudt, 1999). Emission of isoprene from woody plant species was ori-

ginally discovered by Sanadze (1957) (for review see Sanadze, 2004) and later confirmed by Rasmussen (1970). Sanadze *et al.* showed that emission of isoprene responds to light in a similar manner as net assimilation. In addition, they demonstrated that ¹³CO₂ was rapidly incorporated into isoprene and that isolated chloroplasts produced isoprene (Sanadze, 1991). Together, these early studies indicated a close association of isoprene synthesis and photosynthetic processes. However, 50 years after the

discovery of isoprene emission and despite several decades of research addressing biological functions of isoprene emission from plants, details of the functional association of isoprene synthesis and photosynthetic processes are still a topic of some considerable scientific debate (Sharkey and Yeh, 2001).

The emission of isoprene can amount to up to 5–10% of the photosynthetically assimilated carbon (Sharkey and Yeh, 2001). However, despite its importance as a substantial source of carbon emission potentially affecting carbon sequestration rates, the biological function of isoprene for plants is still unclear. As high light intensity and elevated temperature stimulate isoprene formation, Sharkey and Singaas (1995) proposed that isoprene can protect photosynthesis against damage caused by transient high-temperature stress. Subsequent work by Sharkey and coworkers (Sharkey *et al.*, 2001; Singaas and Sharkey, 2000; Singaas *et al.*, 1997), as well as by other groups (e.g. Velikova and Loreto, 2005; Velikova *et al.*, 2005a), provided evidence for this hypothesis. Other work demonstrated that isoprene can also protect plants against oxidative stress caused by ozone, nitric oxide or singlet oxygen radicals (Affek and Yakir, 2002; Loreto and Velikova, 2001; Loreto *et al.*, 2001; Peñuelas *et al.*, 2005; Velikova *et al.*, 2005a). As a result, various hypotheses are currently under debate that attribute functions to isoprene that not only involve protection against transient high temperatures but also protection against oxidative stress, as well as a possible function as a metabolic safety valve to avoid the undue sequestration of phosphates (Rosenstiel *et al.*, 2004).

The mechanism(s) by which a protective function of isoprene is achieved is unclear, but the most likely explanation is that isoprene is embedded in the organelle membranes and increases the stability of membranes and photosynthetic processes by preventing membrane lipid denaturation following oxidative stress (for overview see Owen and Peñuelas, 2005; Sharkey, 2005). Such a mode of action is supported by results from experiments with leaves of stress-exposed plants that were fumigated with isoprene, as well as from experiments in which isoprene biosynthesis was blocked with fosmidomycin (FOS), a competitive inhibitor of deoxyxylulose-5-phosphate-dehydrogenase (DXR), the first committed step of the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (e.g. Singaas and Sharkey, 2000; Singaas *et al.*, 1997; Sharkey *et al.*, 2001; Velikova and Loreto, 2005). However, as inhibition of isoprenoid biosynthesis by FOS not only results in fast suppression of isoprene emission, but also causes inhibition of other parts of terpenoid biosynthesis such as abscisic acid biosynthesis as well as enhanced transpiration, it is clear that FOS treatment can substantially perturb overall leaf physiology (Barta and Loreto, 2006), and results from such inhibitor experiments may be difficult to interpret or ambiguous.

The recent cloning of a poplar isoprene synthase (*ISPS*) gene (Miller *et al.*, 2001) provided new avenues to test biological and eco-physiological functions of isoprene using molecular genetic approaches. In the present work, we genetically engineered poplar plants (*Populus × canescens*) to study functions of isoprene emission by knocking down *ISPS* transcript levels using an RNA interference (RNAi) approach or by over-expression of the *ISPS* gene. The resulting isoprene emitting and non-emitting plants grown in climate chambers and in the field were subjected to transient high-temperature stress events comparable to the treatments applied by Sharkey *et al.* (2001). Thermotolerance was assessed by measuring the recovery of photosynthesis following short stress treatments at 38–40°C. Here we report that transgenic poplars in which isoprene emission is knocked down are negatively affected in thermotolerance of photosynthesis as well as photosynthetic electron transport, providing convincing and unambiguous evidence for an eco-physiological role of isoprene in thermoprotection of photosynthesis.

Results

Metabolic, biochemical and molecular characterization of transgenic poplar lines with modified isoprene emission

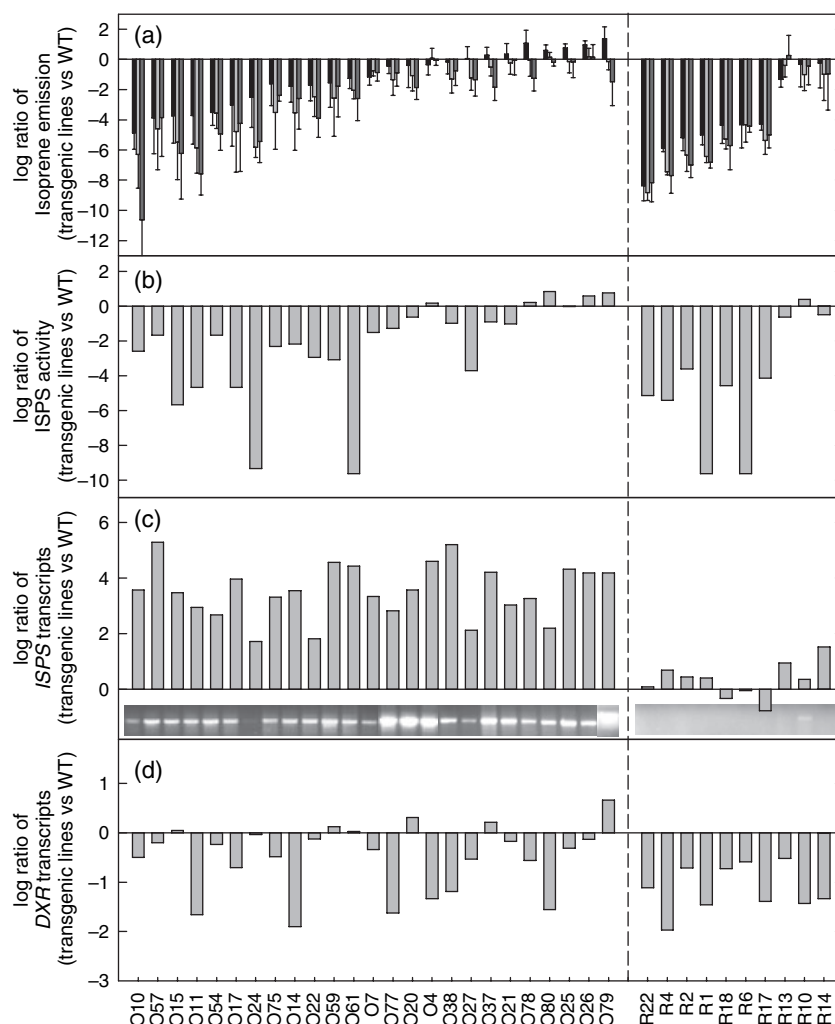
We developed several transgenic poplar lines (*Populus × canescens*) that were designed to over-express the *PcISPS* gene based on transformation with 35S::*PcISPS*-6xHis (O-lines) or to suppress expression of the *PcISPS* gene via RNAi (35S::*PcISPS*-RNAi, R-lines). Functional screening of transgenic lines was performed using proton transfer reaction mass spectrometry (PTR-MS), a very fast and high-throughput method that allows measurement of the amount of isoprene in the head space of gas-tight vials containing excised leaves from shoot cultures. Levels of isoprene emission were modestly increased [up to 150% compared to wild-type (WT)] in seven out of 19 O-lines tested. The observed increases in isoprene emissions were not consistent, as the effect was mostly confined to younger leaves (number 5), whereas older leaves showed repression of isoprene emission (leaves 6 and 7, see Figure 1a). In contrast, the remaining 12 O-lines showed a strong repression of isoprene emission compared to WT plants (Figure 1a). Transformation of poplar plants with the 35S::*PcISPS*-RNAi construct resulted in a strong inhibition of isoprene emission for seven of the ten R-lines. This effect was independent of leaf age.

To further characterize transgenic poplar plants at the level of isoprene biosynthesis, we measured *ISPS* enzyme activity (Figure 1b). The log ratios of *ISPS* enzyme activity correlated significantly ($P < 0.001$; $r^2 = 0.487$) with the isoprene emission rates. Reduced levels of isoprene emission correlated with highly decreased enzyme activity, and

Figure 1. Functional screening of over-expressing (O) and repressing (R) *PcISPS* poplar lines either containing a 35S::PcISPS-6xHis (O) or a 35S::PcISPS-RNA interference (R) construct.

(a) Log ratio ($\pm$ SE) of isoprene emission in transgenic shoot cultures relative to wild-type (WT) emission. Log ratio = 0, similar to WT; log ratio > 0, increased emission compared to WT; log ratio < 0, decreased emission compared to WT ($n = 5$). Single leaves were analyzed *in vivo*. Black bars, leaf number 5 (fully expanded young leaf); light-gray bars, leaf number 6 (adjacent to number 5, fully expanded); dark-gray bars, leaf number 7 (adjacent to number 6, fully expanded). (b) Log ratio (versus WT) of *PcISPS* activity *in vitro* in leaf number 6.

(c) Log ratio (versus WT) of *PcISPS* transcript levels in leaf number 5 determined by quantitative real-time PCR (197 bp fragment amplified); insert shows amplification of full-length sequence of *PcISPS* using gene flanking primers for real-time PCR (1969 bp fragment amplified). (d) Log ratio (versus WT) of *PcDXR* transcript levels in leaf number 5 determined by quantitative real-time PCR.



O-lines with increased emission rates showed an increase in ISPS activity.

At the molecular level, we characterized gene expression of *PcISPS* by quantitative real-time PCR. All O-lines displayed much higher transcript levels of *PcISPS* when compared to WT (Figure 1c). However, this increase in transcript level was not reflected at the biochemical and metabolite levels (Figure 1a,b). Similarly, in six of the R-lines we detected elevated *ISPS* transcript levels, while the biochemical analysis demonstrated a total knock-down of *PcISPS* activity and isoprene emission. As RNAi gene silencing results in degradation of the target mRNA, it is possible that small mRNA fragments may have been detected in our RT-PCR analysis which was designed to amplify a 197 bp fragment of the *PcISPS* gene. To analyze the amount of full-length *PcISPS* mRNA, primers were used for real-time PCR amplifying a 1969 bp long sequence (Figure 1c, insert). This analysis showed that levels of full-length *PcISPS* mRNA were highly increased in all O-lines, whereas no full-length *PcISPS* mRNA was detectable in the R-lines.

In addition to analysis of *PcISPS* transcript levels, we also measured gene expression of *PcDXR*, which encodes the first committed step of isoprenoid biosynthesis via the plastidial MEP pathway. Compared with WT plants, the O-lines did not show a consistent pattern of *PcDXR* gene expression (Figure 1d). While *PcDXR* transcript levels were reduced for the majority of O-lines, other O-lines showed the opposite. In contrast, transcript levels of *PcDXR* were reduced in all R-lines.

Assessment of photosynthesis and chlorophyll fluorescence in WT and transgenic poplars in the absence of temperature stress

To assess effects of altered expression of *PcISPS* at the physiological level, two sets of heat stress experiments were carried out with WT and transgenic poplars: (1) WT poplars (WT_C) and three transgenic lines grown in climate chambers (RNAi lines R1_C, R22_C and O-line O15_C), and (2) two transgenic lines grown under field conditions (C_F, empty vector control,

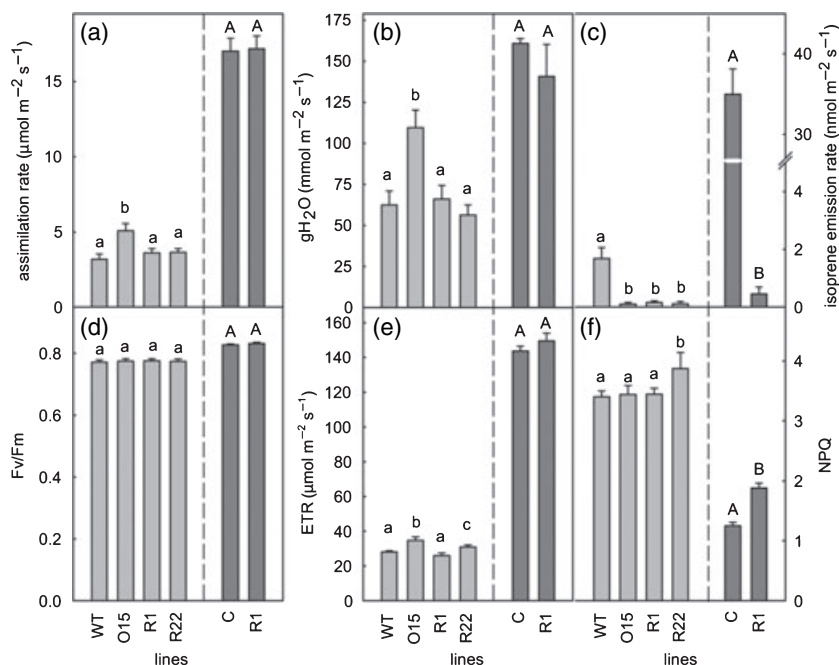


Figure 2. Photosynthetic gas exchange and chlorophyll fluorescence before temperature stress treatment in wild type (WT) and transgenic poplar leaves.

Measurements ($\pm$ SE) reflect initial status before temperature-stress treatment was performed. Plants cultivated under climate chamber conditions and field-grown plants are represented as light-gray bars and dark-gray bars, respectively. (a) Assimilation rates of climate chamber-grown lines [WT, O15 (over-expressed), R1 and R22 (repressed)] and field-grown lines [control (C) and R1 (repressed)].

(b) Water vapor conductance (gH₂O).

(c) Isoprene emission rates.

(d) Maximum photosynthetic quantum yield of PSII (F_v/F_m).

(e) Electron transport rate.

(f) Non-photochemical quenching.

Significant differences between the lines ($n = 6$ leaves) are indicated by labelling with lower-case letters for the climate chamber-grown lines and capital letters for the field-grown lines ($P < 0.05$).

and R1_F). Prior to exposing plants to transient heat stress (see below), leaves were allowed to stabilize gas exchange and isoprene emission at a leaf temperature of 30°C and a PPFD (photosynthetic photon flux density) of 1000 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$, which represents the status of unstressed plants (Figure 2). Field-grown poplars showed fourfold higher net assimilation (A) rates, and twofold higher stomatal conductance (gH₂O) compared to WT plants grown in climate chambers. The rate of isoprene emission detected for control plants grown under field conditions was 17 times higher than the rate of isoprene emission of WT_C plants grown in climate chambers. Under both cultivation conditions, the isoprene emission of lines O15_C, R1_C, R1_F and R22_C was reduced to virtually zero (Figure 2c). When comparing the lines within one experimental group, the gH₂O of controls and WT plants was similar to that of the transgenic lines except for line O15_C, which showed a gH₂O value that was almost twice as high (Figure 2b). These results demonstrate that the absence of isoprene emission in the transgenic lines (O15_C, R1_C, R22_C and R1_F) was not caused by reduced transpiration.

The ratio F_v/F_m reflects the intrinsic or maximum quantum efficiency of photosystem II (PSII), and is commonly used as a sensitive indicator of plant photosynthetic performance, with optimal values of approximately 0.83 (Björkman and Demmig, 1987). Both lines of field-grown poplars (R1_F, C_F) achieved the optimum F_v/F_m value, while plants grown in the climate chamber showed slightly lower values. There was no difference between the different lines within climate chamber and field-grown plants (Figure 2d). In addition to F_v/F_m , we also determined electron transport rates (ETR, Figure 2e) and NPQ (Figure 2f), two parameters for photosynthetic performance in the light-adapted state. ETR meas-

ures the proportion of light absorbed by PSII chlorophyll that is used for photochemistry, whereas NPQ [$(F_m - F_m)/F_m$] describes dissipation of excess light energy as heat. NPQ together with chlorophyll fluorescence are competing processes to photochemistry (Maxwell and Johnson, 2000).

Initial measurement of ETR, before application of heat stress, revealed differences between lines grown in the climate chamber. Compared to WT_C (28.1 $\mu\text{mol electrons m}^{-2} \text{sec}^{-1}$) and R1_C (26.0 $\mu\text{mol electrons m}^{-2} \text{sec}^{-1}$), O15_C and R22_C (34.6 and 30.9 $\mu\text{mol electrons m}^{-2} \text{sec}^{-1}$, respectively) showed significantly enhanced ETR values ($P < 0.05$; Figure 2e). In addition to our observation of an enhanced ETR, line R22_C also showed significantly enhanced NPQ values ($P < 0.05$) compared with the other lines (Figure 2f). Field-grown poplars showed higher initial ETR values of 144 and 150 $\mu\text{mol electrons m}^{-2} \text{sec}^{-1}$ for C_F and R1_F, respectively (Figure 2e), and lower NPQ levels (Figure 2f) compared to the climate chamber-grown poplars, indicating higher PSII efficiency under natural irradiation conditions. The ETR was similar in both field-grown lines, while NPQ values in R1_F were significantly increased ($P = 0.026$) compared to the isoprene-emitting control line (Figure 2f).

Based on these results, we conclude that, in general, transgenic and WT plants grown under the same conditions are similar in their overall photosynthetic performance.

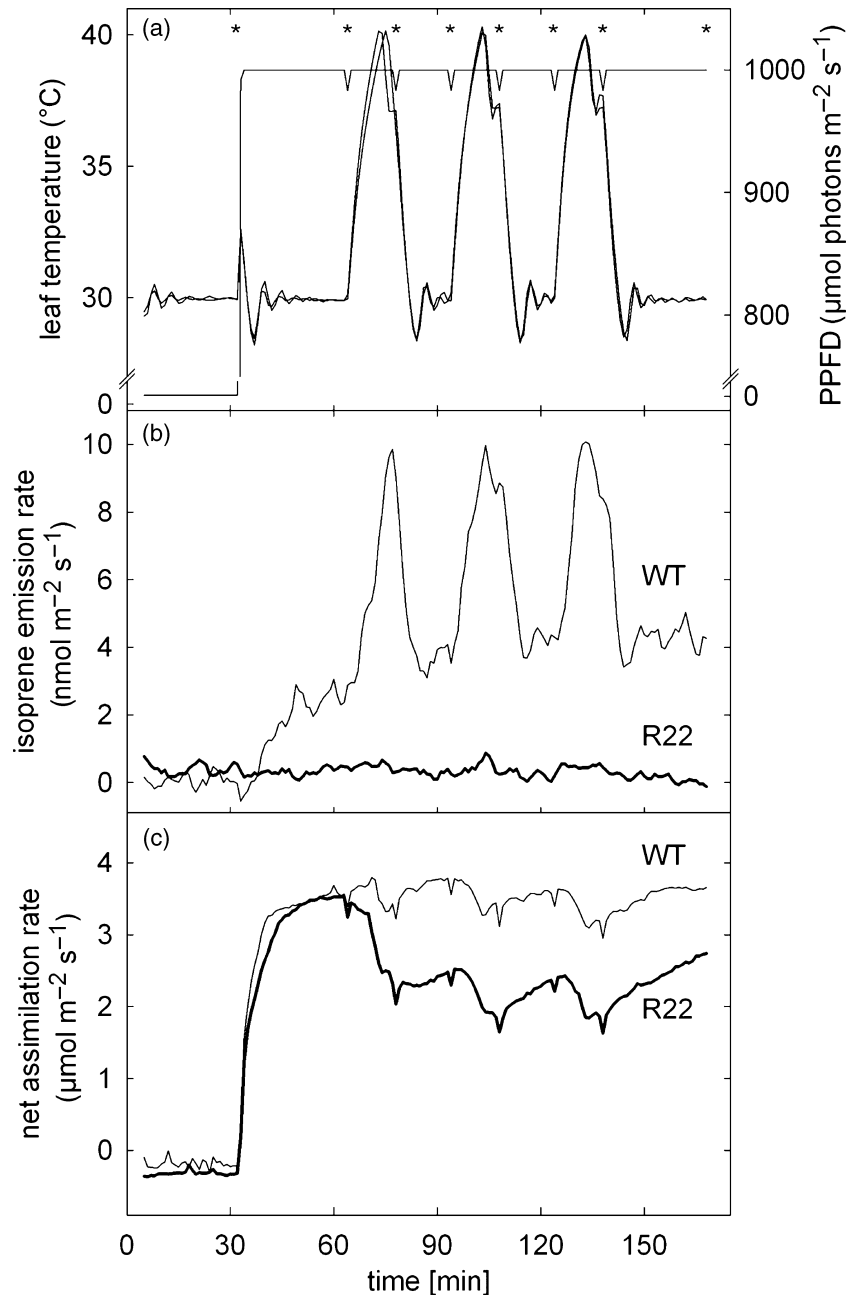
Effects of heat stress on photosynthetic gas exchange in WT and transgenic poplars

Thermal protection of the photosynthetic apparatus is one of the debated functions for isoprene (Owen and Peñuelas,

2005; Peñuelas *et al.*, 2005; Sharkey *et al.*, 2001; Velikova *et al.*, 2006). Based on the hypothesis that isoprene provides protection from damage caused during short periods of high temperature (Singsaas and Sharkey, 2000) or during recovery from heat stress (Velikova and Loreto, 2005), we tested the thermotolerance of isoprene emitters versus transgenic non-emitters by applying a temperature cycle program that involves short periods of high temperature under constant light intensity of $1000 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ PPFD (Figure 3a). In parallel, we determined isoprene emission rates measured by online PTR-MS (Figure 3b) and net assimilation by gas exchange (Figure 3c). Figure 3(b,c) show

representative results obtained with 4-month-old WT_C and R22_C poplars. Isoprene emission of WT_C started after a short delay of about 5 min after onset of illumination, and within a few minutes reached levels close to saturation of $3 \text{ nmol m}^{-2} \text{sec}^{-1}$ at conditions of $1000 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ PPFD and 30°C . With increasing temperature during the heat cycles, the isoprene emission rate also increased, and each temperature maximum (approximately 40°C) was followed within 1–2 min by a maximum emission ($10 \text{ nmol m}^{-2} \text{sec}^{-1}$). During each recovery phase, isoprene emission decreased. The transgenic line R22_C had no detectable isoprene emission at any time during the

Figure 3. Net assimilation rate, isoprene emission and leaf temperature during heat stress in transgenic and wild-type (WT) poplar leaves under climate chamber conditions. Profiles of one typical experiment are presented. WT is presented by thin line, the RNAi line (R22) is represented by bold line. The analyses were performed on leaf number 9 below the apex. (a) Profiles of leaf temperature and PPFD during three heat cycles and recovery phases. Asterisks mark time points of chlorophyll fluorescence determination. (b) Isoprene emission rates. (c) Net assimilation rates.



experiment. Parallel online analysis of other volatile organic compounds by PTR-MS, i.e. acetaldehyde (m45) and C6 wound alcohols (m81 and m99 as major ion products of hexenals, and m83 and m101 originating from hexenyl acetate), showed a rapid transient emission characterized by these ion masses directly after enclosure of the leaf into the cuvette system used for PTR-MS analysis. However, these emissions disappeared during the first 30 min of acclimatization to the cuvette environment. None of these compounds was detected as emission during the heat cycles, indicating that emission of volatiles resulting from lipid degradation did not occur (data not shown).

In contrast to isoprene emission, net assimilation started immediately upon onset of light and reached a similar maximum value within 10 min of about $3.5 \mu\text{mol m}^{-2} \text{sec}^{-1}$ for both WT_C and R22_C. The net assimilation curve of WT_C decreased by approximately 15% during each high-temperature event, and increased again during the recovery phase up to the initial value. In contrast, net assimilation of R22_C declined about 33% during the first high-temperature event and did not recover initially. The second and third temperature increases caused further but less prominent decreases followed by moderate recovery. Overall, these data show that the net carbon assimilation of R22_C is more susceptible to high temperature than that of WT_C.

We also applied the same temperature program to R1_C and O15_C plants and found similar results as with transgenic line R22_C. All three transgenic lines showed similar temperature-dependent fluctuations of isoprene emission (Figure 4a) and net assimilation during the course of the temperature program. Leaf internal isoprene concentrations (C_{iso}) were calculated from gas exchange data according to that method described by Singaas *et al.* (1997) (Figure 4b). Corresponding with isoprene emission rates in WT_C, the C_{iso} values increased with every temperature increase. Internal isoprene concentrations could not be calculated in transgenic lines due to the absence of isoprene emission.

Relative values of net assimilation (related to the individual value of each leaf after 30 min of acclimatization to light and temperature) clearly demonstrated that all lines with repressed isoprene emission (O15_C, R1_C and R22_C) showed a significant and similar ($P = 0.009$) reduction of relative net assimilation (Figure 4c) that was detectable even during the first high-temperature event. A maximum reduction of approximately 45% was reached during the third high-temperature event. Finally, after the last recovery phase, the photosynthetic activity of O15_C, R1_C and R22_C was still reduced by approximately 20% (Figure 4c).

The same type of experiment with applications of short high-temperature events was performed with leaves of poplars grown for one season under field conditions (vector control C_F and RNAi line R1_F; Figure 5). Control plants (C_F) showed high rates of isoprene emission of approximately $50 \text{ nmol m}^{-2} \text{sec}^{-1}$, as well as high levels of leaf internal

isoprene concentrations of approximately $1 \mu\text{L l}^{-1}$ with the same temperature-dependent pattern as observed for WT_C (Figure 5a,b). The RNAi line R1_F was completely repressed

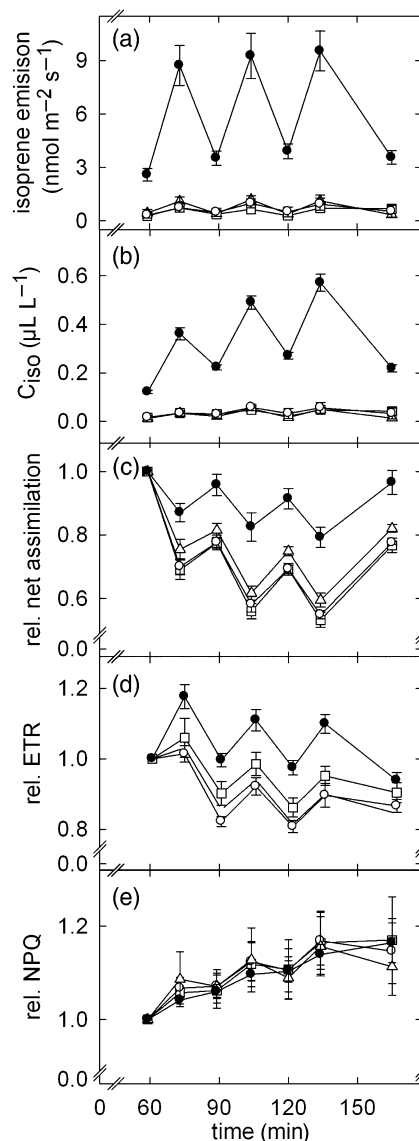


Figure 4. Photosynthetic gas exchange and chlorophyll fluorescence during repeated heat cycles in wild-type (WT) and transgenic poplar leaves cultivated under climate chamber conditions.

The seven measurement points represent the data for the initial status (1st, after 30 min dark and 30 min light adaptation at 30°C), during heat stress (2nd, 4th and 6th) and after some minutes of recovery (3rd, 5th and 7th). For the relative plots, data were normalized relative to the initial values (time point 60 min) at a leaf temperature of 30°C and a PPFD of $1000 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$. Measurements ($\pm \text{SE}$, $n = 6$ leaves) were performed on mature leaves (leaf numbers 9 and 10 below the apex) of 4-month-old poplars. Black circle, WT; open triangle, over-expressed transgenic line O15; open square, RNA interference (RNAi) line R1; open circle, RNAi line R22.

- (a) Isoprene emission rates.
- (b) Calculated leaf internal isoprene concentration (C_{iso}).
- (c) Relative rates of net assimilation during heat stress and recovery phases.
- (d) Relative rates of electron transport during heat stress and recovery phases.
- (e) Relative rates of non-photochemical quenching during heat stress and recovery phases.

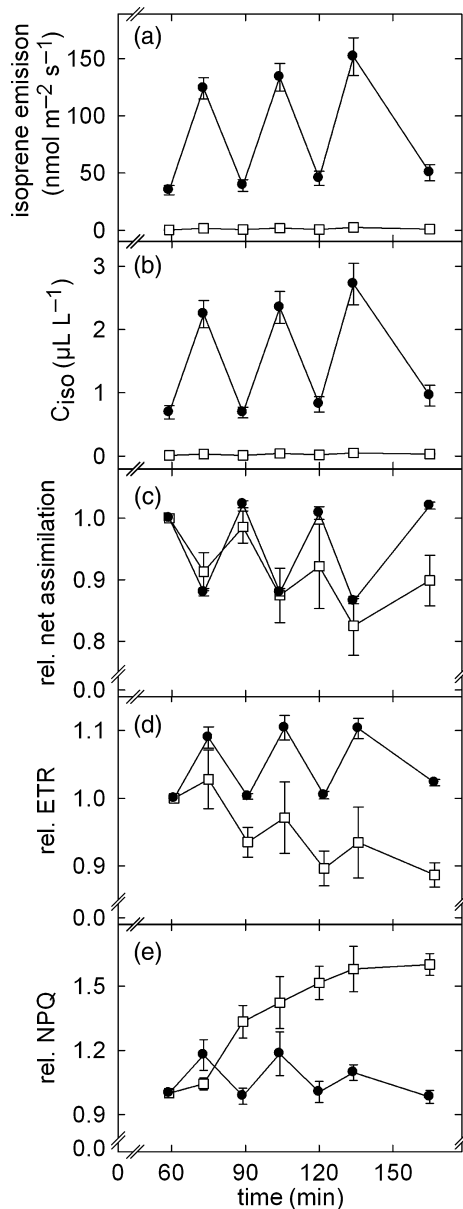


Figure 5. Photosynthetic gas exchange and chlorophyll fluorescence during repeated heat cycles in wild-type (WT) and transgenic poplar leaves cultivated under field conditions.

The seven measurement points represent the data for the initial status (1st, after 30 min dark and 30 min light adaptation), during heat stress (2nd, 4th and 6th) and after some minutes of recovery (3rd, 5th and 7th). For the relative plots, data were normalized relative to the initial values (time point 60 min) at a leaf temperature of 30°C and a PPFD of 1000 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$. Measurements ($\pm$ SE, $n = 5$ leaves) were performed on mature leaves (leaf numbers 6–10 below the apex) of 10-month-old poplars grown under field conditions from May to September 2006. Black circle, binary vector control; open square, RNAi line R1.

(a) Isoprene emission rates.

(b) Calculated leaf internal isoprene concentration (C_{iso}).

(c) Relative rates of net assimilation during heat stress and recovery phases.

(d) Relative rates of electron transport during heat stress and recovery phases.

(e) Relative rates of non-photochemical quenching during heat stress and recovery phases.

in isoprene production and emission. Similar to the temperature-dependent fluctuations of WT_C , net assimilation in C_F decreased by 10% for every high-temperature event, followed by a full recovery up to 100% (Figure 5c). A significant difference in relative photosynthetic capacity between C_F and $R1_F$ was detectable at the last time point, where leaves of $R1_F$ reached 90% of the initial assimilation rate (Figure 5c, $P \leq 0.05$).

When we compared the isoprene-repressed poplar lines grown in climate chambers and in the field, several differences were observed. In general, leaves of the climate chamber-grown poplars ($O15_C$, $R1_C$ and $R22_C$, Figure 4d) showed an earlier and stronger reduction of net assimilation rates than leaves developed in the field. The negative effects of high temperature were observed during each high-temperature event for the climate chamber-grown poplars, whereas the negative impact on field-grown leaves of line $R1_F$ was more pronounced with respect to the plant's ability to recover at the last stage of the stress treatment (Figure 5c).

Effects of short high-temperature events on chlorophyll fluorescence in WT and transgenic poplars

ETR and NPQ were determined in order to gain a more detailed insight into the effects of repeated short high-temperature events on the light reactions of photosynthesis. Under laboratory conditions, ETR reflects the quantum yield of PSII photochemistry and is correlated with CO_2 fixation (Genty *et al.*, 1989). However, this correlation can diminish under conditions when competing processes of CO_2 fixation such as photorespiration or other alternative electron transports increase (Fryer *et al.*, 1998).

The relative values of ETR (Figure 4d) clearly demonstrate that changes in leaf temperature induced fluctuations in ETR with a pattern that matches phases of increasing and decreasing temperature. ETR in the WT_C was stimulated with each high-temperature event up to 20%. In contrast, the ETR of $O15_C$, $R1_C$ and $R22_C$ responded later (during the 2nd and 3rd temperature pulses) and to a lower extent (8%). Consequently, the relative ETR values of WT_C leaves were significantly ($P = 0.004$) higher than ETR values in the isoprene-repressed lines starting with the 3rd time point, except for the last time point where the ETR values decreased below the initial value for WT_C as well.

The temperature response of relative ETR showed the positive effect of heat on ETR (Figure 5d) for control C_F , with significant differences between a heat event and recovery ($P < 0.001$). For $R1_F$, the stimulation of ETR during the high-temperature episodes was weaker, leading to the first significant difference between the two lines at the 3rd time point ($P = 0.038$). Compared to the isoprene-emitting C_F plants, ETR values in $R1_F$ plants were negatively affected by the heat cycles.

Relative NPQ values (Figure 4e) showed a positive response to the temperature episodes for all lines including WT_C. Relative to its initial values, NPQ increased in all plants, with constant levels during the recovery phase, resulting in a relative increase of NPQ values between 11% and 17% throughout the experiment compared to the initial values.

The high-temperature events showed their strongest effect on photosynthesis in terms of the response of NPQ in transgenic versus control leaves of field-grown poplars. During cycles of high-temperature events, relative NPQ values in C_F leaves showed only small fluctuations, whereas relative NPQ values in R1_F increased continuously over the entire experiment, finally ending with a NPQ value of 3.88 reflecting a relative increase of 160% (Figure 5e). Relative NPQ values in C_F were significantly different ($P = 0.008$) from those in R1_F after the 3rd time point, comparable to the results for ETR.

Impact of isoprene repression on DMADP levels and photosynthetic pigment concentration

Dimethylallyl diphosphate (DMADP), which is the substrate for ISPS and the immediate metabolic precursor in the formation of isoprene (Miller *et al.*, 2001), together with its isomer isopentenyl diphosphate (IPP), are the common C5 precursors for all isoprenoids including carotenoids. In isoprene-emitting lines, total DMADP pools varied between

21 pmol mg DW⁻¹ in WT_C (Figure 6a) and 36 pmol mg DW⁻¹ in C_F (Figure 6d). Compared to controls, leaf DMADP levels in the RNAi lines were dramatically increased up to approximately 150 and 700 pmol mg DW⁻¹ in leaves of plants grown in the climate chamber and the field, respectively. DMADP levels in the transgenic line O15_C were similar to those of WT leaves (Figure 6a), although isoprene synthesis was repressed (Figure 3c).

The biosynthesis of isoprene and carotenoid are closely linked through common precursors and intermediates in the MEP pathways of isoprenoid biosynthesis (Lichtenthaler, 1999; Owen and Peñuelas, 2005). Therefore, we tested whether increased levels of DMADP had an effect on levels of carotenoid pigments, specifically the levels of neoxanthin (Nx), lutein (Lut), β -carotenes (β -Car), violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx), as well as total xanthophyll content (Vx, Ax, Zx), and analyzed the de-epoxidation status of the xanthophylls: $([Zx] + 0.5 [Ax]) / ([Zx] + [Ax] + [Vx])$. For poplars grown in climate chambers, the xanthophyll content was significantly lower in lines with reduced isoprene emission (R1_C, R22_C and O15_C) compared to WT_C (Figure 6b). The total amount of carotenoids was reduced only in R1_C and R22_C. However, no obvious differences were observed for any of the carotenoid pigments analyzed (carotenoids, xanthophylls and chlorophylls) when we compared the R1_F and C_F lines grown in the field.

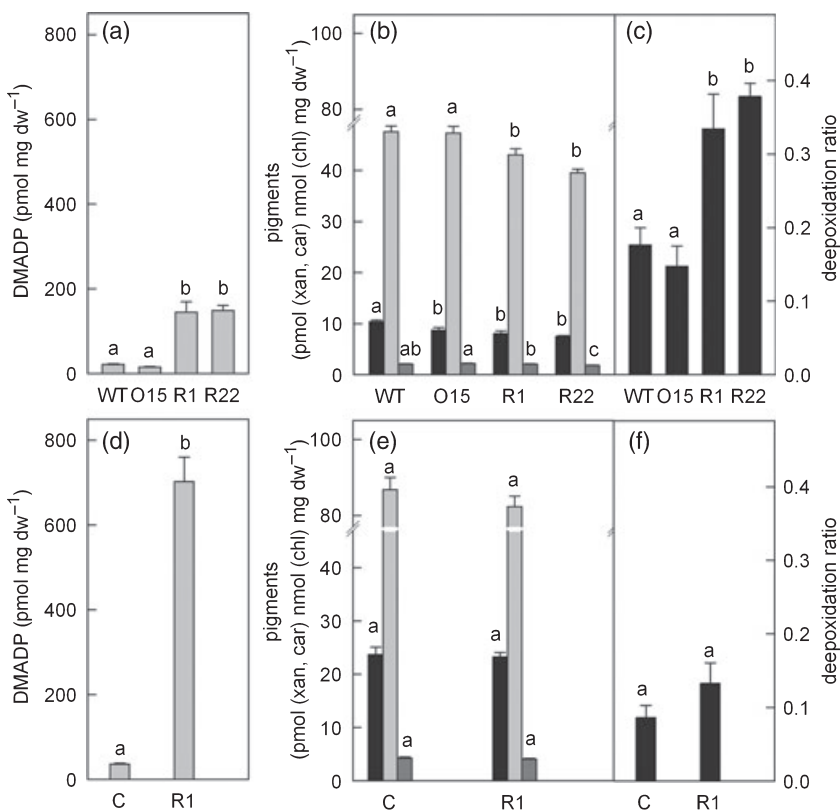


Figure 6. Dimethylallyl diphosphate (DMADP) and photosynthetic pigment contents in wild type (WT) and transgenic poplar leaves. (a) Mid-day DMADP leaf content in WT and isoprene emission-repressed transgenic lines cultivated under climate chamber conditions. (b) Contents of xanthophylls (black bar), carotenoids (light-gray bar) and chlorophylls (chl a and chl b) (dark-gray bar) in leaves of climate chamber-grown saplings. (c) De-epoxidation ratio, calculated as $([Zx] + 0.5 [Ax]) / ([Zx] + [Ax] + [Vx])$, of xanthophylls in climate chamber-grown leaves. (d) Mid-day dimethylallyl diphosphate leaf content in binary vector control plants (C) and the isoprene emission-repressed line R1 cultivated under field conditions during summer 2006. (e) Contents of xanthophylls (black bar), carotenoids (light-gray bar) and chlorophylls (chl a and chl b) (dark-gray bar) in field-grown leaves. (f) De-epoxidation ratio, calculated as $([Zx] + 0.5 [Ax]) / ([Zx] + [Ax] + [Vx])$, of xanthophylls in field-grown leaves. Analyses were performed on leaf numbers 9 and 10 of every plant ($n = 6$). Values are means $\pm$ SE.

An increased de-epoxidation ratio/formation of Zx can reflect enhanced dissipation of excess absorbed light as heat (Maxwell and Johnson, 2000), or can indicate an enhanced level of antioxidants (Havaux and Niyogi, 1999). This effect was clearly detectable and significant ($P < 0.05$) for R1_C and R22_C compared to WT_C, but not for O15_C in the group of climate chamber saplings (Figure 6c). Leaves of the field-grown poplars showed the same trend even though the difference between the C_F and R1_F values was not significant (Figure 6f).

Discussion

Screening of transgenic lines for identification of repressed lines

The present work describes successful approaches to genetically engineer the biosynthesis of isoprene in poplars, thereby enabling physiological studies to test some long-standing hypotheses regarding the biological function of isoprene in thermotolerance. Our transformation of Grey poplar with a construct suppressing the *PcISPS* gene via RNAi successfully resulted in the development of stable lines with virtually no ISPS activity and completely abolished isoprene emission. Even under conditions of high temperatures, when isoprene emission is normally at its maximum, emission of margin was strongly suppressed. Attempts to generate a 'super-emitter' of isoprene by over-expression of the poplar *ISPS* gene under a strong constitutive promoter were not successful, probably due post-transcriptional gene silencing (PTGS) or co-suppression events (Bruening, 1998; Depicker and Van Montagu, 1997; Napoli *et al.*, 1990). Results obtained with the O15_C line indicate that isoprene emission was suppressed to a similar extent in this system as it was in the R22_C and R1_C lines. In contrast to RNAi-silenced lines, the PTGS effect observed in the O-lines was developmentally controlled as expected for PTGS events (Depicker and Van Montagu, 1997).

Isoprene protects photosynthesis and photosynthetic electron transport from effects of transient heat stress

Temperature experiments to test possible functions of isoprene have previously been performed in a number of different ways. In some of the pioneering experiments in this area (Sharkey and Singsaas, 1995; Singsaas *et al.*, 1997), thermal protection of isoprene was studied by gradually increasing leaf temperatures to levels up to 50°C. In other experiments, thermal stress was applied to intact leaves for a period of 1.5 h at 38°C (Velikova and Loreto, 2005), or to excised leaf discs for 30 min at various temperatures (Velikova *et al.*, 2005a,b, 2006). However, when designing relevant experiments to test the role of isoprene in thermotolerance, it is important to bear in mind that Sharkey and

Singsaas (1995) suggested a role of isoprene specifically in the protection of leaves from the damage caused by the rapid and transient high-temperature events that occur naturally in the sun-exposed canopy of trees (Singsaas and Sharkey, 1998). To test their theory under conditions that resemble relevant scenarios of heat stress in nature, Sharkey *et al.* (2001) assessed thermotolerance as recovery of photosynthesis from short-term treatments at 46°C.

A similar approach was chosen in the present work to test isoprene function in poplar leaves under temperature fluctuations ranging from 30–40°C, which are realistic under natural conditions. It is important to keep in mind that leaf heating under natural conditions is a very rapid process with a time constant of approximately 20 sec (Singsaas and Sharkey, 1998). A first temperature-stress experiment was performed with climate chamber-grown poplars. Experiments were then repeated with field-grown poplars to validate data obtained in climate chambers. Due to some differences in growth conditions and different ages of plants, a direct comparison of the two experiments is not possible. For example, different ratios of photosynthetically active biomass to leaf area caused by the different growth conditions and age contributed to enhanced activity for all gas exchange values in the field-grown poplars.

However, both experiments provided similar insights into the effect of transient temperature increases on photosynthesis. Inhibition of photosynthesis has often been attributed to an impairment of electron transport as part of the light reactions of photosynthesis, or to heat sensitivity of Calvin cycle enzymes as part of the dark reactions. In our experiments, the temperature response of ETR in WT_C and C_F was positive, in that ETR was enhanced with every short high-temperature event. This result is consistent with other work (Copolovici *et al.*, 2005; Schrader *et al.*, 2004; Wang *et al.*, 2006) reporting a stimulation of ETR by moderately high temperatures. Simultaneously with the stimulation of ETR, net assimilation decreased during the high-temperature episodes, demonstrating a discrepancy between ETR and CO₂ assimilation under stress conditions. This discrepancy may be caused by enhanced alternative electron transport pathways such as photorespiration or Mehler reactions. Alternatively, it is possible that initial processes of inhibition of net assimilation are associated with Calvin cycle reactions, excluding inhibition of linear ETR. Several studies have shown that the activation of Rubisco by Rubisco activase is already inhibited at moderately high temperatures (< 40°C) and accompanies the decrease in net assimilation rates (Kim and Portis, 2005; Salvucci and Crafts-Brandner, 2004; Salvucci *et al.*, 2001). The inhibition of Rubisco activase at moderately high temperatures is rapidly reversible, which is in agreement with our data showing a fast recovery from reduction of net assimilation after the high-temperature event. Nevertheless, further studies are necessary to explore these possible explanations for the

different patterns of ETR and net assimilation in response to increased temperature.

Using transgenic poplars, we clearly demonstrated a functional relationship between isoprene emission and recovery of photosynthesis from transient heat stress, which is consistent with the earlier findings of Sharkey *et al.* (2001) using Kudzu and oak leaves. In all transgenic non-emitting lines, the absence of isoprene emission is accompanied by reduced recovery of net assimilation rates and inhibited ETR after the short-term high-temperature treatments. In field-grown poplars, linear partial recovery of ETR was observed after the 2nd heat cycle, followed by a delayed decrease in net assimilation rate.

Based on these results, we hypothesize that, in the absence of isoprene, the initial site of high-temperature stress involve membrane-localized processes as indicated by the reduced ETR capacity. Impaired ETR is indeed related to decreased membrane stability or fluidity (Murakami *et al.*, 2000), which can be influenced by oxidative damage and lipid peroxidation. We suggest that these two processes may be enhanced by the absence of isoprene. A membrane-stabilizing function of isoprene under stress conditions has already been suggested by Velikova and Loreto (2005). These authors attributed the protective role of isoprene to an antioxidant action/ROS-scavenging ability. Other studies have demonstrated that isoprene protects membranes against ROS (reactive oxygen species) production and lipid peroxidation under various stress conditions – not only heat, but also ozone and high light intensity (Affek and Yakir, 2002; Loreto and Velikova, 2001; Loreto *et al.*, 2006).

For isoprene-repressed lines (O15_C, R1_C, R22_C) grown in the climate chamber, impairment of ETR and net assimilation occurred concurrently during the first high-temperature event. Therefore, we could not assess an initial site of temperature stress as was possible with field-grown poplars. However, all plants in the climate chamber experiment had relatively low photosynthetic yield and high NPQ values even at the beginning of the experiment. We assume that a PPFD of 1000 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ had already caused high light-intensity stress. Therefore, it is difficult to address the effect of a single stress event in this system.

Along with decreasing ETR, we observed increasing NPQ values, indicating that limited photochemistry resulted in enhanced dissipation of the absorbed light energy as heat. NPQ is dependent on the formation of a pH gradient (ΔpH) across the thylakoid membranes. As the linear ETR is responsible for maintenance of the pH gradient, in the case of reduced linear ETR, alternative mechanisms are required to maintain the necessary ΔpH . As proposed by several authors (Miyake *et al.*, 2004; Pastenes and Horton, 1996; Wang *et al.*, 2006), the cyclic electron transport of photosystem I (PSI) can substitute for repressed linear ETR. Nevertheless, additional studies are necessary to confirm this hypothesis in the isoprene emission knock-down lines.

Knock-down of isoprene affects photosynthetic pigment content and regulation of the MEP pathway

NPQ may be divided into at least three different components according to their relaxation kinetics (for overview, see Müller *et al.*, 2001). The major and arguably most important component is the pH- or energy-dependent qE (photochemical quenching), which is rapidly reversible and inducible. In a large number of plant species, the level of qE correlates with the amount of zeaxanthin (Zx), which is synthesized via antheraxanthin (Ax) from violaxanthin (Vx) in de-epoxidation reactions (Demmig-Adams, 1990). As the formation of antheraxanthin from Vx involves qE (Gilmore and Yamamoto, 1993), it has become common practice to calculate the level of de-epoxidation as the amount of Zx and Ax in comparison to the total pool of Vx, Ax and Zx (Müller *et al.*, 2001). Our analyses of the de-epoxidation ratio revealed an increased de-epoxidation status for the repressed lines R1_C and R22_C cultivated in the climate chamber, and, to a lower extent, for the field-grown poplars R1_F. The samples for the analyses were harvested under the normal climate chamber cultivation conditions (approximately 100 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$, air temperature 27°C) after a few days of re-acclimatization following the heat stress experiments. Thus, neither high temperature nor high light appear to be responsible for the increased de-epoxidation status in the RNAi lines. However, pigment analyses of O15_C revealed an equal de-epoxidation ratio to that in WT_C which cannot be fully explained.

An increased pool of Zx (and Ax) can cause increased NPQ as a regulatory response to an imbalance between light absorption and photochemistry. Additionally, there are studies indicating that Zx functions not only in NPQ, but also in protection of the thylakoid membrane against photo-oxidative damage (summarized in Havaux and Niyogi, 1999). Considering the possible role of Zx as an antioxidant, the RNAi lines may have to cope with an enhanced level of oxidative stress concentrated on the thylakoid membranes (even under normal growth conditions).

Pigment analyses also revealed a significant decrease in the total amount of carotenoids and xanthophylls for the RNAi lines of poplars grown in climate chambers (R1_C and R22_C; $P < 0.05$, see Figure 6c). This phenomenon may be the result of changes in flux through the MEP and isoprenoid pathways as a consequence of DMADP accumulation due to *PcISPS* knock-down. As the MEP pathway has only been discovered in the last decade, very little is known about mechanisms of regulation of this important pathway for isoprenoid biosynthesis. Curiously, along with the knock-down of *PcISPS* transcript levels by the RNAi transformation, *PcDXR* transcript levels were also reduced in all RNAi lines compared to WT. This result indicates that repression of isoprene emission influenced *PcDXR* expression by regulatory mechanisms that remain to be studied in more detail. For example, Guevara-Garcia *et al.* (2005) demonstrated

that the MEP pathway is subject to multiple levels of transcriptional and post-transcriptional control, for example by end-product feedback reactions resulting in coordinated transcription rates of all MEP pathway genes in *Arabidopsis*. Here we demonstrated a high accumulation of DMADP in the RNAi lines as a consequence of *PcISPS* repression. This increased pool of DMADP could function as a regulatory factor, causing a coordinated feedback regulation of gene expression in the MEP pathway as well as a negative feed-forward regulation of genes downstream of DMADP formation that are responsible for carotenoid biosynthesis.

In conclusion, this work with transgenic Grey poplars clearly supports the hypothesis that isoprene emission or its leaf internal concentration have a protective role for membrane-localized processes of photosynthetic electron transport and energy dissipation, keeping net assimilation stable under transient and moderate thermal stress. However, the specific mechanism(s) behind this effect of heat tolerance and its full significance under natural conditions are still unclear. Therefore, future work will involve detailed biochemical/biochemical studies of heat effects on chloroplasts and reactive oxygen species as well as long-term field trials to test whether the isoprene effect represents a positive adaptive trait for isoprene-producing species. In a more general context, poplars that no longer emit isoprene also provide a suitable system to study regulation of the MEP and isoprenoid pathways leading to higher condensed isoprenoids. Moreover, the *PcISPS* knock-down lines will help to identify possible inter-relationships with other pathways (i.e. plastidic shikimate pathway, cytosolic amino acid synthesis) via competition for phosphoenolpyruvate (PEP), a central prerequisite of each pathway, thereby testing the other proposed biological functions of isoprene biosynthesis and emission.

Experimental procedures

Construction of binary vectors for plant transformation

In order to elucidate the physiological function of isoprene biosynthesis, isoprene-overproducing poplar lines and lines knocked down in isoprene formation were established by applying two strategies. For potential over-expression of *ISPS*, the full-length cDNA of *PcISPS* was supplemented by a sequence coding for a C-terminal His tag (for details see Schnitzler *et al.*, 2005), and cloned into the binary vector pBinAR (Bevan, 1984), conferring kanamycin resistance under the control of a 35S promoter. For the knock-down of *PcISPS* transcription, a 160 bp long gene segment of the transit peptide sequence, highly *ISPS*-specific in comparison to monoterpene synthase genes, was selected and cloned as a self-complementary hairpin construct for induction of PTGS or RNA interference (RNAi). Sense and antisense gene fragments were cloned into the pKANNIBAL vector (a gift from Peter Waterhouse of CSIRO Plant Industry, Canberra, Australia) harboring an intron sequence (Wesley *et al.*, 2001), and finally cloned into the binary vector pART27 (Wesley *et al.*, 2001). The antisense sequence was

first amplified via standard PCR using the forward primer IS.Xho.antisense ATTGTGCTCGAGCCGTCCAATC and reverse primer IS.Kpn.antisense CTATTTGGTACCTAATTGGCAG with the *Xho*I and *Kpn*I restriction sites underlined (bold letters indicate changes in the original sequence in order to introduce the restriction site), and cloned according to standard protocols into pCR2.1 (Invitrogen; <http://www.invitrogen.com/>). After digestion with *Xho*I and *Kpn*I, the antisense sequence was then subcloned into pKAN-NIBAL. The sense sequence was amplified in a sticky-end PCR using the forward primers IS.Xba.sense1 CTAGAAATGGCAACTGAATT-ATTGTGC and IS.Xba.senseII AATGGCAACTGAATTATTGTGC and the reverse primers IS.Hind.senseIII TCATAATTGGCAGACCGTCTGG and IS.Hind.senseIV AGCTTCATAATTGGCAGACCGTCTGG. The amplicon was directly cloned into the pKANNIBAL vector harboring the antisense construct. Plasmids were sequenced after each cloning step for verification, according to standard protocols.

Transformation of poplar

Both expression cassettes as well as the empty pBinAR were then used to transform *Agrobacterium tumefaciens* strain C58C1/pMP90. To prepare electrocompetent *A. tumefaciens* cells, a single colony was grown overnight at 28°C in 10 ml of LB medium (Luria-Bertani medium) containing 50 µM kanamycin. This culture was then used to inoculate 150 ml of the same medium. Cells of 0.6 OD₅₅₀ were pelleted by centrifugation (15 000 g), and resuspended in 150 ml sterile bi-distilled water on ice. This step was repeated twice with 50 ml of water. After another centrifugation (15 000 g), the pellet was resuspended in 1 ml of water, washed twice and finally resuspended with water to obtain a viscous solution. Aliquots (50 µl) of this solution was then mixed with 1 µl of DNA in a cold 0.2 cm cuvette. Electroporation was performed on a Bio-Rad Gene Pulser® II (<http://www.bio-rad.com/>) at 25 µF, 400 Ω, 2.5 kV. After the pulse, 1 ml of SOC medium was added, and cells were incubated at 28°C for 1 h and then plated on selective LB-agar medium. Positive clones (checked by PCR) were used to transform poplar plants by stem-internode transformation. Experiments were performed with *Poplar × canescens* (number 7171-B4, Institute de la Recherche Agronomique, Nancy, France). The plants were cultured *in vitro* on modified MS-based medium (Lloyd and McCown, 1980), transformed with the *A. tumefaciens* strain, and regenerated as described by Leplé *et al.* (1992). Regenerated plantlets were maintained on medium containing 50 mg l⁻¹ kanamycin.

Cultivation of transgenic and wild-type poplars

Wild-type and selected transgenic *P. × canescens* lines were amplified by micropropagation as described by Leplé *et al.* (1992) on half-concentrated MS medium (approximately 200 ml), in 1 litre glass containers under standard conditions of 27°C (day) and 24°C (night) and a long photoperiod of 16 h with approximately 100 µmol m⁻² sec⁻¹ PPFD during the light period. After 8 weeks, rooted shoots were transferred to glass containers containing approximately 200 ml of soil substrate [25% v/v Fruhstorfer Einheitserde (Bayerische Gärtner-eigenossenschaft, <http://bgg-cg.+3-kundenserver.de/>), 25% v/v silica sand (particle size 1–3 mm) and 50% v/v perlite (Agriperl Dämmstoff GmbH, http://web2.cylex.de/firma-home/perlite-daemmstoff-gmbh_-co-beratung-u-vertrieb-722726.html)] and 10 g fertilizer [Triabon (Compo, <http://www.compo.de>) and Osmocote (Scotts International (<http://www.scottinternational.com/en/>)) BV, 1:1 v/v per litre of soil], moistened with tap water and adapted to ambient conditions by carefully opening the glass lid. After acclimatization, the plants were planted into 2.2 l pots with the

same soil substrate and further cultivated in the climate chamber for additional 2 months before temperature stress experiments were started.

For the experiment with free-air cultivated plants, 4-month-old shoots were cut, transferred into the S1 cage (a glass roof-free greenhouse covered with wire netting) at the University of Göttingen, Germany, and allowed to re-sprout during the vegetation period in 2006. In August, the plants were transferred into 5 l containers. Temperature-stress experiments were performed in September 2006.

Functional screening

Functional screening on isoprene emission was performed with a head-space analysis system using online PTR-MS (for details, see Lindinger *et al.*, 1998; Tholl *et al.*, 2006). To avoid diurnal influences on emission rates, gene expression and enzyme activities (for details, see Loivamäki *et al.*, 2007), screening experiments were performed at noon. Single leaves of micropropagated 5–6 cm tall shoots were placed in 2 ml vials filled with 200 µl carbonated mineral water, and allowed to stabilize for 30 min on a light bench with a PPFD of approximately 300 µmol photons m⁻² sec⁻¹ and an air temperature of 32–35°C. Subsequently, vials were sealed gas-tight and further incubated for 60 min. After incubation, vials were immediately transferred to darkness to stop light-dependent isoprene formation. For transfer of samples into the PTR-MS, the head space of the vials was transferred into a 10 ml injection loop by flushing the vials with 10 ml N₂, and the samples were subsequently injected directly into the online MS with a flow rate of 250 ml min⁻¹. PTR-MS detected protonated isoprene (m⁺) at mass 69. Using this high-throughput system, up to eight transgenic poplar lines were analysed in parallel per day. Calibration of the system was performed using 2 ml vials filled with a calibration standard (10.9 ppmv isoprene in N₂, Messer Griesheim, <http://www.airliquide.de/loesungen/produkte/gase/>).

For emission screening, three subsequent mature leaves (leaf numbers 5–7 below the apex, 8-week-old shoots) of five plants per line were used. After emission measurement, leaf FW was determined for normalization of the emission. Leaves of the same age were pooled, frozen in liquid N₂, and stored at -80°C for further analysis of *PcISPS* activity (leaf number 6), transcript levels (leaf number 5) and photosynthetic pigment concentration (leaf number 7).

RNA isolation and cDNA synthesis

Total RNA from frozen poplar leaves was isolated with a Qiagen RNeasy Minikit™ (www.qiagen.com/) following the manufacturer's standard protocol. The amount and purity of isolated RNA was determined with a NanoDrop spectrometer (Peqlab Biotechnologie GmbH, <http://www.airliquide.de/loesungen/produkte/gase/>). For first-strand cDNA synthesis, 3 µg of total RNA were reverse-transcribed using oligo(dT) primers and Superscript II™ reverse transcriptase (Invitrogen) in a total volume of 20 µl according to the manufacturer's protocol. cDNA was stored at -20°C prior to analysis.

Quantitative real-time PCR

Quantitative measurements of transcription rates of *PcISPS* and *PcDXR* were performed as described by Mayrhofer *et al.* (2005) with a fivefold dilution of cDNA. For these quantitative PCR measure-

ments, the following oligonucleotide primer sets were used: for *PcISPS*, forward 5'-TTGCCTACTTTGCCGTGGTTCAAAC-3' and reverse 5'-TCCTCAGAAATGCCTTTTGACGCATG-3'; for *PcDXR*, forward 5'-GCATATGTCTTTCCAGCTTCTATTGC-3' and reverse 5'-GGAATAGTAGGTTGCGCAGGC-3'. The resulting PCR segment lengths were 197 bp (*PcISPS*) and 65 bp (*PcDXR*). For internal normalization, transcription rates of poplar β-tubulin (*PcTUB*) (EMBL accession number AY353093) were determined using forward primer 5'-GATTGTCCCTCGCGCTGT-3' and reverse primer 5'-TCGGTATAATGACCCTTGCC-3'.

Determination of isoprene synthase (*PcISPS*) activity and DMADP content

Isoprene synthase activity was assayed as described by Mayrhofer *et al.* (2005). An aliquot of 200 mg PVPP was added to 4.0 ml poplar-adapted plant extraction buffer (PEB: 100 mM Tris/HCl, pH 8.0, 20 mM MgCl₂, 100 mM CaCl₂, 1.5% w/v PEG1500, 5% v/v glycerol, 0.1% v/v Tween-80 and 20 mM DTT) prior to use, and stirred for 15 min with 200 mg of homogenized leaf material. Protein concentrations were determined by the Bradford assay with BSA as standard. Determination of DMADP was performed as described by Brüggemann and Schnitzler (2002).

Determination of photosynthetic pigments

For pigment extraction, frozen leaf material was homogenized with a mortar and pestle in liquid N₂, 50 mg of fine powder were transferred into 2 ml reaction tubes, and pigments were extracted in the dark at room temperature for 10 min using 1 ml pure acetone (HPLC grade; VWR, <http://de.vwr.com/app/Home>) and the suspension was centrifuged (10 min at 15 000 g and 4°C). The pellet was extracted once more with 500 µl of acetone, centrifuged again, and both supernatants were unified.

Pigments were analyzed according to the method described by Kirchgeßner *et al.* (2003) using a Beckman HPLC System Gold (<http://www.beckmancoulter.com/localization/subinfo/germany/Germany.asp>). Detection was at 440 nm using a UV/visible diode-array detector (Beckman Model 168). System calibration was performed using purified chlorophylls (chl *a* and chl *b*), violaxanthin and β-carotene isolated by flash chromatography. The dissolved pigments were quantified spectrophotometrically using published extinction coefficients (Jeffrey *et al.*, 1997). Pigments were identified by their retention times, spectral properties and increased concentrations observed under dark–light transitions. Xanthophylls (lutein, neoxanthin, antheraxanthin and zeaxanthin) were quantified using the data obtained with violaxanthin as standard.

Analysis of photosynthetic gas exchange, chlorophyll fluorescence and isoprene emission

The gas exchange analysis was performed with the portable gas exchange fluorescence system GFS-3000 (Walz, <http://www.walz.com>). For *in situ* fluorescence measurements, the system was equipped with a LED array/PAM (Pulse-Amplitude-Modulation) combination module. The PTR-MS technique (for details see Tholl *et al.*, 2006) was used for online monitoring of isoprene emission. A bypass was inserted in the outlet air of the cuvette and an air stream of 100 ml min⁻¹ was transferred to the PTR-MS. Isoprene was detected at protonated molecular mass 69. In addition, ion signals of other masses were collected to monitor potential emission of

acetaldehyde (m45), fragments of C6 wound compounds (m81 and m99 as major ion products of hexenals, and m83 and m101 originating from hexenyl acetate; for details, see Fall *et al.*, 1999). For calibration of the PTR-MS, a gas standard (Apel-Riemer, Broomfield, CO) with a continuous flow (0–50 ml min⁻¹) of a mixture of volatile compounds, including isoprene at 1.05 ppmv, was diluted into inlet air of the GFS3000 and flushed through the empty cuvette at the beginning and at the end of experiments.

For the temperature-stress experiments, a fixed time program was set up to allow maximum repeatability (see Figure 3). Prior each measurement, the empty cuvette was run for 20 min to determine the background level of all ion masses and to adjust the CO₂ and H₂O channels of the infra-red gas analyzer. After that period, a mature poplar leaf (leaf number 9 or 10 below the apex) was inserted and analyzed for 30 min at a leaf temperature of 30°C. After that dark phase, light was switched on and held constant at a PPFD of 1000 µmol photons m⁻² sec⁻¹ for the remainder of the experiment. Under these conditions, gas exchange and isoprene emission were allowed to stabilize for an additional 30 min. Subsequently, three cycles of temperature stress were applied. Each cycle consisted of a fast increase of leaf temperature up to 38–40°C, followed by a fast decline down to 30°C, and a recovery period at that leaf temperature. The recovery periods after the 1st and 2nd temperature cycle were 12 min and that after the 3rd was 30 min. Chlorophyll fluorescence parameters were determined after 30 min of dark adaptation (F_0/F_m), after 30 min of light (yield, ETR, quenching parameters), and subsequently three times during the temperature cycles, always at the temperature maximum and the end of the recovery period.

For comparison of data and statistical analysis, gas exchange parameters and isoprene emission rates for the last 5 min preceding each fluorescence measurement were averaged for each individual leaf.

Statistical analysis

Statistical (multi-variant ANOVA) and correlation analysis was performed with SPSS for Windows NT (release 8.0.0) and SIGMAPLOT for Windows (version 9.0) (both from SPSS Inc., <http://www.spss.com>).

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