# Evidence That Light, Carbon Dioxide, and Oxygen Dependencies of Leaf Isoprene Emission Are Driven by Energy Status in Hybrid Aspen[1]

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Leaf isoprene emission scales positively with light intensity, is inhibited by high carbon dioxide ($CO_2$) concentrations, and may be enhanced or inhibited by low oxygen ($O_2$) concentrations, but the mechanisms of environmental regulation of isoprene emission are still not fully understood. Emission controls by isoprene synthase, availability of carbon intermediates, or energetic cofactors have been suggested previously. In this study, we asked whether the short-term (tens of minutes) environmental control of isoprene synthesis results from alterations in the immediate isoprene precursor dimethylallyldiphosphate (DMADP) pool size, and to what extent DMADP concentrations are affected by the supply of carbon and energetic metabolites. A novel in vivo method based on postillumination isoprene release was employed to measure the pool size of DMADP simultaneously with the rates of isoprene emission and net assimilation at different light intensities and $CO_2$ and $O_2$ concentrations. Both net assimilation and isoprene emission rates increased hyperbolically with light intensity. The photosynthetic response to $CO_2$ concentration was also hyperbolic, while the $CO_2$ response curve of isoprene emission exhibited a maximum at close to $CO_2$ compensation point. Low $O_2$ positively affected both net assimilation and isoprene emission. In all cases, the variation in isoprene emission was matched with changes in DMADP pool size. The results of these experiments suggest that DMADP pool size controls the response of isoprene emission to light intensity and to $CO_2$ and $O_2$ concentrations and that the pool size is determined by the level of energetic metabolites generated in photosynthesis.

Plants interact with the atmosphere mainly through carbon dioxide ($CO_2$), oxygen ($O_2$), and water fluxes but also through various trace gas fluxes. Plant-generated volatile organic compounds are significant players in tropospheric photochemistry (Trainer et al., 1987; Monson et al., 1991), catalyzing the synthesis of ozone and other oxidants as well as prolonging the lifetime of the greenhouse gas methane (Monson et al., 2007). Plant emissions of isoprene are estimated worldwide to be the largest source of biogenic volatile organic compound emissions (Guenther et al., 2006; Arneth et al., 2008). Therefore, predicting plant isoprene fluxes is of key relevance in simulating atmospheric reactivity.

Apart from the atmospheric significance, isoprene plays an important role in protecting plants from heat and oxidative stresses (Fang et al., 1996; Litvak et al., 1996; Sharkey, 1996; Sharkey et al., 1996; Loreto et al., 2001; Loreto and Velikova, 2001). As only some specific plant species are able to produce isoprene, the capacity of plants to make isoprene can alter plant competitive relations under heat and ozone stresses (Lerdau, 2007; Darbah et al., 2008). It has further been suggested that the isoprenoid synthesis pathway can act as a metabolic protection valve for dissipation of excess assimilatory and reductive power (Fall, 1999; Rosenstiel et al., 2004; Sanadze, 2004; Magel et al., 2006). This information collectively emphasizes the need to gain mechanistic insight into the determinants of isoprene emission.

Despite the rich experimental evidence accumulated over several decades, there is still no general agreement about the mechanisms controlling the rate of isoprene synthesis under different environmental conditions. At present, the models of isoprene emission include a great deal of empiricism (Guenther et al., 1993; Monson et al., 2007; Grote and Niinemets, 2008; Wilkinson et al., 2009), and although they provide numerically good fits to the data, the capacity of these models to predict isoprene fluxes in future conditions and under stress is uncertain (Monson et al., 2007; Arneth et al., 2008). Isoprene emission rate in the emitting species depends on temperature, light intensity, and the concentrations of $CO_2$ and $O_2$ (Sanadze, 1969, 1990; Sanadze and Tarhnishvili, 1986; Monson and Fall, 1989; Loreto and Sharkey, 1990; Monson et al., 1991), but there is no consensus on what drives these dependencies.

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Early discoveries of the light dependence of isoprene emission (Sanadze, 1969), the rapid appearance of $^{13}C$ label from $^{13}C$-labeled $CO_2$ in the isoprene signal (Sanadze et al., 1972; Mgalobilishvili et al., 1978), and the observation that isolated chloroplasts are capable of isoprene emission (Mgalobilishvili et al., 1978) have provided conclusive evidence of functional interdependence between photosynthesis and isoprene emission. Further studies have shown strong positive relationships between the photosynthetic carbon assimilation and isoprene synthesis mediated by photosynthetic metabolites that provide carbon skeletons for and photosynthetic ATP and NADPH energetically supporting the chloroplastic 2-C-methyl-erythritol 4-phosphate (MEP) pathway (Loreto and Sharkey, 1990, 1993; Eisenreich et al., 2001; Sharkey and Yeh, 2001). However, after the discovery of isoprene synthase (IspS) catalyzing the formation of isoprene from its immediate precursor dimethylallyldiphosphate (DMADP), light regulation of isoprene synthase activity has been hypothesized to be responsible for the light dependence of isoprene emission (Silver and Fall, 1991; Fall and Wildermuth, 1998; Wildermuth and Fall, 1998; Logan et al., 2000). At present, there is no consensus on whether the light response of isoprene emission is driven by changes in the activity of isoprene synthase (Wiberley et al., 2008) or by the availability of energetic cofactors and carbon intermediates that determine the availability of DMADP for IspS (Monson and Fall, 1989; Rosenstiel et al., 2002).

Another key uncertainty is what drives the $CO_2$ response of isoprene emission. While net assimilation rate scales positively with the $CO_2$ concentration, high $CO_2$ concentrations inhibit isoprene release (Monson and Fall, 1989; Loreto and Sharkey, 1990). This has been hypothesized to reflect inhibition of the activity of IspS or another enzyme of the MEP pathway (Potosnak, 2002). However, DMADP pool size varies throughout the $CO_2$ response of isoprene emission (Rosenstiel et al., 2003). Based on this variation, the alternative hypothesis suggests that transport of the intermediate of the MEP pathway, phospho*enol*pyruvate (PEP), from cytosol becomes increasingly limited at elevated $CO_2$ due to enhanced cytosolic PEP carboxylation at higher $CO_2$ concentrations (Rosenstiel et al., 2003, 2004, 2006; Wilkinson et al., 2009). In fact, a negative correlation between PEP carboxylase activity and isoprene emission rate has been observed across a variety of environmental treatments (Loreto et al., 2007). Finally, it has been suggested that high $CO_2$ concentrations inhibit isoprene emission due to limited availability of ATP for isoprene synthesis (Loreto and Sharkey, 1990, 1993; Sharkey et al., 1991b). Provided that the effective $K_m$ for ATP of the isoprenoid synthesis pathway is large, isoprenoid synthesis at high $CO_2$ may become limited as a result of a drawdown of leaf ATP level. Such a drawdown commonly occurs due to increased consumption of ATP for carbon reduction that is typically limited by electron transport activity at higher $CO_2$ (Farquhar et al., 1980) and because of sequestration of chloroplastic phosphate into sugar phosphates, thereby feedback inhibiting photosynthetic electron transport (Loreto and Sharkey, 1990, 1993; Sharkey et al., 1991b).

The least studied and most controversial is the influence of $O_2$ on isoprene emission. Although mechanistically important, there has been little interest in $O_2$ effects because of only moderate variations in its atmospheric concentration during and between seasons (Keeling and Shertz, 1992) and in the recent geological past (Kump, 2008). Among the available studies on $O_2$ effects, some have reported the increase of isoprene emission under low $O_2$ of 1.5% to 2% relative to the ambient level of 21% (Sanadze, 1966; Sanadze and Tarhnishvili, 1986; Hewitt et al., 1990). In other studies, isoprene emission was either enhanced or reduced by $O_2$ concentration in dependence on ambient $CO_2$ concentration (Loreto and Sharkey, 1990, 1993). Finally, short-term stimulation and long-term (>40–60 min) inhibition of isoprene emission by low $O_2$ have been reported (Monson and Fall, 1989). As $O_2$ importantly alters the share of the photosynthetic limitations between electron transport and carbon input, an analysis of $O_2$ dependence can provide fundamental information on the key limitations of isoprene emission.

The controversies in the literature partly arise from the circumstance that it has been difficult to estimate the intermediates of the isoprene emission pathway simultaneously with foliage physiological characteristics. We have previously developed a novel in vivo method for the measurement of the immediate isoprene precursor DMADP pool responsible for isoprene emission (presumably the chloroplastic pool) by integrating the postillumination isoprene emission (Rasulov et al., 2009) that overcomes this difficulty. In this study, we combine the measurements of foliage photosynthetic traits, isoprene emission, and DMADP pool size at different light intensities and $CO_2$ and $O_2$ concentrations in hybrid aspen (*Populus tremula* × *P. tremuloides*) to ask (1) whether the alterations in isoprene synthase activity or in DMADP pool size are responsible for the short-term controls by these driving variables on isoprene emission, and (2) to what extent substrate concentrations are affected by the emission rate and the supply of carbon and energetic metabolites? The results of these experiments suggest that the variation in the DMADP pool size controls the response of isoprene emission to light intensity and $CO_2$ and $O_2$ concentrations and that the pool size is determined by the level of energetic metabolites (ATP) generated in photosynthesis.

## RESULTS

### Light Responses of Net Assimilation, Isoprene Emission, and DMADP Pool Size

Both the rate of isoprene emission and net assimilation scaled curvilinearly with quantum flux density

($Q$), but net assimilation rate saturated at lower $Q$ than isoprene emission (Fig. 1). Fitting the data by Equation 1 suggested that the isoprene emission rate at the highest $Q$ achieved by the system (550 $\mu$mol m$^{-2}$ s$^{-1}$) was approximately 70% of the light-saturated value and the net assimilation rate was 85% of the light-saturated value, while the isoprene emission rate was 80% and the net assimilation rate was 95% of the rates predicted at $Q$ = 1,000 $\mu$mol m$^{-2}$ s$^{-1}$. The pool size of DMADP measured kinetically as an integral of the postillumination isoprene emission at each light intensity (Rasulov et al., 2009) increased in parallel with the rate of isoprene emission (Fig. 2). This relationship had an initial slope (DMADP pool range of 150–500 nmol m$^{-2}$) of 0.034 s$^{-1}$ (the maximum turnover rate of the DMADP pool) and the slope was 0.021 s$^{-1}$ for higher DMADP pool sizes (600–1,000 nmol m$^{-2}$), demonstrating that this dependence was nearly linear.

Temporal kinetics of dark/light activation of foliage physiological characteristics were studied after a 10-h dark period (Fig. 3A). Fitting the induction data by a single-exponential model (Eq. 2) indicated that net assimilation was induced with the fastest rate ($k_1$ = 0.077 min$^{-1}$), followed by stomata ($k_1$ = 0.042 min$^{-1}$ for the transpiration rate) and isoprene emission ($k_1$ = 0.036 min$^{-1}$; Fig. 3B). However, the induction of isoprene emission was delayed for about 5 min. In addition, the induction of isoprene emission continued for more than 80 min, while the rates of transpiration and net assimilation reached a saturation in approximately 30 min after switching on the light. In fact, a biphasic, double-exponential model (Eq. 3) better fitted the isoprene emission data (Fig. 3B; $r^2$ = 0.99) than the monophasic, single-exponential model (Fig. 3B; $r^2$ = 0.96).

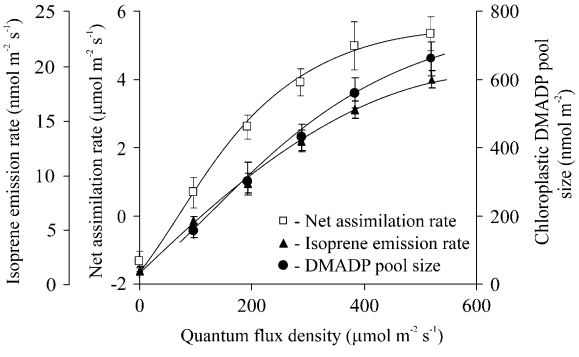


**Figure 1.** Dependencies of the rates of isoprene emission and net assimilation, and the chloroplastic DMADP pool size, on incident photosynthetic quantum flux density in hybrid aspen (clone 200) leaves. The measurements were carried out at an ambient $CO_2$ concentration of 390 $\mu$mol mol$^{-1}$, $O_2$ concentration of 210 mmol mol$^{-1}$, and leaf temperature of 28°C to 30°C. An in vivo method based on the postillumination isoprene release was used to determine the chloroplastic DMADP pool size concomitant with leaf physiological measurements (Rasulov et al., 2009). Error bars show SD ($n$ = 5). Data were fitted by Equation 1 ($r^2 > 0.98$, $P < 0.001$ for all).

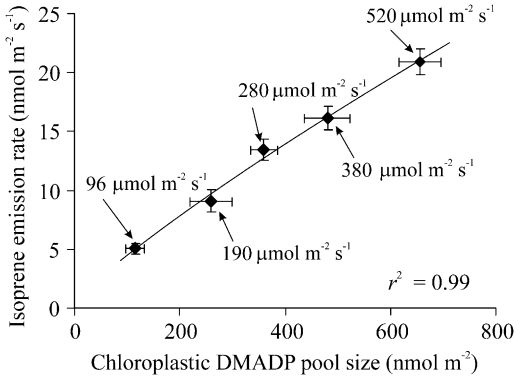


**Figure 2.** Correlation between the isoprene emission rate and the chloroplastic DMADP pool size (the same data as in Fig. 1). Data were fitted by linear regression. Arrows with the values denote the quantum flux density ($\mu$mol m$^{-2}$ s$^{-1}$) corresponding to each measurement. The pool size of DMADP was determined according to an in vivo method based on postillumination isoprene release (Rasulov et al., 2009).

### Effects of Ambient $CO_2$ Concentration on Net Assimilation, Isoprene Emission, and DMADP Pool Size

Net assimilation rate responded to ambient $CO_2$ concentration according to a classical hyperbola, exhibiting photorespiratory $CO_2$ evolution in the absence of external $CO_2$, with $CO_2$ compensation point at about 75 $\mu$mol mol$^{-1}$ and approaching $CO_2$ saturation at concentrations above 1,000 $\mu$mol mol$^{-1}$ (Fig. 4A). In contrast, the $CO_2$ response of isoprene emission was a curve with a maximum that occurred close to the $CO_2$ compensation point of photosynthesis. The reduction of isoprene emission toward lower $CO_2$ concentrations (minimum ambient $CO_2$ concentration achieved was 8–12 $\mu$mol mol$^{-1}$, corresponding to a $CO_2$ concentration in substomatal cavities of 20–30 $\mu$mol mol$^{-1}$) was about 20%. The reduction of isoprene emission toward higher $CO_2$ concentrations was much larger, with the emission rate at the highest $CO_2$ concentration being only approximately 10% of its maximum rate.

DMADP pool size responded to $CO_2$ concentration analogously with the isoprene emission rate (Fig. 4B), and DMADP pool size and isoprene emission rate were strongly correlated throughout the entire $CO_2$ response curve (Fig. 4B, inset). The initial slope of the isoprene emission versus DMADP pool size (DMADP pool turnover rate) was 0.029 s$^{-1}$.

### $O_2$ Responses of Net Assimilation, Isoprene Emission, and DMADP Pool Size

$O_2$ concentration was altered between 2% and 21% at different $CO_2$ concentrations and light intensities. At close to ambient $CO_2$ concentration of 350 $\mu$mol mol$^{-1}$ and light intensity of 550 $\mu$mol m$^{-2}$ s$^{-1}$, a typical Warburg effect was observed (i.e. an increase of net assimilation rate by about 30% to 40% at 2% $O_2$ compared with 21% $O_2$; Fig. 5A). An even more pronounced positive response was observed in iso-

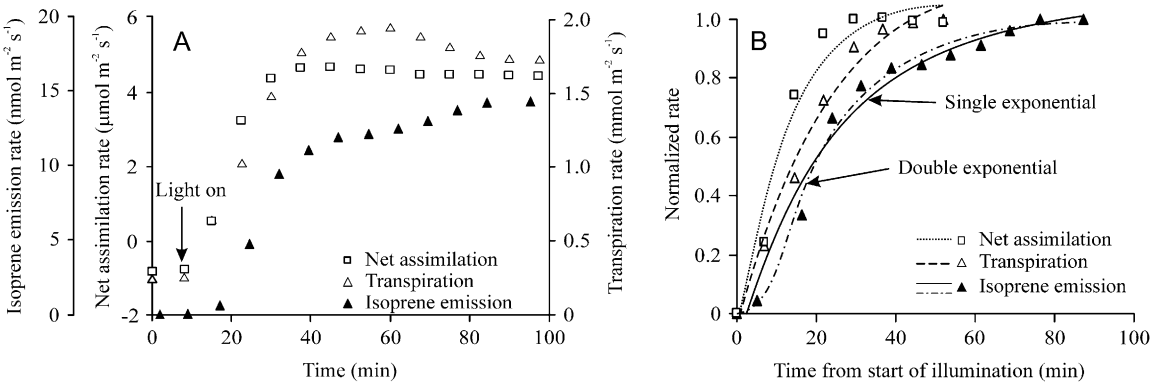


**Figure 3.** Time courses of the light activation of leaf photosynthesis, isoprene emission, and transpiration rates in hybrid aspen plants dark conditioned for 10 h (A), and normalized induction responses with single (Eq. 2) and double (Eq. 3) exponential data fits (B). After the dark period, light (550 $\mu$mol $m^{-2}$ $s^{-1}$) was switched on and leaf physiological characteristics were continuously monitored until full induction. To directly compare the shapes of the induction curves in B, all data were normalized such that the normalized rate was 0 at the beginning of the transient and 1 at the steady state. Leaf temperature was 24°C in the darkness and increased to 28°C to 30°C in the light.

prene emission, which increased 1.5- to 1.7-fold in 2% $O_2$ relative to 21% $O_2$. The enhancement of isoprene emission at the low $O_2$ concentration was persistent for 20 to 40 min and completely reversible (Fig. 5, A and D).

At the low light intensity of 200 $\mu$mol $m^{-2}$ $s^{-1}$, the enhancement of isoprene emission by low $O_2$ concentration (1.07-fold increase in 2% $O_2$; Fig. 5B) was statistically nonsignificant. Under these conditions, net assimilation rate also did not significantly respond to low $O_2$ (Fig. 5B). The low $O_2$ effect was also small, but statistically significant, at the saturating $CO_2$ concentration of 1,150 $\mu$mol $mol^{-1}$ (1.13-fold increase in 2% $O_2$; Fig. 5C), but net assimilation rate was not significantly affected by low $O_2$ (Fig. 5C).

The strong positive effect of low $O_2$ concentration on isoprene emission at high light/normal ambient $CO_2$ concentration was paralleled by a significant increase in DMADP pool size under low $O_2$ (Fig. 6). The enhancement in DMADP pool size was small, on the order of 10% in low $O_2$ treatments, resulting in minor enhancement of isoprene emission (low light, high $CO_2$; Fig. 5, B and C).

Transient responses of isoprene emission to changing $O_2$ concentration demonstrated that the response was not immediate but took approximately 2 to 3 min for full induction, consistent with the time required to alter the DMADP pool size (Fig. 7). Under low $O_2$, isoprene emission could be further increased by reducing $CO_2$ in agreement with the steady-state patterns (compare with Figs. 4A and 7), but again there was a certain delay of 2 to 3 min before reaching the steady state.

## DISCUSSION

### Is the Variation in Isoprene Emission Rate Driven by Changes in Substrate Availability after Light Changes?

To gain insight into the role of variations in DMADP concentrations in altering isoprene emission rate, we measured isoprene emission in parallel with the chloroplastic DMADP pool at different light intensities and $CO_2$ and $O_2$ concentrations. For the measurement of the in vivo DMADP pool, we used a nondestructive kinetic method based on integration of the postillumination isoprene emission (Rasulov et al., 2009). The dependence of isoprene emission on light intensity (Fig. 1) was reported already 40 years ago (Sanadze, 1969), but the mechanism of light-dependent regulation of isoprene emission is still a matter of discussion. After the discovery of the chloroplastic pathway of isoprene synthesis and the stromal and thylakoid-bound forms of IspS catalyzing the formation of isoprene from DMADP (Silver and Fall, 1991; Kuzma and Fall, 1993; Wildermuth and Fall, 1998), two major possibilities leading to light dependence have been suggested: light-induced activation of the rate-limiting IspS enzyme via changes in chloroplastic $Mg^{2+}$ concentrations (Wildermuth and Fall, 1996; Fall and Wildermuth, 1998; Logan et al., 2000; Sasaki et al., 2005), and light-dependent changes in the availability of the substrate DMADP (Loreto and Sharkey, 1993). In the case of entirely enzymatic control, variation in isoprene emission rate would result from changes in enzyme activity at the saturating level of the DMADP pool. In the case of full substrate control, isoprene emission rate would vary in dependence on the changes in DMADP pool size at a constant level of enzyme activity. Although the positive correlations between isoprene synthase activity and isoprene emission rates in leaves of different ages and from different growth conditions have been found (Kuzma and Fall, 1993; Wildermuth and Fall, 1998; Lehning et al., 1999; Scholefield et al., 2004), to our knowledge, there is currently no experimental information on the activation of isoprene synthase in response to rapid light/dark changes. In fact, in vitro-measured IspS activity was similar between light- and dark-adapted leaves in *Salix discolor* (Wildermuth and Fall, 1998) and did not vary during the day in *Quercus robur* (Lehning et al.,

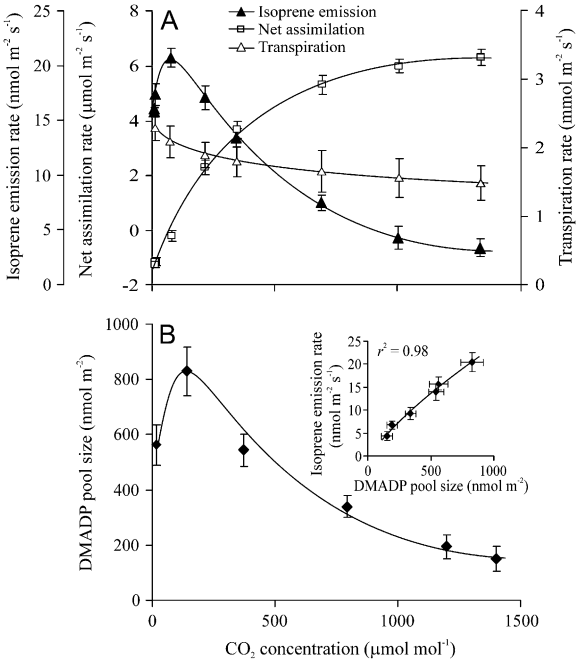


**Figure 4.** Dependencies of the rates of net assimilation, isoprene emission, and transpiration (A) and the size of the chloroplastic DMADP pool (B) on ambient $CO_2$ concentration ($C_a$) at saturating light (550 $\mu$mol $m^{-2}$ $s^{-1}$) and ambient $O_2$ concentration (210 mmol $mol^{-1}$) in hybrid aspen leaves. Leaf temperature was 28°C to 30°C. $CO_2$ concentration in the substomatal cavities was 26 $\mu$mol $mol^{-1}$ at the lowest $C_a$ and 1,230 $\mu$mol $mol^{-1}$ at the highest $C_a$ (calculated from stomatal conductance, assimilation rate, and $C_a$). The inset in B demonstrates the dependence of isoprene emission rate on DMADP pool size. Chloroplastic DMADP pool size was estimated on the basis of an in vivo method using postillumination isoprene release (Rasulov et al., 2009). Error bars show SD ($n$ = 5).

1999). Nevertheless, transcription of the isoprene synthase gene is light dependent (Sasaki et al., 2005), and the transcription of the isoprene synthase gene and the amount of protein can vary during the day and between days (Loivamäki et al., 2007) and depending on long-term light availability (Schnitzler et al., 1997; Lehning et al., 1999), thereby affecting isoprene emission rate over longer time scales (Lehning et al., 1999; Loivamäki et al., 2007). For instance, it has been demonstrated that decreasing the light intensity by 50% by shading individual branches for 3 d decreased IspS activity by 60% (Lehning et al., 1999).

Different from the enzyme-level activation theory, the substrate-level control theory assumes that the variation in the synthesis of DMADP is responsible for the light dependence. In fact, the pool size of DMADP does vary and is commonly higher at higher quantum flux density (Brüggemann and Schnitzler, 2002; Rosenstiel et al., 2002; Mayrhofer et al., 2005; Kaiser et al., 2007; Rasulov et al., 2009). The level of chloroplastic DMADP is generally well below the level needed for the saturation of isoprene synthase (Nogués et al., 2006; Behnke et al., 2007; Rasulov et al., 2009). These observations of light-dependent variation in and overall low DMADP pool sizes demonstrate that substrate-level control of isoprene emission is principally possible. In our study, we observed a strong positive scaling of isoprene emission rate with DMADP pool size (Fig. 2). This relationship fitted close to a straight line, with only a small offset for residual isoprene emission and minor curvilinearity. Given the large $K_m$ value of approximately 3.8 $\mu$mol $m^{-2}$ determined in vivo for *Populus* (Rasulov et al., 2009), substrate concentration was not saturating for isoprene synthesis in any part of this relationship. For mixed enzyme activity/substrate concentration control of isoprene emission, the emission rate would also less strongly respond to changes in substrate concentration at low light availability than at high light availability, because of low synthase activity under such conditions. In fact, the initial slope of isoprene emission rate versus DMADP pool size (Fig. 2) was actually somewhat larger at low DMADP than at high DMADP, as expected for typical Michaelis-Menten kinetics. Thus, this evidence suggests that the enzyme activity did not change over the range of light intensities applied. These results call into question the possibility that the activation state of isoprene synthase is regulated in a light-dependent manner in the time scale of tens of minutes. We conclude that changes in enzyme activity cannot be responsible for the light dependence of isoprene emission in the steady state.

### Slow Turnover of DMADP Pool Size after Light Alteration

The numeric value of the slope of the isoprene emission rate versus DMADP pool size (Fig. 2) provides the rate constant (reciprocal of lifetime) for the conversion of the DMADP molecule to isoprene. We obtained a value of 0.034 $s^{-1}$, corresponding to the average lifetime of DMADP of 30 to 40 s. Such a lifetime is rather long for a typical metabolic intermediate. However, the slow turnover of the DMADP pool observed in our study is in agreement with values recalculated from other reports: 0.01 $s^{-1}$ (Brüggemann and Schnitzler, 2002; Rosenstiel et al., 2003) and 0.026 $s^{-1}$ (Wildermuth and Fall, 1998). The lifetime of the DMADP pool is expected to depend reciprocally on the amount of the active enzyme, likely explaining the differences found in the literature.

The in vitro kinetic constant (turnover rate) of IspS is also rather small, 0.007 to 0.22 $s^{-1}$ (Mayrhofer et al., 2005) or 0.08 $s^{-1}$ (Sharkey et al., 2005). Thus, the kinetic constant of isoprene synthase and the average lifetime of the substrate DMADP are of the same order of magnitude. This suggests that the number of substrate molecules is of the same magnitude as the number of active sites of the fully activated enzyme. This correspondence between substrate and fully activated enzyme is akin to the situation with primary photosynthetic enzyme, Rubisco, and its substrate ribulose-1,5-bisphosphate (RuBP), where the number of active sites binding RuBP, up to 60 $\mu$mol $m^{-2}$, forms a significant fraction of the total RuBP pool of 100 to 200 $\mu$mol $m^{-2}$

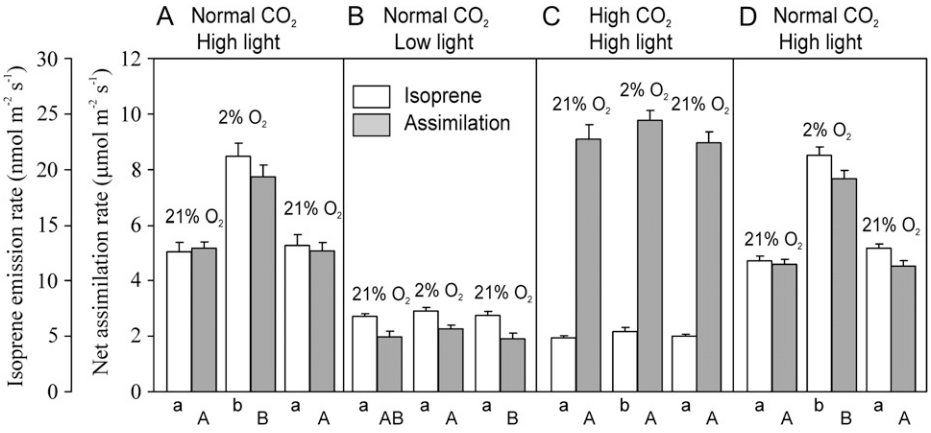


**Figure 5.** Influence of $O_2$ concentration on isoprene emission (white bars) and net assimilation (gray bars) rates at different quantum flux densities and ambient $CO_2$ concentrations in the hybrid aspen leaves. High light corresponds to 550 $\mu$mol $m^{-2}$ $s^{-1}$ and low light to 200 $\mu$mol $m^{-2}$ $s^{-1}$, while normal $CO_2$ concentration corresponds to 350 $\mu$mol $mol^{-1}$ and high $CO_2$ to 1,150 $\mu$mol $mol^{-1}$. Leaf temperature was 28°C to 30°C in all experiments. Error bars denote SE ($n$ = 5). Lowercase letters indicate significant ($P < 0.05$) differences among the isoprene emission rates within a given panel (paired samples $t$ test), while uppercase letters denote the differences among the net assimilation rates. The order of bars denotes the sequence of environmental changes for each individual leaf. Each leaf was kept at each set of environmental conditions until the steady-state rates of isoprene emission and net assimilation rate were observed. The conditions for A and D were the same to test whether the physiological status of the leaf changed during the experiment. In none of the pairwise comparisons were the average isoprene emission and net assimilation rates different between the corresponding treatments among A and D ($P > 0.6$).

(Eichelmann and Laisk, 1999; von Caemmerer, 2000). Provided that the affinity of the enzyme to the substrate is high, the comparable concentrations of enzyme sites and substrate molecules define a linear dependence of the reaction rate on the substrate concentration (Farquhar, 1979), as is visible in Figure 2.

The presence of a large DMADP pool size with slow turnover explains the significant release of isoprene for 3 to 5 min after switching off the light (Rasulov et al., 2009). Filling up this pool after a long-term dark period also likely explains the delay of approximately 3 to 5 min in isoprene emission after switching on the light (Fig. 3). Alternatively, light-dependent activation of isoprene synthase can be responsible for the delay in emission induction, but current analytical techniques cannot monitor isoprene synthase activity under transient conditions. Even if the early light activation of isoprene synthase activity is partly responsible for the delayed isoprene emission rate after switching on the light, current data suggest that changes in isoprene synthase activity were not responsible for differences in isoprene emission in the steady state, as the isoprene emission rate did not essentially vary at a given DMADP pool size. However, our observation of the absence of regulation of IspS activity during the steady-state light responses does not rule out the possibility of longer term regulation (e.g. changes resulting from the synthesis/degradation of IspS protein as stated above).

### $CO_2$ and $O_2$ Responses of Isoprene Emission in Relation to Substrate Availability

Reduction in isoprene emission rate at higher $CO_2$ concentrations has been reported in several studies (Loreto and Sharkey, 1990, 1993; Rosenstiel et al., 2003, 2006; Wilkinson et al., 2009), and this reduction is accompanied by decreased DMADP pool size (Rosenstiel et al., 2003, 2006). In our study, we further observed that the isoprene emission rate declines at lower $CO_2$ concentrations (Fig. 4A). Despite the nonmonotonic $CO_2$ response, the isoprene emission rate and DMADP pool size were strongly correlated throughout the entire $CO_2$ response (Fig. 4B). Again, the relationship between isoprene emission rate and DMADP pool size observed throughout the entire $CO_2$

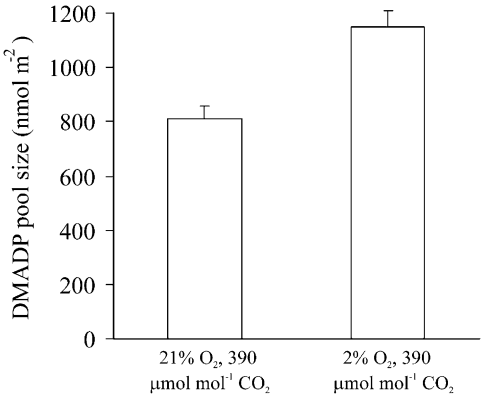


**Figure 6.** Effect of $O_2$ concentration on the chloroplastic DMADP pool size in the hybrid aspen leaves at a quantum flux density of 550 $\mu$mol $m^{-2}$ $s^{-1}$, ambient $CO_2$ concentration of 390 $\mu$mol $mol^{-1}$, and leaf temperature of 28°C to 30°C. DMADP pool size was estimated according to the in vivo method based on postillumination isoprene release (Rasulov et al., 2009).

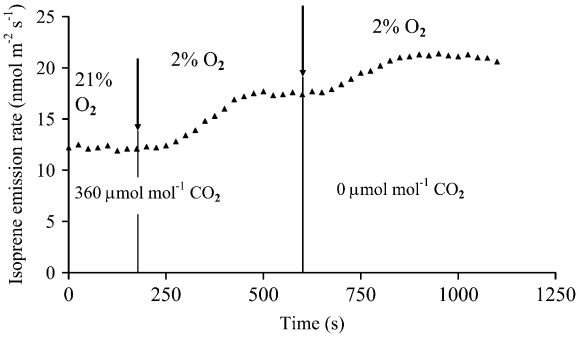


**Figure 7.** Typical time courses of isoprene emission rate after changes in ambient $O_2$ and $CO_2$ concentrations in hybrid aspen leaves. The times of alteration of ambient air composition are indicated by arrows.

response curve was almost linear (Fig. 4B), indicating that isoprene synthase activity did not change over the $CO_2$ concentration range. The slope of isoprene emission versus DMADP pool size obtained, 0.029 $s^{-1}$ (Fig. 4B), was only slightly lower than the slope obtained from the light responses (Fig. 2), demonstrating similar turnover rates of the DMADP pool during both light and $CO_2$ responses. In addition, analogous positive scaling of isoprene emission with DMADP pool size was observed for different $O_2$ concentrations (Figs. 5 and 6). Similar correspondence between isoprene emission rate and DMADP pool size has been observed even throughout leaf development from expanding to senescing leaves (Rasulov et al., 2009).

These data collectively suggest that changes in DMADP pool size rather than in the activation state of isoprene synthase were responsible for the observed $CO_2$ and $O_2$ effects on isoprene emission. Lack of $CO_2$-related regulation of isoprene synthase activity has been reported by Kaiser et al. (2007) and Calfapietra et al. (2007), further confirming the importance of substrate effects in $CO_2$ responses of isoprene emission.

### Is the Control of Isoprene Synthesis by Substrate Due to Limited Availability of Carbon Skeletons or Energetic Cofactors?

The chloroplastic isoprene synthesis pathway (MEP pathway) begins with a condensation of glyceraldehyde 3-phosphate and pyruvate to form 1-deoxy-D-xylulose 5-phosphate. Pyruvate needed in this reaction is presumed to be formed by dephosphorylation of PEP. The availability of carbon skeletons for the pathway is determined by the availability of PEP and glyceraldehyde 3-phosphate. Farther along the pathway, NADPH and ATP are involved in the formation of a series of phosphorylated and reduced intermediates, such as MEP, 4-diphosphocytidyl-2-C-methyl-erythritol, and 2-C-methyl-erythritol 2,4-cyclophosphate, suggesting that the pathway can be controlled by the energetic cofactors NADPH and ATP, pending the effective $K_m$ values for energetic cofactors of the pathway. The energetic cofactors, as well as the carbon skeletons, are produced by leaf photosynthesis. Under nonstressed conditions, about 90% of the carbon in isoprene originates from the early products of photosynthesis (Delwiche and Sharkey, 1993; Karl et al., 2002; Funk et al., 2004).

The key question in understanding the environmental controls on isoprene emission is whether the rate of synthesis of DMADP is controlled by the availability of carbon skeletons or by energetic cofactors. Relying on the positive correlation between the pool size of ATP and isoprene emission rate at different external conditions, some authors have concluded that the synthesis of DMADP is limited by the availability of energetic cofactors (Sanadze and Baazov, 1985; Loreto and Sharkey, 1990, 1993; Niinemets et al., 1999), while others suggest that the supply of carbon skeletons is limiting (Brüggemann and Schnitzler, 2002; Rosenstiel et al., 2002; Mayrhofer et al., 2005). Especially in explaining the $CO_2$ responses of isoprene emission, the supply of carbon skeletons has been considered to be the dominant limitation (Rosenstiel et al., 2003, 2006; Monson et al., 2007; Wilkinson et al., 2009).

Already the early measurements of isoprene emission suggested that only very low photosynthetic rates are needed to support a relatively fast isoprene emission rate (Tingey et al., 1981). Isoprene emission is significantly suppressed during the complete absence of $CO_2$ and $O_2$, but about 50 to 100 $\mu$mol $CO_2$ $mol^{-1}$ in the ambient air is sufficient to support a high rate of isoprene emission (Loreto and Sharkey, 1993). Our measurements demonstrating that the maximum isoprene emission rate occurred around the $CO_2$ compensation point and decreased only to a minor extent when $CO_2$ was completely removed from the ambient air (Fig. 4A), confirming the previous observations. This evidence indicates that carbon skeletons are sufficiently abundant for isoprene synthesis already when the net carbon supply is slightly positive. During the absence of photosynthesis in the dark, the isoprene precursor pools were depleted to the extent that a delay of about 5 min was needed to refill the MEP pathway intermediate pools, despite the fact that photosynthesis was already running fast (Fig. 3). These observations, along with the direct measurements of the carbon intermediate pools in the leaves (Badger et al., 1984), encourage us to focus on the regulation of the MEP pathway mainly by energetic cofactors.

An observation of an Emerson-type enhancement effect of isoprene emission when far-red and blue light were provided simultaneously (Sanadze and Baazov, 1985) has led to the suggestion that isoprene emission is controlled by energy supply. From the measurements of the ATP pool size, a direct relationship between ATP level and isoprene emission has been established (Loreto and Sharkey, 1993). The light-induced activation of isoprene emission observed in this work is consistent with the hypothesis of ATP-controlled DMADP pool size. In addition, saturation of isoprene emission rate at higher light than is needed

to saturate photosynthesis (Fig. 1; Loreto and Sharkey, 1990, 1993; Harley et al., 1996, 1997) also supports the dominant role of energy supply. No further increase of the carbon pools is expected when photosynthetic $CO_2$ uptake becomes light saturated, but the transthylakoid proton gradient, directly related to the ATP/ADP ratio, can still increase (Stitt, 1986; Maxwell et al., 1998).

### $CO_2$-Dependent Limitation of DMADP Synthesis: Energetic Cofactors or PEP?

How can the $CO_2$ responses of isoprene emission and DMADP synthesis be explained on the basis of energetic cofactors? All known mechanisms explaining $CO_2$ saturation of photosynthesis are based on limitation by ATP and/or NADPH. The theory of electron transport limitation of photosynthesis (Farquhar and von Caemmerer, 1982) assumed that the turnover of cytochrome $b_6f$ was the rate-limiting step of linear electron transport at high $CO_2$ concentration, inducing a deficit in both ATP and NADPH. Experimental estimations of ATP level do demonstrate that its pool size is lower under high than under ambient $CO_2$ (Cardon and Berry, 1992; Delwiche and Sharkey, 1993). The third player, phosphate (end-product synthesis) limitation, may sometimes occur at high $CO_2$ concentrations (Sharkey, 1985). However, during phosphate limitation, the level of free inorganic phosphate, $P_i$, becomes limiting for ATP synthesis in the chloroplast, leading to the drop of ATP level, although the proton gradient may remain high (Harley and Sharkey, 1991). Thus, the phosphate limitation is actually also a situation with limited supply of energetic cofactors. As experimental evidence demonstrates, such reductions of ATP level due to limited $P_i$ are associated with reduced isoprene emissions: addition of Man, which binds $P_i$, dramatically decreased both photosynthesis and isoprene emission (Monson and Fall, 1989).

On the other hand, at lower $CO_2$, below the compensation point, photosynthetic electron transport rate becomes inhibited due to a lack of electron acceptors, resulting in reduced supply of ATP and NADPH (Laisk and Sumberg, 1994; Miyake et al., 2005). Such reduction in energy status can explain the suppression of DMADP synthesis under low $CO_2$. Thus, we conclude that the available pieces of evidence collectively are consistent with the hypothesis that the level of energetic cofactors matches the rate of DMADP synthesis throughout the $CO_2$ response curve (Fig. 4).

An alternative hypothesis to explain the inhibitory effect of high $CO_2$ concentration on isoprene emission is the regulation of the isoprenoid synthesis pathway by the availability of cytosolic PEP that is presumed to be transported to chloroplasts by PEP/$P_i$ translocator (Streatfield et al., 1999), where it is converted to the MEP pathway substrate pyruvate. As PEP is used in multiple processes, and the distribution of PEP between different pathways can depend on $CO_2$ concentration, the competition for PEP can be responsible for the $CO_2$ dependence of DMADP formation. In particular, it has been postulated that PEP carboxylation in cytosol, which likely strongly scales with $CO_2$ concentration, draws increasingly more PEP away from the MEP pathway at higher $CO_2$ concentrations (Rosenstiel et al., 2002, 2003, 2004; Affek and Yakir, 2003; Loreto et al., 2007). So far, the information regarding the regulation of PEP carboxylation in $C_3$ plants is very limited, but there are several lines of evidence against the PEP control of DMADP synthesis under different $CO_2$ levels. First, the rate of PEP carboxylation in $C_3$ plants is overall very much slower than the rate of the photosynthetic metabolism, being at most 10% of the rate of photosynthesis (Roeske et al., 1989; Laisk and Sumberg, 1994). Second, cytosolic PEP that can be carboxylated by $CO_2$ is a derivative from 3-phosphoglycerate (3-PGA) via 2-PGA. During photosynthesis at saturating $CO_2$ concentrations, 3-PGA is available in great amounts (von Caemmerer and Edmondson, 1986) and the chloroplastic and cytosolic PGA pools are in equilibrium (for review, see Flügge and Heldt, 1991). Third, Ala, an amino acid formed mainly in chloroplasts from PEP via dephosphorylation and transamination (Schulze-Siebert et al., 1984), is synthesized at increased rates when photosynthesis becomes $CO_2$ saturated (Platt et al., 1977; Keerberg et al., 1983; Murray et al., 1987), suggesting that PEP concentration in chloroplasts is high under $CO_2$ saturation. In fact, the PEP pool is larger at higher $CO_2$ concentrations (Platt et al., 1977). Fourth, given that PEP carboxylation scales positively with $CO_2$ concentration, limited PEP availability would be unable to explain the decrease of isoprene emission and DMADP pool size at low $CO_2$ concentrations (Fig. 4). Fifth, the PEP carboxylase competition hypothesis assumes that PEP and finally pyruvate for isoprene synthesis must come from cytosol. However, chloroplasts possess all enzymes of the glycolytic pathway that lead to the synthesis of pyruvate (Liedvogel and Bäuerle, 1986; Hoppe et al., 1993; Givan, 1999; Eastmond and Rawsthorne, 2000; Baud et al., 2007), although for some species, there is evidence that the chloroplasts of mesophyll cells may be deficient in terminal enzymes of glycolysis, such as enolase and phosphoglyceromutase (Plaxton, 1996). Alternatively, pyruvate may be formed by $\beta$-elimination of phosphate from the carbanion intermediate in the Rubisco reaction (Andrews and Kane, 1991) in sufficient amounts needed for isoprene synthesis (Sharkey et al., 1991a). In fact, the early experiments of Sanadze and colleagues with isolated chloroplasts have demonstrated that cytosolic PEP is not needed for high isoprene fluxes (Sanadze and Dzhaiani, 1972; Mgalobilishvili et al., 1978). High isotopic labeling observed in $^{13}CO_2$ addition experiments (see above) is also not consistent with the extrachloroplastic origin of a large fraction of PEP used in isoprene synthesis. Depending on the contributions of chloroplastic and cytosolic PEP pools, PEP carboxylase should not necessarily interfere with DMADP synthesis at all. Finally,

we note that PEP carboxylation is activated in parallel with light activation of photosynthesis (Samaras and Manetas, 1988; Pfeffer and Peisker, 1998). If PEP carboxylase would efficiently compete for PEP, isoprene synthesis rate would not increase with light intensity, contrary to the results obtained by us (Fig. 1) and others.

On the basis of this evidence, we conclude that the regulation of DMADP synthesis by energetic cofactors is the most likely explanation for the $CO_2$ dependence of isoprene synthesis. However, the conclusion that isoprene emission is controlled at the level of energetic cofactors is valid for short-term (tens of minutes) regulations. During long exposures of plants to unfavorable conditions like low temperature (Sharkey et al., 2008), during the development of young leaves (Mayrhofer et al., 2005; Wiberley et al., 2008), during adjustments to changed light intensity (Lehning et al., 1999; Sasaki et al., 2005), and during other stresses (Brilli et al., 2007), the control of isoprene emission may shift over to the IspS transcription and to carbon sources (Fortunati et al., 2008).

### How Does $O_2$ Affect Isoprene Emission?

Our study (Figs. 5–7) confirms the previous observations of the enhancement of isoprene emission at low concentrations of $O_2$ (Sanadze and Tarhnishvili, 1986; Loreto and Sharkey, 1990). Our results here show that the enhancement of isoprene emission at low $O_2$ levels is the result of an increase in the DMADP pool size by about 35% (Fig. 6). The larger DMADP pool was not the result of increased carbon supply from the carbon reduction-oxidation cycles (CROC; $CO_2$ assimilation and photorespiration), since isoprene emission increased after $CO_2$ was removed from the ambient air in the presence of 2% $O_2$. Under such conditions, CROC activity could only decrease. Although the enhanced isoprene emission in $CO_2$-free atmosphere may not be persistent, as the depletion of CROC carbon pools would finally neutralize the positive effect (Loreto and Sharkey, 1990), the immediate positive response of isoprene emission on the removal of $CO_2$ (Fig. 7) cannot be explained on the basis of carbon limitation.

The lack of carbon limitation under low $O_2$ is also confirmed by $^{13}C$ labeling experiments (Karl et al., 2002). In these experiments, the labeling of isoprene decreased but isoprene emission rate increased under low $O_2$ (Karl et al., 2002). Thus, PEP labeling decreased due to decreased PGA level in the absence of RuBP oxygenation, but an unlabeled cytosolic PEP source immediately replaced the PEP of photosynthetic origin. Thus, there was no evidence of limitation of the MEP pathway by carbon sources. The lack of control of isoprene emission by carbon pools was demonstrated also in experiments where the addition of endogenous deoxyxylulose did not enhance isoprene emission under low $O_2$ concentration (Wolfertz et al., 2003).

Taken together, the simplest explanation of our results is consistent with the ATP-related regulation of isoprene synthesis. The total turnover rate of CROC significantly decreased after RuBP oxidation was suppressed at the low $O_2$ concentration. This resulted in increased pressure of the energetic cofactors ATP and NADPH, as reflected in enhanced DMADP synthesis and isoprene emission. The effect was smaller under lower light (Fig. 5B), where the increase of electron pressure was less due to an overall lower electron transport rate. Analogously, high $CO_2$ can inhibit photosynthetic electron transport due to phosphate sequestration (Sharkey et al., 1986; Harley and Sharkey, 1991), resulting in a small effect of low $O_2$ on isoprene emission (Fig. 5C). Even under ambient $CO_2$, but at longer time scales (more than 30 min), photosynthetic electron transport rate can become inhibited under low $O_2$ as the result of feedback inhibition due to sequestered $P_i$, and the isoprene emission rate can decline after the initial 20 to 30 min of increase under low $O_2$ (Monson and Fall, 1989; Loreto and Sharkey, 1990).

## CONCLUSION

We have shown that foliage isoprene emission is tightly related to photosynthesis via the supply of energetic cofactors. These data do not support short-term enzymatic limitation of the MEP pathway. Isoprene synthase usually operates with a nonsaturating pool of its substrate DMADP (Figs. 2 and 4B; Brüggemann and Schnitzler, 2002), whose concentration is even less than the concentration of the enzyme active sites (Rasulov et al., 2009). Even very low photosynthesis is sufficient to saturate the need for carbon skeletons for isoprene emission (Tingey et al., 1981; Wolfertz et al., 2003). Therefore, the variation in isoprene emission rates due to changes in ambient air composition occurs differently from carbon uptake rates. In contrast, the substrate DMADP for IspS is almost proportionally dependent on energetic and reductive equivalents, suggesting that the effective $K_m$ values for ATP and NADPH are large for the isoprenoid synthesis pathway. For developing process-based models of isoprene emission, the effective $K_m$ values for ATP and NADPH should be determined for the entire series of reactions leading to DMADP.

## MATERIALS AND METHODS

### Plants and Growth Conditions

One-year-old seedlings of hybrid aspen (*Populus tremula* × *P. tremuloides*) clone 200 (for a description of the genotype, see Vahala et al., 2003) were grown in a Percival AR-95 HIL growth chamber (CLF PlantClimatics) under photosynthetic quantum flux density of 500 $\mu$mol $m^{-2}$ $s^{-1}$ (14-h d) and relative humidity of 60%. Air temperature in the growth chamber was maintained at 26°C during the day and 20°C during the night. The plants were grown in plastic pots filled with 4 kg of a sand and peat mixture (1:1) and watered daily to field capacity with distilled water. Once a week, macroelements according to Knopp's and microelements according to Hoagland's standard solutions were added to the irrigation water.

### Gas-Exchange System

The measurements were accomplished with a custom-made open gas-exchange system described in detail by Rasulov et al. (2009). In short, the measurement chamber of 3 $dm^3$ was made of glass and was equipped with a

fan for efficient air mixing in the chamber. Other parts of the system were made of Teflon (DuPont) or stainless steel. Four halogen lamps positioned at different sides of the chamber provided essentially uniform illumination of all leaves in the chamber. Light intensity could be dynamically regulated between 0 and 550 $\mu$mol $m^{-2}$ $s^{-1}$ by changing the lamp voltage. The air flow rate through the chamber was 3 $dm^3$ $min^{-1}$, resulting in the response half-time of approximately 40 s. Either the outside air low in pollutants ($O_3$ concentrations $< 20$ nmol $mol^{-1}$, NO $< 5$ nmol $mol^{-1}$, isoprene concentration below the detection limit) taken through a 50-$dm^3$ buffer volume (experiments with constant ambient $O_2$ and $CO_2$ concentrations) or air mixed from pure $N_2$, $O_2$, and $CO_2$ using capillary mixers (Laisk and Oja, 1998) was used. In all cases, a constant air humidity of 60% was achieved using a custom-made thermostatted humidifier. The temperature inside the chamber was continuously measured with a negative temperature coefficient thermistor (model -001; RTI Electronics). Leaf temperature determined from leaf energy balance was within ±1°C of the air temperature in the chamber. In all experiments, leaf temperature was maintained between 28°C and 30°C. Experiments with widely varying temperature demonstrated that fluctuations of leaf temperature over this limited range did not qualitatively affect any of the relationships reported (data not shown). The gas-exchange system was computer operated using an A/D converter board ADIO 1600 (Kontron) and custom-made computer software.

## Determination of Net Assimilation, Transpiration, and Isoprene Emission Rates

All measurements were conducted with fully mature leaves (25–35 d after bud burst) with stable photosynthetic capacity and isoprene emission rate (for age-dependent modifications in isoprene emission rate, see Monson et al., 1994). In our previous study, we have observed isoprene emission rates of up to approximately 35 nmol $m^{-2}$ $s^{-1}$ immediately after the cessation of leaf expansion (Rasulov et al., 2009). As these peak rates were supported only for a limited period of time, 2 to 3 d (data not shown), we used the fully mature nonsenescent leaves with stable (at least for 10 d) assimilation rates and isoprene emission potentials of approximately 25 nmol $m^{-2}$ $s^{-1}$.

Incoming and outgoing air $CO_2$ and water vapor concentrations were measured with an infrared gas analyzer (LI-6262; Li-Cor). Isoprene concentration was measured with the Fast Isoprene Analyzer (Hills Scientific) specifically modified to optimize the sensitivity and stability of isoprene concentration measurements. This device is based on counting the photons emitted as the result of reactions between ozone and isoprene, resulting in chemiluminescence as the isoprene degradation product, electronically excited formaldehyde, reaches the ground state (Hills and Zimmerman, 1990). For this purpose, ozone generated from molecular $O_2$ is continuously added to the gas stream entering the measurement cell. However, a significant background of photons is generated by direct ozone-ozone recombination reaction. The latter reaction is sensitively inhibited by water vapor, making the background count rate dependent on gas humidity. Since the ozone-ozone reaction is proportional to the square of ozone concentration, but the ozone-isoprene reaction follows the first-order kinetics, the humidity-dependent background was optimized by decreasing ozone concentration in the cell. For this purpose, we adjusted the $O_2$ flow rate to 0.4 $dm^3$ $min^{-1}$, while the measurement gas flow was 0.8 $dm^3$ $min^{-1}$. For optimization and maximum stabilization of the background count level, the dew point of the gas entering the isoprene analyzer was set at 23°C using a custom-made humidifier. The isoprene analyzer was regularly calibrated with a standard gas (5.74 $\mu$mol $mol^{-1}$ isoprene in $N_2$).

Light response curves were measured starting with the highest light and decreasing stepwise the light intensity. Preliminary experiments demonstrated that the isoprene emission rate achieved at each light level did not depend on the order of measurements (i.e. there was no significant difference whether the measurements were started from the lowest or from the highest light). At each light level, the measurements were taken after steady-state values of isoprene emission and net assimilation rates had been observed, usually 15 to 20 min after changing the conditions. The light ($Q$) responses of net assimilation and isoprene emission were fitted by a hyperbolic relationship previously used to simulate photosynthetic characteristics and isoprene emission as a function of light (Harley and Tenhunen, 1991; Guenther et al., 1993):

$$R_i = \frac{\alpha Q}{\sqrt{1+\frac{\alpha^2 Q^2}{R_{i,max}^2}}} + R_{i,D} \quad (1)$$

where $R_i$ is either the isoprene emission or the net assimilation rate, $R_{i,max}$ is the light-saturated value, $R_{i,D}$ is the rate in the darkness, and $\alpha$ is the initial quantum yield. As isoprene emission potentials are commonly assessed at $Q$ = 1,000 $\mu$mol $m^{-2}$ $s^{-1}$ (Guenther et al., 1993), Equation 1 was also used to predict the emission rates and net assimilation rates at this $Q$. This allowed us to determine how far the measurements were from full light saturation. In addition to the response curve measurements, dark/light activation of isoprene emission was studied after a 10-h dark period. The time-dependent activations of isoprene emission, photosynthesis, and transpiration rates were fitted by single-exponential and double-exponential functions to gain information on the rate constants ($k$). For the single-exponential function, the rate, $R_i$, varies in time ($t$) as:

$$R_i = A_0 - A_1 \exp(-k_1 t) \quad (2)$$

while for the double exponential function:

$$R_i = A_0' - A_1' \exp(-k_1' t) - A_2 \exp(-k_2 t) \quad (3)$$

where $A_0$, $A_1$, $A_0'$, $A_1'$, and $A_2$ are the scaling constants and $k_1$, $k_1'$, and $k_2$ are the rate constants.

To measure the $CO_2$ responses, the leaf was stabilized at an ambient $CO_2$ concentration of 390 $\mu$mol $mol^{-1}$, and after the steady-state net assimilation and isoprene emission rates had been reached under the ambient $CO_2$ concentrations, $CO_2$ concentration was changed to the target value. Again, enough time was allowed to attain the new steady-state conditions, and the rates of isoprene emission and net assimilation were recorded. After the measurements at given $CO_2$ concentrations, the $CO_2$ concentration was switched back again to 390 $\mu$mol $mol^{-1}$ and the leaf was stabilized until previous steady-state values were reached before taking the next point of the $CO_2$ response curve. An analogous stabilization procedure was followed for $O_2$ responses.

Each measurement at different light, $CO_2$, and $O_2$ levels was combined with postillumination isoprene release measurements. After reaching the steady-state value under given environmental conditions, light was switched off and postillumination isoprene release was measured for 5 min to estimate the in vivo DMADP pool size as detailed below.

In all cases, at least five replicate experiments with different plants were conducted and averages were calculated. After each experiment, leaves were scanned and leaf area was calculated. All gas-exchange characteristics were calculated per unit of leaf area according to von Caemmerer and Farquhar (1981).

## In Vivo Estimation of the DMADP Pool Responsible for Isoprene Formation

The DMADP pool responsible for isoprene emission (chloroplastic isoprene pool) was measured kinetically in intact plants as described by Rasulov et al. (2009). These measurements are based on the observation that after darkening, isoprene emission continues for 3 to 5 min (Monson and Fall, 1989; Rasulov et al., 2009). Assuming that the synthesis of DMADP stops rapidly (within seconds) in the dark due to the lack of ATP and NADPH, and the isoprene emission can continue in the darkness only at the expense of the existing DMADP pool, the integral of the isoprene emission during the 3 to 5 min after the darkening gives the size of the DMADP pool responsible for isoprene emission. The pool size of DMADP estimated this way is in good correspondence with the chemical estimation of chloroplastic DMADP pool size (difference in DMADP pools in light- and dark-adapted leaf samples), although the chemical estimations provide approximately 10% larger estimates, possibly reflecting the DMADP that is used in other chloroplastic reactions occurring simultaneously with isoprene formation such as carotenoid biosynthesis (Rasulov et al., 2009). The advantage of the in vivo method is that the DMADP pool size responsible for isoprene emission can be repeatedly estimated for the same leaves, making it possible to investigate the influence of environmental drivers on and linking the emission measurements with the DMADP pool size for the same leaves. In practice, in vivo DMADP pool size estimations also require consideration of the response time of the gas-exchange system, as explained in detail by Rasulov et al. (2009) and done in this study.

Simultaneous measurements of isoprene emission rate and DMADP pool size were further used to determine the turnover rate of DMADP pool size ($s^{-1}$) as the initial slope of isoprene emission rate versus the DMADP pool size.

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