Increased Thermostability of Thylakoid Membranes in Isoprene-Emitting Leaves Probed with Three Biophysical Techniques

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Three biophysical approaches were used to get insight into increased thermostability of thylakoid membranes in isoprene-emitting plants. Arabidopsis (Arabidopsis thaliana) plants genetically modified to make isoprene and Platanus orientalis leaves, in which isoprene emission was chemically inhibited, were used. First, in the circular dichroism spectrum the transition temperature of the main band at 694 nm was higher in the presence of isoprene, indicating that the heat stability of chiral macrodomains of chloroplast membranes, and specifically the stability of ordered arrays of light-harvesting complex II-photosystem II in the stacked region of the thylakoid grana, was improved in the presence of isoprene. Second, the decay of electrochromic absorbance changes resulting from the electric field component of the proton motive force (photosystem II in the stacked region of the thylakoid grana, was improved in the presence of isoprene. Second, the decay of electrochromic absorbance changes resulting from the electric field component of the proton motive force (ΔA515) was evaluated following single-turnover saturating flashes. The decay of ΔA515 was faster in the absence of isoprene when leaves of Arabidopsis and Platanus were exposed to high temperature, indicating that isoprene protects the thylakoid membranes against leakiness at elevated temperature. Finally, thermoluminescence measurements revealed that S2Qβ charge recombination was shifted to higher temperature in Arabidopsis and Platanus plants in the presence of isoprene, indicating higher activation energy for S2Qβ redox pair, which enables isoprene-emitting plants to perform efficient primary photochemistry of photosystem II even at higher temperatures. The data provide biophysical evidence that isoprene improves the integrity and functionality of the thylakoid membranes at high temperature. These results contribute to our understanding of isoprene mechanism of action in plant protection against environmental stresses.

Vegetation is a source of large quantities of biogenic volatile organic compounds emitted into the atmosphere, of which isoprene is the most abundant (Guenther et al., 2006). Owing to the high reactivity and fast oxidation by hydroxyl radicals in the atmosphere, isoprene can increase the formation of atmospheric ozone (Monson and Holland, 2001; Kleindienst et al., 2007), organic nitrates (O’Brien et al., 1995), and peroxyacetyl nitrates (Sun and Huang, 1995). Isoprene is also a significant factor in secondary aerosol formation, with direct and indirect effects on the global radiation balance of the atmosphere (Claeys et al., 2004; Matsunaga et al., 2005; Kroll et al., 2006; Ervens et al., 2007), organic nitrates (O’Brien et al., 1995), and peroxyacylnitrates (Sun and Huang, 1995). Isoprene is also a significant factor in secondary aerosol formation, with direct and indirect effects on the global radiation balance of the atmosphere (Claeys et al., 2004; Matsunaga et al., 2005; Kroll et al., 2006; Ervens et al., 2007).

Besides having significant influences on atmospheric chemistry, many biogenic volatile organic compounds play an important role in plant biology. Experimental evidence shows that isoprene protects photosynthesis under thermal and oxidative stress conditions (for review, see Vickers et al., 2009a; Loreto and Schnitzler, 2010). It was demonstrated that leaves in which isoprene biosynthesis was blocked by the methyl erythritol pathway inhibitor fosmidomycin, were more sensitive to high temperature and ozone exposure, and developed stronger oxidative damage, compared to isoprene-emitting leaves (Loreto and Velikova, 2001; Sharkey et al., 2001; Velikova and Loreto, 2005). Plants fumigated with isoprene suffer...
less damage when exposed to oxidative stresses (Loreto et al., 2001) and recover more rapidly from heat stress than untreated controls (Singsaas et al., 1997; Sharkey et al., 2001; Velikova et al., 2006). Genetic approaches to develop isoprene-emitting species from nonemitting wild types (Sharkey et al., 2005; Loivamäki et al., 2007; Sasaki et al., 2007; Vickers et al., 2009a) or to suppress isoprene synthesis in strong emitters (Behnke et al., 2007) have also been used to clarify the role of isoprene in plant protection. Studies with genetically modified plants mostly confirmed improved tolerance associated with the capacity to form and emit isoprene. Recently important compensatory responses, such as the activation of alternative defensive biochemical pathways, e.g. phenolics biosynthesis, to cope with stressful conditions, were also highlighted (Fares et al., 2010), especially when isoprene biosynthesis is knocked out (Behnke et al., 2009).

Several hypotheses have been put forward to explain the physiological mechanism(s) by which isoprene protects the photosynthetic apparatus (Loreto and Schnitzler, 2010). The oldest and most widely accepted idea is that isoprene stabilizes chloroplast membranes (Sharkey and Singsaas, 1995). Thylakoid membranes become leaky at high temperature (Fasten and Horton, 1996; Bukhov et al., 1999; Schrader et al., 2004; Zhang et al., 2009). It was suggested that the positive effect of isoprene might be due to the hydrophobic nature of the molecule, the localization of isoprene synthase enzyme near the thylakoid membranes (Widermus and Fall, 1998; Schnitzler et al., 2005), and the high octanol/water partitioning coefficient (Copolovici and Niinemets, 2005). Lipophilic isoprene partitioned into membranes might prevent the formation of water channels responsible for the membrane leakiness at high temperature, or may prevent the formation of nonlamellar aggregates, or help stabilize the photosynthetic complexes embedded in thylakoid membranes (Singsaas et al., 1997; Sharkey et al., 2001). Isoprene could also enhance hydrophobic interactions within thylakoids and thereby stabilize interactions between lipids and/or membrane proteins during episodes of heat shock or high-temperature stress conditions (Sharkey and Yeh, 2001). Based on molecular dynamics simulations of phospholipid bilayers with and without isoprene, Siwko et al. (2007) suggested that isoprene enhances the packing of lipid tails. The authors suggested that the role of isoprene as a membrane stabilizer can be related to the fact that it fits well into the available pockets of the free volume inside the membrane, and adds cohesiveness, amplifying membrane packing, while not affecting the dynamics of phospholipid bilayers (Siwko et al., 2007). However, Logan and Monson (1999) working with reconstituted liposomes were not able to prove that isoprene improves the thermal stability of membranes.

Another hypothesis to explain the generally positive role of isoprene in plant metabolism is based on the high reactivity of volatile isoprenoids with radicals and other reactive compounds. Loreto et al. (2001) first suggested that isoprene might operate as a volatile molecule, scavenging reactive oxygen species in the intercellular spaces of the leaf mesophyll. More recently, it was proven that isoprene also removes reactive nitrogen species from the mesophyll (Velikova et al., 2008). Vickers et al. (2009a) reviewed the possible mechanisms of isoprene function and suggested that the molecule may have a general antioxidant role. A demonstration of effects of isoprene on biophysical measurements of thylakoid function at high temperature could help in working toward a resolution of the primary mechanism of isoprene action. Here three well-known techniques in biophysical studies of thylakoid membrane function were tested for the effect of isoprene at high temperature. Two plant systems were used. Arabidopsis (Arabidopsis thaliana), which does not normally make isoprene, was engineered with an isoprene synthase gene from kudzu (Pueraria lobata) so that wild-type plants (nonemitting) could be compared to the transformed plants that do make isoprene. Leaves of Platanus (plane tree) normally do make isoprene but this was inhibited by fosmidomycin so that emitting (water-fed) and nonemitting (fosmidomycin-fed) leaves could be compared. The thermal stability of the thylakoid membranes was characterized with biophysical approaches not previously used in isoprene studies, namely circular dichroism (CD) spectroscopy, electrochromic absorbance transients (ΔA/Δt), and thermoluminescence (TL). These measurements revealed that in the presence of isoprene, the macroorganization of the pigment-protein complexes in the membranes were more stable to elevated temperature, the membranes were better able to maintain a light-induced transmembrane electric field at elevated temperatures, and the recombination of the PSII donor and acceptor side charges occurred up to higher temperature.

RESULTS
Suitability of Plant Material

Genetic manipulation of Arabidopsis plants did not affect their photosynthetic performance. IspS and wild-type plants were characterized by similar photosynthesis (Fig. 1; absolute values 7.0 ± 0.3 μmol m−2 s−1, n = 14). Inhibition of isoprene emission in Platanus leaves by the chemical inhibitor also did not influence photosynthesis. Absolute values of photosynthesis in Platanus leaves were 5.3 ± 0.4 μmol m−2 s−1, n = 14. Isoprene emission was not detectable in wild-type plants of Arabidopsis, whereas in fosmidomycin-fed Platanus leaves isoprene emission was inhibited to about 10% of the original level (Fig. 1).

CD Spectroscopy

CD spectra in wild-type and IspS Arabidopsis plants showed considerable differences at 20°C. In particular,
Isoprene and Thermal Resistance of Leaves

Flash-Induced Electrochromic Shift at 515 nm

To test the membrane integrity, the electrochromic absorbance changes at 515 nm (ΔA515) induced by single-turnover, saturating flashes was recorded. The half-times of the decay of ΔA515 transients characterizing the membrane permeability are shown in Table I. We found that the decay times on IspS and wild-type Arabidopsis leaves were essentially identical at 20°C, suggesting that the thylakoid membrane of these plants possess similar permeability; however, after a 5-min-long 40°C treatment, the decay of ΔA515 became faster in the wild type, while remained unaffected in IspS (Table I).

In Platanus leaves that were maintained at 25°C the decay half-times of ΔA515 were also similar in isoprene-emitting and isoprene-inhibited leaves (Table I). The heat treatment at 45°C (4 h in weak light) resulted in stronger decrease of the half-time of ΔA515 decay in isoprene-inhibited than in isoprene-emitting leaves. Two representative traces registered after high-temperature treatment (40°C for Arabidopsis and 45°C for Platanus) are shown in Supplemental Figure S1 to exemplify the change driven by the high-temperature treatment.

Flash-Induced TL from Arabidopsis and Platanus Leaves

TL measurements were performed to assess the possibility of isoprene-induced alterations to PSII primary photochemistry for direct estimation and comparison of redox properties of PSII. Upon illumination by a single flash at 1°C, the wild-type Arabidopsis leaves showed a main TL B band (S2Qh−) peaking at 23.0°C ± 1.3°C (Fig. 4A). In IspS leaves, the peak position of the B band was up shifted by about 10°C, to 32.6°C ± 1.0°C (Fig. 4C), showing a significant increase in the activation energy for S2Qh− charge recombination. Similarly, higher emission temperatures were recorded when two (compare with Fig. 4, A and C) and more (data not shown) flashes were given, confirming the role of S3 oxidation state in charge stabilization upon multiple turnovers. The higher emission temperatures were 40.1°C and 49.4°C in wild-type and IspS leaves, respectively (Fig. 2C).

Measurements of CD spectra were also performed on isoprene-emitting and isoprene-inhibited Platanus leaves (Fig. 3). The CD spectra of Platanus leaves at 25°C were not affected by the fosmidomycin treatment suppressing isoprene biosynthesis (compare Fig. 3, A and B, black lines). At elevated temperatures, the amplitudes of the (−)675 and (+)694 nm CD bands were higher in isoprene-emitting (Fig. 3A, gray line) than in isoprene-inhibited leaves (Fig. 3B, gray line). The CD band at (+)694 nm was completely missing in isoprene-inhibited leaves already at 55°C and in isoprene-emitting leaves over 60°C (data not shown). The transition temperature was shifted to lower value (46.4°C) in isoprene-inhibited leaves, compared to isoprene-emitting leaves (55.3°C; Fig. 3C).

Figure 1. Photosynthesis and isoprene emission in Arabidopsis (A) and P. orientalis (B) leaves. Values represent means ± se (n = 7) and are expressed as percent of photosynthesis and isoprene emission of wild-type Arabidopsis and isoprene-emitting Platanus. Measurements were performed at growth conditions: 22°C and 150 μmol photons m−2 s−1 for Arabidopsis, and 25°C and 350 μmol photons m−2 s−1 for Platanus. In Platanus isoprene emission was inhibited by adding fosmidomycin (5 μM) to the water. Asterisks indicate significant differences (*P < 0.01). n.d. identifies emissions that were not detectable (<0.05 nmol m−2 s−1).

it was found that the amplitude of the main CD band (at [+]694 nm) was lower in wild-type than in IspS plants (compare Fig. 2, A and B, solid lines), suggesting that constitutive presence of isoprene might determine structural changes in the thylakoid membranes.

To investigate the possible role of isoprene in the conformational stability of chloroplast membranes subjected to high temperatures, measurements of CD spectra were performed in wild-type and IspS Arabidopsis leaves after 10 min incubations at 20°C, 30°C, 40°C, 45°C, 50°C, 55°C, and 60°C. At 40°C the amplitude of the main band at (+)694 nm was considerably lower in wild-type than in IspS leaves (compare Fig. 2, A and B, gray lines), while the weaker, excitonic bands at (+)440 and (−)650 nm and the excitonic band pair (+)482/(−)470 were not affected by isoprene. The transition temperature of the band at (+)694 nm, was shifted to higher temperature in IspS leaves; the transition temperatures were 40.1°C and 49.4°C in wild-type and IspS leaves, respectively (Fig. 2C).
temperature of TL B band in isoprene-emitting plants indicates a more stably stored S<sub>2</sub>/Q<sub>B</sub> charge pairs.

When both wild-type and isoprene-emitting plants were illuminated by a flash series, the intensity of TL B band exhibited a period four-oscillation pattern with maximum on the second flash (data not shown), typical for active PSII. TL oscillations are related to dark distribution of the S states of oxygen-evolving complex and Q<sub>B</sub>/Q<sub>B</sub> ratio (Rutherford et al., 1984), thus suggesting no differences in these parameters between the wild-type and isoprene-emitting plants.

In Platanus leaves, the TL traces could not be recorded in frozen material and therefore samples that were not previously frozen were used. The inhibition of isoprene emission by fosmidomycin did not cause significant changes in the main B-band temperature (Fig. 4, B and D). The B band induced by a single flash in isoprene-inhibited and isoprene-emitting leaves peaked at 33.1°C ± 2.0°C and 35.8°C ± 2.5°C, respectively. These values were similar to the B-band temperature observed in isoprene-emitting Arabidopsis plants (Fig. 4C).

**Figure 2.** CD spectra measured in Arabidopsis wild type, which do not emit isoprene (A), and isoprene-emitting (B) plants. CD was recorded in leaves at 20°C (black line) and 40°C (gray line). Temperature dependences of the intensity of the (+)694 nm band for the wild type (white circles) and isoprene-emitting plants (black circles) are shown in C. All measurements were done on 35-d-old plants. Measurements were repeated on seven leaves from different plants; representative spectra are shown in A and B. All spectra were corrected for a flat baseline obtained by setting the CD values at 400 and 750 nm to zero. In C, the temperature at which the intensity of the CD band is 50% of its value at 20°C (transition temperature, T<sub>t</sub>) is shown. Statistical significance of differences between T<sub>t</sub> in wild-type and isoprene-emitting leaves (P < 0.01) was determined by Student’s t test. Means ± st are given (n = 7), for the replicates at 40°C. rel. u., Relative units.

**Figure 3.** CD spectra measured in isoprene-emitting (A) and isoprene-inhibited (B) Platanus leaves. CD was recorded in leaves at 25°C (black line) and 50°C (gray line). Temperature dependences of the intensity of the (+)694 nm band for the isoprene-emitting (black circles) and isoprene-inhibited, fosmidomycin-treated leaves (white circles) are shown in C. Measurements were repeated on seven leaves from different plants; representative spectra are shown in A and B. All spectra were corrected for a flat baseline obtained by setting the CD values at 400 and 750 nm to zero. In C, the temperature at which the intensity of the CD band is 50% of its value at 20°C (transition temperature, T<sub>t</sub>) is shown. Statistical significance of differences between T<sub>t</sub> in wild-type and isoprene-emitting leaves (P < 0.01) was determined by Student’s t test. Means ± st are given (n = 7), for the replicates at 50°C. rel. u., Relative units.
The half-times were obtained by fitting the decay phase with single exponential decay functions. Asterisks (* (Fig. 5, C and D).

than in isoprene-inhibited leaves of the two plants reduced by the heat treatment, but significantly less (Fig. 5, A and B); the intensity of the B-band peak was B-band peak emission temperature was unchanged leaves (Fig. 5, A and B); the intensity of the B-band peak was reduced by the heat treatment, but significantly less than in isoprene-inhibited leaves of the two plants (Fig. 5, C and D).

DISCUSSION

CD

CD measures the difference in the extinction of left-handed versus right-handed circularly polarized light. CD spectroscopy is used to study chiral molecules and their assemblies of all types and sizes, and it is particularly important in the study of hierarchically organized biological samples. A primary use is in analyzing the secondary structure or conformation of macromolecules, particularly proteins, and because secondary structure is sensitive to its environment, e.g. temperature or pH. In plants, CD spectroscopy in the visible range originates from pigment-pigment interactions and hence is a valuable tool for probing the molecular architecture of the photosynthetic complexes and supercomplexes and their macroorganization in the membrane system (Garab, 1996; Garab and van Amerongen, 2009). CD can originate from different levels of complexity in the sample. Short-range (excitonic) interactions are observed for pigment-pigment interactions within one pigment-protein complex or in supercomplexes between molecules on adjacent complexes. In leaves, characteristic excitonic CD bands are typically observed around (+)440 and (-)650 nm, originating from PSI and light-harvesting complex II (LHCl), respectively. Additionally the band pair (+) 482/(-)470 nm has been shown to correlate with the trimeric organization of LHCl (Garab et al., 2002).

Long-range (polymer- or salt-induced or $\Psi$-type) interactions are due to pigment interactions in densely packed chirally organized macroaggregates with dimensions of hundreds of nanometers, membrane domains, also called chiral macromdomains. $\Psi$-Type aggregates are abundant in biological systems; e.g. nuclei, chromosomes, viruses, and numerous other highly organized systems. In these biological systems, $\Psi$-type CD signals are determined by the size, handedness, and strength of long-range coupling of the

Table 1. Half-times (t1/2, ms) of the decay kinetics of the flash-induced electrochromic absorbance changes at 515 nm in detached Arabidopsis and P. orientalis leaves

<table>
<thead>
<tr>
<th>Arabidopsis</th>
<th>Wild Type</th>
<th>P. orientalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IspS</td>
<td>Isoprene Emitting</td>
<td>Isoprene Inhibited</td>
</tr>
<tr>
<td>20°C</td>
<td>25°C</td>
<td>20°C</td>
</tr>
<tr>
<td>57.8 ± 6.5</td>
<td>83.3 ± 5.4</td>
<td>65.2 ± 2.4</td>
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TL measurements were compared on leaf discs that were maintained at 25°C and on leaf discs incubated at 40°C for 5 min. The effect of the heat treatment on B-band peak temperature and intensity is shown in Figure 5. The heat treatment of wild-type Arabidopsis and isoprene-inhibited Platanus leaves reduced the B-band peak emission temperature (Fig. 5, A and B) and intensity (Fig. 5, C and D). In isoprene-emitting leaves (IspS and isoprene-emitting Platanus leaves) the B-band peak emission temperature was unchanged (Fig. 5, A and B); the intensity of the B-band peak was reduced by the heat treatment, but significantly less than in isoprene-inhibited leaves of the two plants (Fig. 5, C and D).

Figure 4. TL curves of wild-type (A) and IspS (C) Arabidopsis leaves and of isoprene-inhibited (B) and isoprene-emitting (D) Platanus leaves after illumination with one or two single-turnover flashes of white saturating light at 1°C. TL was measured during heating of the samples to 70°C in darkness with a ramp of temperature at constant rate of 0.6°C s-1. Curves are representative of measurements on 10 leaves per treatment from different plants. a.u., Arbitrary units.
chromophores (Keller and Bustamante, 1986). In thylakoids the CD bands at (−)675 and (+)694 nm that posses such Ψ-type features are usually correlated with the macroorganization of LHCII (Garab and Mustárdy, 1999) and with the presence of ordered arrays of LHCII-PSII supercomplexes, as shown also by advanced electron microscopy (Kovács et al., 2006).

CD can be used to observe how secondary structure of proteins changes with environmental conditions or on interaction with other molecules. In fact, our CD results show that the heat stability of the chiral macrodomains in the grana, i.e. of the ordered arrays of LHCII-PSII in the stacked regions, was positively affected by the presence of isoprene. Probably, in the IspS leaves, isoprene significantly modifies the organization of the pigment-protein complexes in the thylakoid membranes. At physiological temperatures (20°C), however, the Ψ-type CD at (+)694 nm was increased in IspS leaves. This last Ψ-type CD feature might indicate the presence of better-ordered arrays of LHCII-PSII supercomplexes or of LHCII macrodomains. This interesting observation requires further investigations since it might indicate a modified two-dimensional organization of the LHCII-PSII supercomplexes in the stacked membranes of plants that are transformed to emit isoprene (compare with Kovács et al., 2006). In contrast, Platanus leaves in which isoprene emission was chemically inhibited did not show changes of (+)694 nm CD band with respect to isoprene-emitting leaves at physiological temperatures (25°C). This indicates that the inhibition of isoprene biosynthesis does not affect the macroorganization of the photosynthetic complexes that have been formed when isoprene was actively synthesized during the ontogeny of leaves, or that the small amount of isoprene still being made by chemically inhibited Platanus leaves was enough to stabilize the LHCII-PSII supercomplexes revealed by the (+)694 nm band.

CD spectroscopy is also a useful tool for probing the thermal stability of the macrodomains formed by the photosynthetic complexes in the membranes (Garab et al., 2002; Dobrikova et al., 2003). Our data demonstrated that modified membrane structure in IspS leaves is related to the increased thermostability of the supercomplexes in isoprene-transformed Arabidopsis plants, and that thermal stability of the chiral macrodomains in the thylakoid membranes is higher in isoprene-emitting Platanus leaves compared to isoprene-inhibited leaves. Under increasing temperatures, the transition temperature that revealed the disassembly of the protein macrodomain constituted by the LHCII-PSII supramolecular organization (as monitored by the (+)694 nm CD band), was about 8°C higher in Isp5 Arabidopsis and isoprene-emitting Platanus leaves compared to the plants that do not emit isoprene. In general, heat- or light-induced reorganizations occur mostly at the supramolecular level of structural complexity, rather than at the level of the building

Figure 5. TL B-band (S2QB) peak temperature (A and B) and TL maximum intensity (C and D) in wild-type and IspS Arabidopsis (A and C) and in isoprene-emitting and isoprene-inhibited Platanus leaves (B and D). The measurements were done after a 5-min incubation at 25°C (white bars) or 40°C (gray bars). Values are means of five different leaves exposed to each treatment ± se. The significant differences between means at different temperatures were determined by Student’s t test. Means significantly different at P < 0.05 and 0.01 confidence levels are labeled by one or two asterisks, respectively. a.u., Arbitrary units.
blocks of macroassemblies. For example, the stability of the single constituents of pigment-protein complexes has been shown to be considerably higher than the stability of their macroassemblies (Cseh et al., 2000, 2005; Dobrikova et al., 2003). The experiment using CD spectroscopy therefore confirms that a higher thermal stability is conferred by isoprene to thylakoid membranes, and suggests that this might be due to a sustained stability of the ordered arrays of supercomplexes in the thylakoid membranes, rather than to a specifically induced resistance in a single photosystem component.

It was shown that the structural flexibility of LHCII macrodomains depends largely on the lipid environment of the complexes (Simidjiev et al., 1998). The positive stabilizing effect of isoprene on membranes could therefore be related to changes in the lipid phase and/or alterations induced by isoprene in the phase behavior of lipids. The mixed lipid phases can have significant effects on the organization of thylakoid membranes (Williams and Quinn, 1987). Lateral organization of membranes into domains can depend critically on temperature and small molecules have been found to significantly affect large-scale phase separations. Molecules that affect the miscibility of different phases will significantly alter the stability of different membrane domains under heat stress (Veatch, 2007). As under physiological conditions the CD spectrum at (+)694 nm is similar in isoprene-emitting and isoprene-inhibited Platanus leaves, we infer that the chemical inhibition of isoprene does not alter the macromolecular structure of the membranes. If isoprene simply alters the lipid phase of membranes, then the protective effect may be more rapidly lost after isoprene inhibition. This would explain why the protective effect of isoprene is seen on both Arabidopsis ispS plants and in isoprene-emitting Platanus under heat stress, independently of the stability of the LHCII-PSII supramolecular organization and of the way isoprene emission is manipulated. Recent data have shown that the dgd1 Arabidopsis mutant, which is deficient in the bilayer lipid digalatosyldiacylglycerol and enriched in the nonbilayer monogalactosyldiacylglycerol, display considerably lower thermal stability of the chiral macrodomains, measured with the temperature dependence of the main Ψ-type CD bands (Krumova et al., 2010).

In whole leaves and intact granal chloroplasts, usually the Ψ-type bands are dominating the spectra and the much weaker excitonic bands become visible only upon disintegration of the macrodomains, e.g. at hypotonic low-salt medium (Garab et al., 1991) or at elevated temperatures (Cseh et al., 2000). Interestingly, the capacity to emit isoprene does not seem to affect significantly the excitonic (non-Ψ-type) bands arising from supercomplexes as also monitored by CD spectroscopy. Namely, the trimer-to-monomer transition temperatures of LHCII, between 55°C and 60°C, as tested with the excitonic band pair (+)482/(-)470 (Garab et al., 2002) was unchanged (data not shown), indicating that the trimeric LHCII is resistant to heat independently of isoprene presence, in both tested species.

Electrochromic Shift and Membrane Conductance

Electrochromic absorbance changes at 515 nm (ΔA315) are used to monitor ion permeability of membranes (Junge, 1977; Witt, 1979; Peters et al., 1984) because of the effect on the electrical potential across the thylakoid membranes. In particular, the decay kinetics of the absorbance changes are proportional to the ion flux across the thylakoid membranes and are sensitive indicators of thylakoid membrane intactness (Peters et al., 1984), i.e. of the ability of membranes to maintain the light-induced transmembrane electric field. In dark-adapted leaves the ATP synthase is turned off and so membrane effects are more prominent than in light-adapted leaves. The decay is slower in dark-adapted leaves than light-adapted leaves but is variable among species. Therefore, the absolute decay rate cannot be compared among species but the decay for one species can be compared under different conditions such as moderate or high temperature and with or without isoprene present.

Increased permeability has been found to occur particularly at high temperatures, concurrently with denaturation of membrane lipids (Bukhov et al., 1999; Schrader et al., 2004; Zhang and Sharkey, 2009; Zhang et al., 2009). The increased membrane permeability of heat-treated samples can be due to conformational changes of membrane lipids, opening of ion channels, or changes in lipid-lipid interactions (Santarius, 1980). At moderate (20°C–25°C) temperature, the thylakoid membranes exhibit similar electrochromic absorbance changes at 515 nm (ΔA315), in spite of the presence (ispS Arabidopsis, and control water-fed Platanus leaves) or absence (wild-type Arabidopsis, and fosmidomycin-fed Platanus leaves) of isoprene. Thus, the membrane permeability to ions is low and is not affected by isoprene presence at growth temperature. However, at high temperature (40°C–45°C) wild-type Arabidopsis membranes, and isoprene-inhibited Platanus membranes became more permeable to ions, whereas isoprene-emitting Platanus membranes were less affected, and ispS membranes remained unaffected. Thus, isoprene presence is associated with better membrane integrity under heat-stress conditions, as shown by lower ion leakage at high temperatures.

TL

TL measurements also support the thermotolerance hypothesis, demonstrating the protective role of isoprene in increasing the membrane thermostability. TL is a sensitive and reliable tool for monitoring the functionality of PSII donor and acceptor side components (Sane and Rutherford, 1986; Vass and Inoue, 1992; Ducruet, 2003; Sane, 2004). Illumination of dark-adapted samples with single-turnover flashes generates charge pairs within PSII reaction centers that are
energetically stabilized on the primary and secondary quinone acceptors of the chloroplast’s electron transport chain. The samples are rapidly cooled down to trap separated charges that, on heating, yield a TL light emission at characteristic temperatures (for details, see Sane, 2004). Two major TL bands, B and Q, appear in TL curves as a result of the recombination of the trapped electrons and stabilized positive holes on the reduced quinone acceptors (QA or QB) and on the S2 (or S1) oxidation state of the water-splitting enzyme. The B band is generated by the recombination of QB- with S2 state of the water-splitting complex. Even small changes in the redox properties of the radical pairs affect the intensity and the peak position of TL bands. This complexity of information of TL emission curves is widely used for detection of structural changes in both the donor and acceptor side of PSII.

We tested with TL measurements the impact of isoprene presence in leaves that were exposed to physiological or high temperatures before running the assay. In our experiment with Arabidopsis plants exposed to physiological temperatures, the peak position of the B band was up shifted by about 10°C in IspS leaves, compared to wild-type leaves. Alterations in the peak positions of a TL band indicates changes in the stability, redox potential, or activation energy of the recombining charge pairs, while the peak amplitude is proportional to the concentration of the corresponding charge pairs (Rutherford et al., 1984). The observed stabilization of the charge separation state between the redox components on PSII donor and acceptor side in IspS Arabidopsis clearly indicates an isoprene-induced modification that may enable plants to perform efficient primary photochemistry of PSII at higher temperatures. In Platanus leaves exposed to physiological temperatures, however, the B band was not shifted to lower temperatures when isoprene was chemically inhibited, as we would have expected. As in the case of CD spectroscopy, we attribute this result to the possibility that the chemical inhibition of isoprene does not interfere with the molecular structure of membrane architecture that was assembled when isoprene was still available. Specifically, our results might indicate that the isoprene-related membrane modulation affecting PSII reaction centers, and generating the shift in the energy levels of the S2QB, is a long-term, structural effect that cannot be altered by fast removal of isoprene. Alternatively, however, the low rate of isoprene synthesis in the fosmidomycin-fed Platanus leaves might be sufficient to stabilize charge separation. In any case, a change in TL peak position is not directly associated with the presence of isoprene under physiological conditions.

When TL measurements were repeated in samples previously exposed to heat stress, the B band clearly peaked at a lower temperature in plants that do not emit isoprene, whereas the B-band peak position was not affected in isoprene-emitting leaves of both Arabidopsis and Platanus. Absence of isoprene caused a strong reduction of the intensity of the B band in both plant species that were subjected to the heat treatment. It has been shown that the peak temperature of B band increases in stress-tolerant photosynthetic organisms; for example in thermophilic cyanobacteria or desiccation-tolerant species (Govindjee et al., 1985; Sass et al., 1996; Maslenkova and Homann, 2000; Peeva and Maslenkova, 2004). It has also been shown that stresses do not affect the energetic levels of the stabilized S2QB- charge pairs in stress-tolerant plants (Sass et al., 1996; Georgieva et al., 2005).

It is of interest that the heat stress also affected TL parameters in isoprene-inhibited Platanus leaves that did not show TL changes at physiological temperatures. However, the TL intensity of the B band in isoprene-inhibited Platanus leaves was less affected after heat treatment, than in wild-type Arabidopsis that constitutively do not emit isoprene. We interpret this result as showing that a continuous biosynthesis of isoprene is needed to maintain membrane stability under heat stress, whereas the residual isoprene that is likely stabilizing membranes under physiological temperatures in isoprene-inhibited Platanus (see above) may only partially fulfill this role under elevated temperatures.

Since the production of isoprene is strongly stimulated at elevated temperatures in the range of 30°C to 40°C (Monson et al., 1992; Singsaas and Sharkey, 2000), we suggest that isoprene emission represents an adaptive mechanism of membrane stabilization under high temperatures. Isoprene is highly lipophilic so that it may partition into chloroplast membranes and therefore it could act within the thylakoid lipid phase by rendering membranes more viscous and changing the features of secondary electron acceptor QA. It was shown that this plastoquinone molecule is the most sensitive to the lipid environment (Gombos et al., 2002). However, this does not rule out other volatile isoprenoids (e.g. monoterpenes, sesquiterpenes), zeaxanthin synthesis, accumulation of soluble thermoprotectants such as sugars or free amino acids, or changes in membrane lipid composition involvement in membrane stabilization (Ducruet et al., 2007).

CONCLUSION

There currently is a controversy about the mechanism of action of isoprene (Loreto and Schnitzler, 2010). Vickers et al. (2009b) suggests that the mechanism could be interaction/quenching of reactive oxygen species that allows plants to tolerate heat and oxidative stress. Sharkey et al. (2008) suggest that isoprene may affect membrane organization and function and that this allows plants to tolerate heat and reduces the formation of reactive oxygen. The molecular dynamics modeling of Siwko et al. (2007) and the octanol/water partitioning (Copolovici and Niinemets, 2005) show that isoprene will accumulate inside membranes but until now there were no data showing that this could affect biophysical signals of membrane function.
Three biophysical techniques, which are often used to study thylakoid membrane function, show that isoprene can extend thylakoid membrane functionality to higher temperature while having little or no effect at moderate temperature. While the three techniques specifically investigated different aspects of the photochemical apparatus, they converge in indicating that isoprene improves the integrity of the photochemistry of photosynthesis under heat-stress conditions. The CD spectra in particular show that isoprene can affect large-scale thylakoid membrane organization while not affecting the short-range interactions that arise from pigment-protein complexes and their supercomplexes.

As more is learned about how heat affects photosynthetic function and what mechanisms improve thermotolerance it will be possible to interpret these biophysical measures of isoprene effects in terms of improved thermotolerance and ultimately in terms of the evolutionary pressure that favors isoprene emission from plants in some conditions. These experiments show that there are biophysical mechanisms by which the integrity and functionality of the thylakoid membranes are better preserved at high temperature in the presence of isoprene. This strongly supports the idea that isoprene improves thermotolerance of plants (Sharkey and Singsaas, 1995; Loreto and Schnitzler, 2010) and favors effects on membrane function as the reason. This suggests that isoprene-emitting plants will cope better with temperature extremes that may become more frequent in the near future due to current climate change (e.g. De Boeck et al., 2011).

**Plant Material and Growth Conditions**

Wild-type Arabidopsis (*Arabidopsis thaliana*; ecotype Wassilewskija), which does not naturally emit isoprene (wild type), and genetically modified Arabidopsis plants (*ispS*) producing isoprene as a natural metabolite were used in this study. Arabidopsis *ispS* plants were transformed inserting the kudzu (*Pueraria lobata*) ISPS genomic sequence under the control of a constitutive promoter (Sharkey et al., 2009). The T4 generation was used for the experiments. Plants were grown in a climate chamber (Percival Scientific) under a photoperiod of 8 h light at 22°C and 16 h dark at 20°C. Light intensity was 150 μmol photons m⁻² s⁻¹ and relative humidity was 50% to 60%. Fully grown rosettes were used for the experiments.

Three-year-old Platanus (*Platanus orientalis*) plants were grown in 5-L pots with sand and peat-based commercial soil (1:1) in a greenhouse under controlled environmental factors. In particular the greenhouse was thermostated with air conditioning modules and the light intensity was supplemented, if needed, with artificial lamps characterized by a solar spectrum (Osram PowerStar HQI-TS 150W). Growth temperature was 25°C/22°C (day/night), daily light intensity was 350 μmol photons m⁻² s⁻¹, with a 12-h photoperiod, and relative humidity was maintained around 65%. The experiments were performed on fully expanded leaves.

Plants were regularly watered to pot water capacity and fertilized once a week with full-strength Hoagland solution.

Fosmidomycin and High-Temperature Treatments of *Platanus* Leaves

To inhibit isoprene emission from *Platanus* leaves, fosmidomycin, a specific inhibitor of 2-deoxysylulose 5-phosphate reductoisomerase (Zeidler et al., 1998), was used. *Platanus* leaves were cut under water and maintained with the petiole in a glass vial with 15 ml of distilled water for 1 h. Fosmidomycin (5 μM) was then added to the water and taken up by the leaf through transpiration stream. After 1 h of incubation isoprene emission was inhibited to approximately 10% of the original level, as monitored by gas exchange and gas chromatography (Loreto and Velikova, 2001). Cut leaves with the petiole immersed in a vial with distilled water only were used as a control (isoprene-emitting leaves). Isoprene-emitting and isoprene-inhibited leaves were used for all measurements.

**Measurements**

**Gas-Exchange and Isoprene Emission Measurements**

CO₂ and water exchange were measured by using a LI-7000 infrared gas analyzer (LI-COR). Two entire Arabidopsis plants were placed in a 1.7-L glass cuvette. Plants were exposed to synthetic air made by mixing O₂, N₂, and CO₂ from cylinders deprived of contaminants. The absence of contamination by trace gases that could interfere with isoprene measurements was tested every time a cylinder was changed, using the spectrometric analysis outlined below.

Atmospheric concentrations of the three gases (20%, 80%, and 390 μmol mol⁻¹, respectively) were set with mass flow controllers. The air flow was set at 2 L min⁻¹. During gas-exchange measurements leaf temperature, light intensity, and relative humidity were maintained at constant levels close to growth conditions (22°C, 150 μmol m⁻² s⁻¹, and 56%, respectively).

Steady-state photosynthesis, stomatal conductance, and transpiration in *Platanus* leaves were measured by a portable gas-exchange system (LI-6400) equipped with a 6-cm² cuvette. Measurements were made in ambient CO₂ concentration (390 μmol mol⁻¹) on individual leaves enclosed into a leaf cuvette under a rate of 0.44 L min⁻¹ air flow, relative humidity within the cuvette at 50% to 55%, a leaf temperature of 25°C, and 500 μmol photons m⁻² s⁻¹ light intensity.

Isoprene emission was measured on both Arabidopsis and *Platanus* plants online, by diverting the air at the exit of the gas-exchange cuvettes into a proton transfer reaction-mass spectrometer (Ionicon). Details on isoprene analysis by proton transfer reaction-mass spectrometer can be found in Tholl et al. (2006).

Data represent means ± s.e. of measurements on seven different plants. The significant differences between means were determined by Student’s t test. Means significantly different at P < 0.05 and 0.01 confidence levels are labeled by one or two asterisks, respectively.

**CD Spectroscopy**

CD was measured in a JobinYvon CD6 dichrograph (JobinYvon ISA) equipped with a thermostated sample holder on Arabidopsis leaves, and with Jasco 815 dichrograph equipped with Peltier sample holder on *Platanus* leaves. The spectra were recorded between 400 and 750 nm in 1-nm steps with an integration time of 0.3 s and a band pass of 2 nm. For the measurements of thermal stability, Arabidopsis or *Platanus* leaves were placed in the sample holder and were dark adapted for 10 min at 20°C. Then the temperature was gradually increased up to 80°C (60°C in Arabidopsis due to higher thermal sensitivity of this plant) in 5°C steps. The leaves were incubated for 10 min before measurements at each temperature. The transition temperature (Tt) is the temperature at which the intensity of the CD band is decreased by 50% of its value at 25°C (Cseh et al., 2000). The transition temperatures for the main Φ-type CD band at around (+)694 nm were calculated from the inflection point of the fitted curve of the temperature dependence of the CD amplitude. The experiment was repeated on seven leaves of different plants per treatment, on each one of the two species. The mean transition temperatures of isoprene-emitting and nonemitting leaves were separated by Student’s t test and positively tested against a P < 0.01. The spectra were corrected for a flat baseline obtained by setting the CD values at 400 and 750 nm to zero.

**Electrochromic Absorbance Changes (ΔA515)**

Electrochromic absorbance changes (ΔA515, also named electrochromic shift) induced by saturating single-turnover flashes were measured at 515 nm on detached leaves in a setup described earlier (Horváth et al., 1979; Barábás et al., 1985; Büchel and Garab, 1995). Briefly, the single-beam kinetic spectrophotometer consists of a tungsten light source, a grating monochromator, and
a photomultiplier with an optical filter for the blue spectral region, and is equipped with two xenon lamps (Strobosilave, General Electric) providing red flashes of 3 μs half-duration. For recording the signal and data acquisition, a differential amplifier and a digital averaging storage scope (Tektronix AM502 and 2224, respectively) are used in combination with a home-built timer unit and a personal computer. The Arabidopsis plants used for these measurements were dark adapted at 20°C for 20 min. Then, detached wild-type and csp5 leaves were incubated for 5 min either at 20°C or 40°C in the dark, and thereafter measured at 20°C. In *Platanus*, control (isoprene-emitting) and fosmidomycin-treated (isoprene-inhibited) leaves were exposed to 25°C or 45°C for 4 h in weak white light (40 μmol photons m⁻² s⁻¹). The treatment with *Platanus* leaves was much longer than in the case of Arabidopsis and was carried out in dim light to allow complete infiltration of fosmidomycin in the leaves. In the last 20 min of the heat treatment, the light was turned off to ensure the dark-adapted state of the leaves. Kinetic traces (*n* = 64) were collected with a repetition rate of 1 s⁻¹, and averaged; the time constant of the measurements was adjusted to 100 μs. The measurements were repeated at least six times on different leaves. The half-times of the decay were obtained by fitting the curves with single exponential decay functions.

**TL**

TL was detected with a photomultiplier tube (Hamamatsu R943-02, Hamamatsu Photonics) linked to an amplifier in a home-built apparatus. A detailed description of the block diagram of the TL equipment, handling, temperature regulation, personal computer-based TL data acquisition, and graphical simulation is in Zeinalov and Maslenkova (1996). After a 30-min dark incubation at room temperature leaf segments were placed on a sample holder and covered with a thin plastic plate. TL was excited by one or two saturating (4) single-turnover flashes (approximately 10 μs half-band with 1-Hz frequency) given at 1°C. After the flash exposure, the sample from Arabidopsis leaves was quickly cooled to −50°C with the aid of a metal block cooled by liquid nitrogen before being warmed to 70°C with 0.6°C/s heating rate. TL emission from *Platanus* leaves was recorded similarly, but samples were not frozen before warming from 0°C to 70°C. This way the distortion of the TL signal during freezing that was observed in *Platanus* as well as in some other species (Homann, 1999), was avoided. The nomenclature of Vass and Govindjee (1996) was used for characterization of the main TL curve peaks. TL B-peak parameters (temperature positions and amplitude; Fig. 5) were recorded following the same procedure as outlined above. Means and SEs for each plant species. TL curves shown in Figure 4 are representative of 10 measurements on leaves from different plants. In another experiment, Arabidopsis and *Platanus* leaves were incubated for 5 min at 25°C or 40°C before TL measurements. TL emission was then recorded following the same procedure as outlined above. Means and SEs for TL B-peak parameters (temperature positions and amplitude; Fig. 5) were calculated from five replicates of each nontreated and high-temperature-treated leaf samples from different plants. The significant differences between means at different temperatures were determined by Student’s *t* test. Means significantly different at *P* < 0.05 and 0.01 confidence levels are labeled by one or two asterisks, respectively. Analysis of the TL curves was carried out using a Microcal Origin v.6.0 software package (Microcal Software).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Representative traces of electrochromic absorbance changes measured at 515 nm in isoprene-emitting and nonemitting leaves.

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**LITERATURE CITED**


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