Circadian Rhythms of Isoprene Biosynthesis in Grey Poplar Leaves[1]

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Isoprene (2-methyl-1,3-butadiene) emission varies diurnally in different species. In poplar (*Populus* spp.), it has recently been shown that the gene encoding the synthesizing enzyme for isoprene, isoprene synthase (ISPS), displays diurnal variation in expression. Working on shoot cultures of Grey poplar (*Populus* × *canescens*) placed under a different light regime in phytochambers, we showed that these variations in *PcISPS* gene expression, measured by quantitative real-time polymerase chain reaction, are not only due to day-night changes, but also are linked to an internal circadian clock. Measurement of additional selected isoprenoid genes revealed that phytoene synthase (carotenoid pathway) displays similar fluctuations, whereas 1-deoxy-D-xylulose 5-phosphate reductoisomerase, possibly the first committed enzyme of the 1-deoxy-D-xylulose 5-phosphate pathway, only shows light regulation. On the protein level, it appeared that PcISPS activity and protein content became reduced under constant darkness, whereas under constant light, activity and protein content of this enzyme were kept high. In contrast, isoprene emission rates under continuous irradiation displayed circadian changes as is the case for gene expression of *PcISPS*. Furthermore, binding assays with Arabidopsis (*Arabidopsis thaliana*) late elongated hypocotyl, a transcription factor of Arabidopsis involved in circadian regulation, clearly revealed the presence of circadian-determining regulatory elements in the promoter region of *PcISPS*.

Rhythms in tune with the day-night cycle of earth are observed in each organism, in metabolism, physiology, or even behavior. In plants, such rhythms have been detected at the physiological level for numerous processes, such as closing of flowers or leaves, chloroplast movements, photosynthetic capacity, stomatal conductance, cell division, and many others (for review, see Johnson, 2001). These events are not simple responses to the external environment and continue under constant conditions. Such phenomena with approximately 24-h periodicity are controlled by an endogenous oscillator, the circadian clock. It allows organisms to anticipate daily changes in the environment, providing them with an adaptive advantage.

Circadian clocks have often been divided into three major components: input (resetting the clock), the oscillator itself, and output (physiological phenomena; Somers, 1999; Devlin, 2002). Underlying the physiological rhythms, genes encoding diverse enzymes or regulatory and structural proteins show circadian rhythm in their expression (Johnson et al., 1984; Piechulla, 1993). However, a few genes at a time were characterized until two groups, using microarrays, each representing around 8,000 Arabidopsis (*Arabidopsis thaliana*) genes, gave a global idea of clock-controlled genes (Harmer et al., 2000; Schaffer et al., 2001), thus confirming that many genes involved in photosynthetic processes and in carbon allocation are circadian regulated.

However, if these primary metabolic processes are relatively well characterized for rhythms, secondary metabolites still must be analyzed. Concerning terpenoids, reports on circadian biosynthetic rhythms are sparse and have been limited mainly to in planta chemical analysis and emission profiles (Helsper et al., 1998; Dudareva et al., 2003, 2005). However, some studies about circadian rhythms also exist on a molecular level: Lu et al. (2002) showed cyclic expression of a β-pinene synthase gene in leaves of *Artemisia annua* under continuous conditions; Simkin et al. (2004) a carotenoid cleavage dioxygenase implicated in β-ionone synthesis in petunia (*Petunia hybrida*) leaves and flowers; and Dudareva et al. (2003) β-ocimene and myrcene synthases in snapdragon (*Antirrhinum majus*) petals. However, no information exists as to whether biosynthesis of isoprene (2-methyl-1,3-butadiene), the simplest isoprenoid (terpenoid) compound, is regulated by circadian rhythm. Isoprene is a highly volatile organic compound (VOC) naturally emitted from many tree species (Kesselmeier and Staudt, 1999) with significant influence on atmospheric chemistry (Thompson, 1992; Biesenthal et al., 1997; Derwent et al., 1998). For

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the plant itself, isoprene emission is thought to prevent leaf metabolic processes from thermal (Sharkey and Loreto, 1993; Singsaas et al., 1997; Loreto et al., 2001) and oxidative stress (Loreto and Velikova, 2001; Affek and Yakir, 2002) or serve as an overflow mechanism for excess carbon intermediates (Rosenstiel et al., 2004) or photosynthetic energy (Sharkey and Yeh, 2001).

Variations of isoprene emissions during the day can be explained by synthesis of isoprene synthase (ISPS) substrates, mainly originating from recently fixed CO_2 (Schnitzler et al., 2004), and by the temperature dependence of ISPS activity and other enzymes of the plastidic 1-deoxy-D-xylulose 5-P (DOXP) pathway (Brüggemann and Schnitzler, 2002; Wolfertz et al., 2003), thus reflecting the temperature response of isoprene emission (Monson et al., 1992).

Sparse information is present on the regulation of genes related to isoprene biosynthesis. There are indications that light and temperature stimulate gene expression of DOXP synthase (*DXS*), the starting enzyme of the DOXP pathway (Sprenger et al., 1997; Eisenreich et al., 2001), DOXP reductoisomerase (*DXR*), the first committed enzyme of this pathway (Takahashi et al., 1998), proposed to be one of its key regulators (Carretero-Paulet et al., 2002), and ISPS (Carretero-Paulet et al., 2002; Hsieh and Goodman, 2005; Sasaki et al., 2005). A recent study in poplar (*Populus* spp.) shows partial correlation of *ISPS* gene expression and enzyme activity during the season (Mayrhofer et al., 2005). Diurnal variations of *PcISPS* and *PcDXR* (Mayrhofer et al., 2005) gene expression were also reported, expression peaking in the morning for *PcISPS* and in the afternoon for *PcDXR*. However, no information exists on the factors responsible for these diurnal changes and whether circadian components are involved in that regulation.

In this work, we tried to answer the questions of (1) whether diurnal variations of isoprene emission and *PcISPS* expression are due to circadian regulation or simply related to light cycle-fluctuating day-night conditions and (2) whether other isoprenoid biosynthesis-related genes follow a similar expression pattern. Hence, we measured isoprene emission, ISPS activity, and protein content, as well as gene transcript levels of *PcISPS*, *PcDXR*, and phytoene synthase (*PSY*) in Grey poplar (*Populus* × *canescens*) shoot cultures under different light regimes. These activities were accompanied by isolation and analysis of the *PcISPS* promoter where two regions, which are targets for circadian regulatory proteins, were identified.

RESULTS

Expression of Isoprenoid Biosynthesis-Related Genes under Continuous Light and Darkness

Transcript levels of the three genes were determined at different time points during the day over 3 d for shoot culture plants exposed to long-day (LD) conditions (Figs. 1A, 2A, and 3A for *ISPS*, *DXR*, and *PSY*, respectively) in the climate chamber. It is striking that all gene expressions fluctuate, being at low levels during the night and at higher levels during the day. Diurnal fluctuations were also detected under short-day conditions (8-light/16-h dark; data not shown). Each gene presents a distinct pattern.

PcISPS transcript levels appeared to be high in the morning (Fig. 1A). *PcDXR* expression (Fig. 2A) seems to peak later in the afternoon. The expression levels of *PcPSY* (Fig. 3A) tend to peak in the morning, go down at midday, and exhibit a second, less intense, peak in the evening. These 3-d experiments also showed the repeatability of these diurnal variations, underlined by the stable conditions within our climate chambers.

Because all three studied genes showed diurnal variations in expression level, the hypothesis of a circadian element involved in their transcriptional regulation was tested by placing the poplar shoot cultures in either continuous light (LL) or continuous darkness (DD) and sampling over 3 d.

A striking feature was the dramatic decrease of the level of expression when the plants were placed in DD (Figs. 1C, 2C, and 3C). No significant fluctuation was observed under these conditions.

Under LL, two patterns of expression were revealed, namely, no significant variations in the level of transcription for *PcDXR* (Fig. 2B), but rhythmic fluctuations for both *PcISPS* (Fig. 1B) and *PcPSY* (Fig. 3B).

For *PcISPS* (Fig. 1B), mean transcript levels were clearly higher (about twice) under LL than under LD light periods (Fig. 1A) for both experiments, confirming light as an enhancer of this gene expression. For *PcPSY* (Fig. 2, A and B), however, the expression level was equivalent under LD and LL conditions. Therefore, if light is essential to trigger the expression, it may not be the only regulating factor for *PcPSY*. The most obvious feature of *PcISPS* and *PcPSY* expression levels is their rhythmic fluctuation. To identify possible circadian rhythms, the values of expression were made relative (minimal value brought to 0, maximal value brought to 1), the mean of the six values of the two experiments was calculated, and these fluctuations were tested against a sinusoidal curve. The results obtained are represented in Figures 1D and 3D for *PcISPS* and *PcPSY*, respectively. Separately for each experiment, the correlation between the observed fluctuations and the calculated curve is also high for both *PcISPS* (adjusted $R^2 = 0.9416$, $P < 0.005$ for experiment 1 and adjusted $R^2 = 0.9652$, $P < 0.05$ for experiment 2; data not shown) and *PcPSY* (adjusted $R^2 = 0.9076$, $P < 0.001$ for experiment 1 and adjusted $R^2 = 0.977$, $P < 0.05$ for experiment 2; data not shown). This indicates that a defined period exists within the observed fluctuations. Interestingly, this period is about 24 h and therefore defines circadian fluctuations. It could be observed that these fluctuations are shifted in phase when compared to LD conditions. Under LL, the maximal values of *PcPSY* are reached at times corresponding to subjective evening and maximal values of *PcISPS* are reached at times corresponding to subjective night.

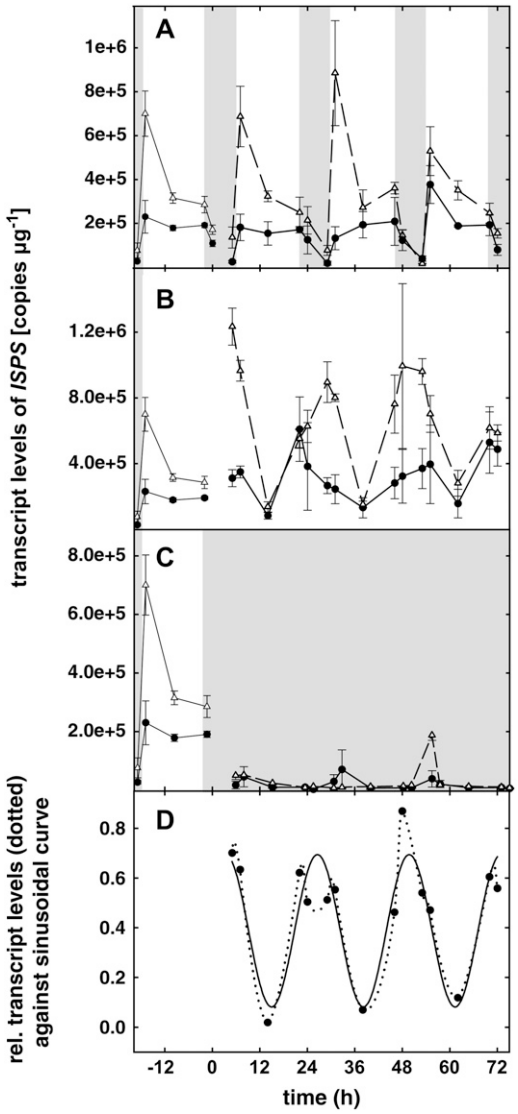

Figure 1. *PcISPS* gene expression under LD (16-h light/8-h dark) conditions (A), under LL conditions (B), and under DD conditions (C). In each graph, the two curves represent two independent experiments ($n = 3 \pm$ SE) and the curves shown in the first 24 h of each graph represent the mean of 3-d expression levels under LD conditions. D, Sinusoidal ($y = y_0 + a \sin [2\pi x/b + c]$; with $a = 0.3058$; $b = 23.8008$; $c = 1.3433$; $y_0 = 0.3920$) regression on relative data under LL conditions (mean of both experiments; adjusted $R^2 = 0.9690$, $P < 0.05$). Darkness is represented as shaded areas. Transcript levels are related to RNA quantity.

Transcript levels of β-tubulin (*TUB*) were also measured as a control (housekeeping gene), as shown in Figure 4. Fluctuations in gene expression of this gene were not as regular as for the three isoprenoid genes under LD conditions, the peaks up and down being observed at different times for different days, even if always in the evening. Under DD, the *PcTUB* expression level stays constant as well as expression under LL in the first experiment. In the second experiment, slight fluctuation of expression can be observed under LL. However, showing a different pattern than the genes of interest, *PcTUB* testifies for the specificity of the observed patterns of the isoprenoid biosynthesis-related genes.

ISPS Protein Concentration and Activity and Isoprene Emission under Different Light Regimes

Because we observed circadian regulation of the level of transcript for *PcISPS*, we wanted to check whether these variations were also present at the level of PcISPS protein concentration, activity, and, finally, emission of isoprene, knowing that emission shows daily variations under day-night cycles (Mayrhofer et al., 2005).

The results of protein concentration and enzyme activity measurements are presented in Figure 5 for the three light regimes tested (LD [A and B]; LL [C and D]; and DD [E and F]) for both experiments (A, C, and E

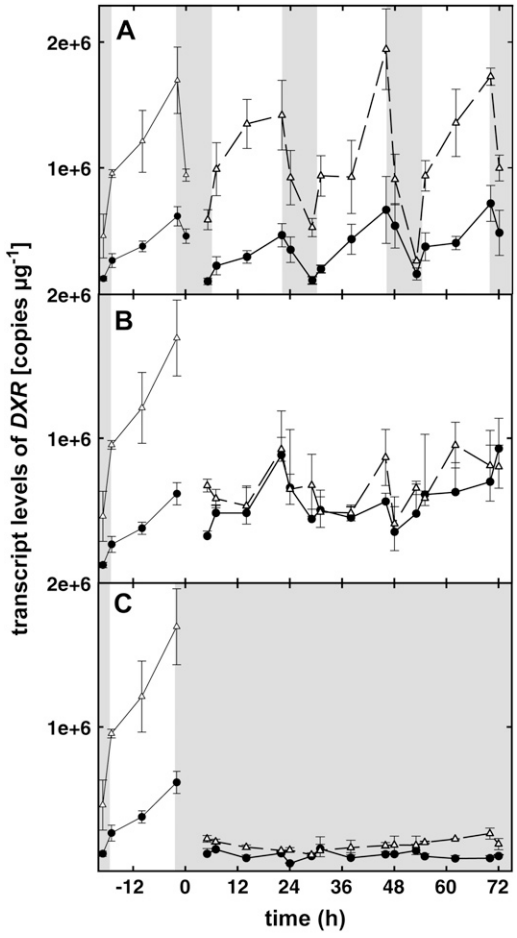

Figure 2. *PcDXR* gene expression under LD (16-h light/8-h dark) conditions (A), under LL conditions (B), and under DD conditions (C). In each graph, the two curves represent two independent experiments ($n = 3 \pm$ SE) and the curves shown in the first 24 h of each graph represent the mean of 3-d expression levels under LD conditions. Darkness is represented as shaded areas. Transcript levels are related to RNA quantity.

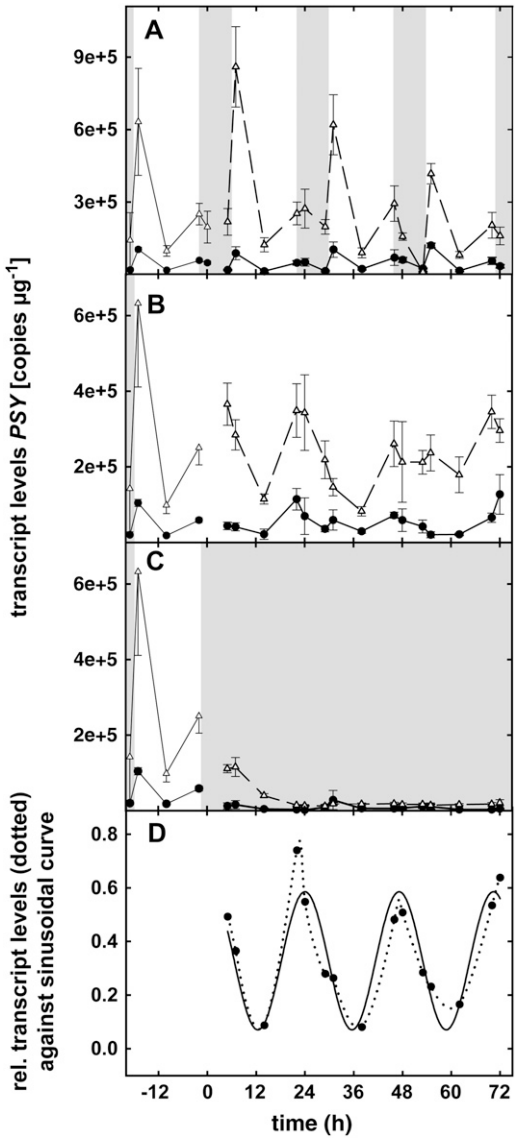

Figure 3. *PcPSY* gene expression under LD (16-h light/8-h dark) conditions (A), under LL conditions (B), and under DD conditions (C). In each graph, the two curves represent two independent experiments ($n = 3 \pm$ SE) and the curves shown in the first 24 h of each graph represent the mean of 3-d expression levels under LD conditions. D, Sinusoidal regression ($y = y_0 + a \sin [2\pi x/b + c]$; with $a = 0.2574$; $b = 23.1706$; $c = 1.3461$; $y_0 = 0.3279$) on relative data under LL conditions (mean of both experiments; adjusted $R^2 = 0.9695$, $P < 0.001$). Darkness is represented as shaded areas. Transcript levels are related to RNA quantity.

and B, D, and F), respectively. It became evident that ISPS protein concentration as well as activity was lower under DD than LL, reaching a maximum of 6 ng mg^{-1} protein for the concentration and 0.45 μkat kg^{-1} protein for the activity in the dark, and being over 4 or 7 ng mg^{-1} protein and 0.6 or 0.4 μkat kg^{-1} protein in the light (values for both experiments, respectively). This difference between values under dark and light conditions was not as obvious under LD conditions. For one experiment (Fig. 5A), it seemed that during the late night both protein concentration and enzyme activity go down slightly. However, these variations are not significant and could not be observed in the second experiment (Fig. 5B). Globally, we observed a tendency for both activity and protein concentration to peak around midday, but no clear pattern could be statistically extracted. In addition, under LL conditions, enzyme activity and protein levels of ISPS seem to increase over time (Fig. 5, C and D).

Another interesting feature is the correlation between protein level and PcISPS activity when both experiments are analyzed separately, the activity-to-protein ratio indeed being lower in the second experiment (Fig. 5, B, D, and F) than in the first one (Fig. 5, A, C, and E). However, some exceptions to this correlation occur, particularly under LD conditions of the first experiment. Indeed, on the second day, protein levels

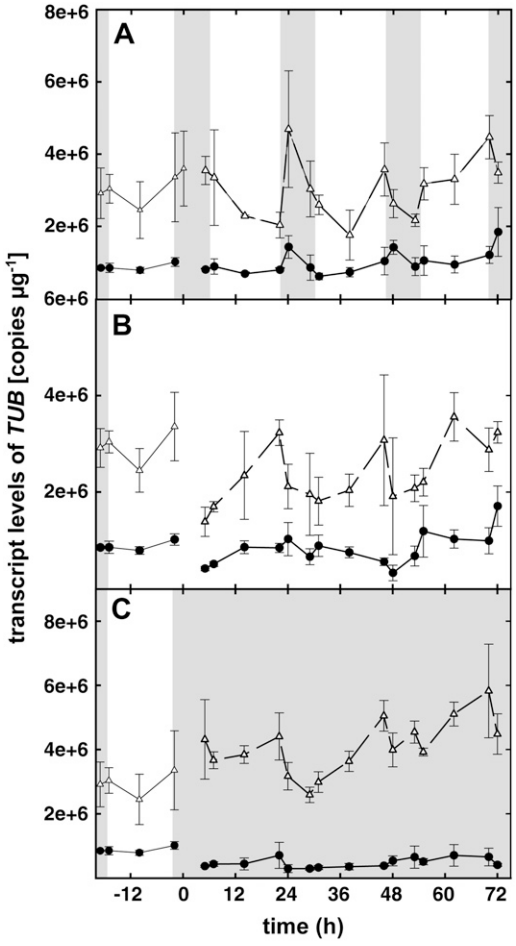

Figure 4. *PcTUB* gene expression under LD (16-h light/8-h dark) conditions (A), under LL (B) conditions, and under DD conditions (C). In each graph, the two curves represent two independent experiments ($n = 3 \pm$ SE) and the curves shown in the first 24 h of each graph represent the mean of 3-d expression levels under LD conditions. Darkness is represented as shaded areas. Transcript levels are related to RNA quantity.

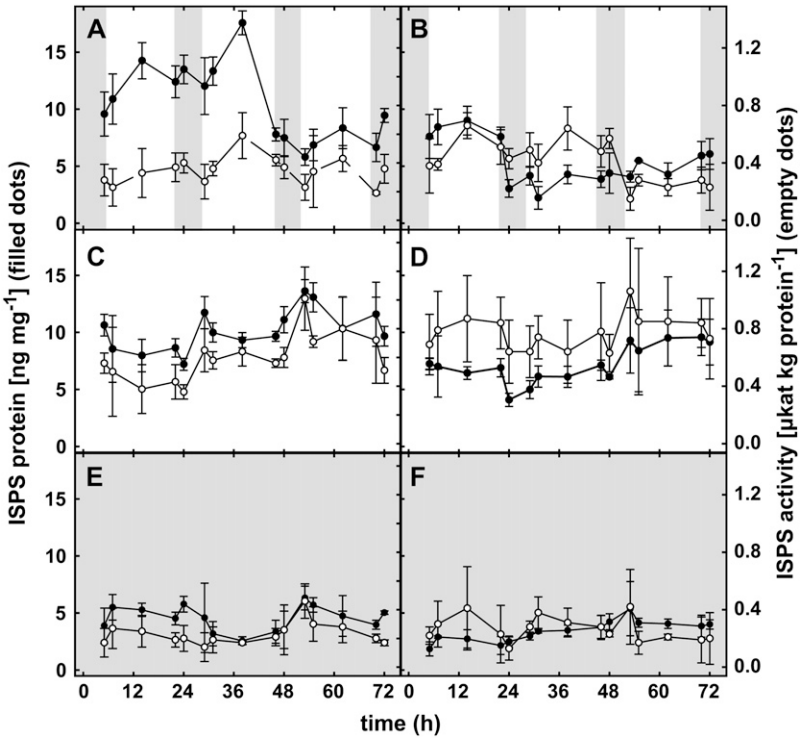

Figure 5. PcISPS protein quantity and enzyme activity under LD (16-h light/8-h dark) conditions in the first (A) and second (B) experiment, under LL conditions in the first (C) and second (D) experiment, and under DD conditions in the first (E) and second (F) experiment, respectively. PcISPS protein quantity (●) and enzyme activity (○; $n = 3 \pm$ SD). Darkness is represented as shaded areas.

stayed low (after a decrease during the night), whereas the level of activity appears as high as during the first day. At the third day, protein and activity were both at lower levels. These discrepancies between protein level and enzyme activity resulted in different turnover (K_{cat}) values ranging from 3.7 to 5.1 mmol isoprene mol^{-1} ISPS under both darkness and LD conditions and under LL, respectively.

Emission of isoprene from the shoot cultures followed a clear diurnal pattern under LD conditions, as shown in Figure 6, A and B (first 48 h) for representative samples, being low overnight and high and stable over the course of the day. When shoots were placed in DD (Fig. 6B, after the first 48 h), isoprene emission dropped down very quickly after switching off the light and, subsequently, declined with a slower rate over the following 3 d. This is not surprising because gene expression, protein concentration, and enzyme activity are all switched down under such conditions. Remarkably, isoprene emission of shoot cultures does occur, even if at a low rate, under darkness. Under LL (Fig. 6A, after the first 48 h), we detected fluctuating isoprene emission rates with a 24-h period between two peaks, therefore defining circadian rhythm. Figure 6C shows the mean of relative values for three independent shoot cultures. Under LL conditions, isoprene emission displayed clear, daily changes on the third and fourth day after onset of LL. On these days, emission was at its strongest (at about 86 h), approximately 20% higher than emission at its lowest (at about 78 h) during the previous 24-h cycle and approximately 30% higher (at about 114 h) than the lowest (at about 100 h) emission in the last 24-h cycle. Relative values (minimal value brought to 0, maximal to 1) of fluctuation were tested against a sinusoidal curve, but, because of the decline in isoprene emission during the first 2 d, it was only possible to fit a curve on the data of the last 2 d. The fluctuation of isoprene emission during these days was highly significant (adjusted $R^2 = 0.9951$, $P < 0.0001$; Fig. 6C), clearly testifying for circadian rhythm of isoprene emission. The highest rate of emission always occurred in the subjective afternoon, showing a switched circadian rhythm phase of isoprene emission under LL.

It is obvious that addition of water onto the agarose surface, even without touching the leaves and disturbing the gas flow, reduced the isoprene emission rate. Gas-exchange analysis of culture glasses after removal of the green tissue parts (stem and leaves) revealed that isoprene emission (background) was approximately 10% of the initial values.

Circadian Regulation of the *PcISPS* Gene Promoter

To get more insight into the circadian regulation of *PcISPS*, we isolated the promoter region of this gene by gene walking and primers designed for putative sequences available in the poplar genome databank and other databases (Fig. 7). The isolated sequence (accession no. AJ294819) of 1,612 bases (1,434 in front of the ATG codon) was aligned with the putative sequence from *Populus trichocarpa* (obtained from the draft genome) and with the published sequence from *Populus tremuloides* (accession no. AY341431). This alignment

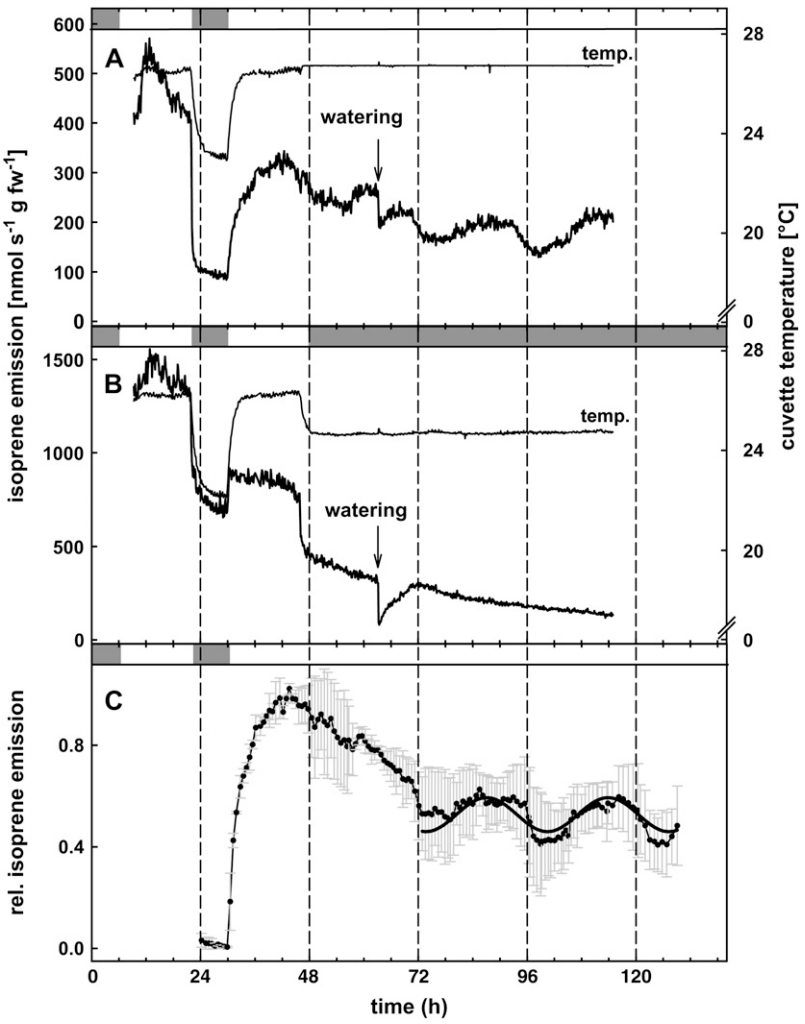

Figure 6. Isoprene emission from cell-cultured poplars under LL conditions (A) and DD conditions (B). C, Related isoprene emission data under LL conditions (15 min means; $n = 3 \pm$ SD) with sinusoidal regression ($y = y_0 + a \sin [2\pi x/b + c]$; with $a = 0.0670$; $b = 26.7854$; $c = 6.2800$; $y_0 = 0.5269$) on related data under LL conditions (mean of both experiments; adjusted $R^2 = 0.9951$, $P < 0.001$) for the last 2 d. After two day-night cycles, plants were placed either under LL (A) or DD (B; 3-min means). During the third day, water was added to the cuvettes to maintain humidity. The following decrease in emission is indicated. A and B, One experiment out of three replicates. Dark and light periods are presented with gray and white boxes above the figures.

explains the failure of some forward primers designed according to the *P. trichocarpa* sequence and extending on variable regions. In general, different sequences show a high degree of similarity (on the common parts), ranging from 90% between Grey poplar and *P. trichocarpa* to 94% between Grey poplar and *P. tremuloides*, respectively. However, some small regions are diverging because of gaps and/or insertions.

Regulatory elements were searched within the Grey poplar promoter sequence. Putative TATA and CAAT boxes are indications of the functionality of this sequence, TATA boxes being one type of core-promoter element essential for transcription initiation (Roeder, 1996). Using the databases plantCARE (Rombauts et al., 1999) and PLACE (Prestridge, 1991; Higo et al., 1999), many putative regulatory sequences were identified, particularly light, heat stress, and circadian-related ones. Moreover, two circadian elements (AAAAATCT) nearer to the start codon were found that are known to be recognized by late elongated hypocotyls (LHYs) and circadian clock-associated 1 (CCA1), two regulation factors enhancing morning expression of genes with such promoter elements (for review, see Devlin, 2002). In particular, this finding corresponds to the observation of the early expression of *PcISPS* in Figure 1A.

To prove the functionality of the cloned putative promoter, it was fused to the reporter genes coding for the enzyme β-glucuronidase and enhanced green fluorescent protein. This construct was then introduced into Arabidopsis and reporter genes were expressed in transgenic plants (data not shown), testifying that the *PcISPS* promoter is active in Arabidopsis plants.

For analyzing whether LHY may have a role in transcription of the *PcISPS* gene, fragment 1 and fragment 2 (Fig. 7) were amplified by PCR, each fragment carrying one putative binding box for this circadian regulatory protein. Heterologously expressed AtLHY protein was used to perform an electrophoretic mobility shift assay (EMSA) to test whether this factor binds to the identified circadian elements on the *PcISPS* promoter. As presented in Figure 8, lines 2 and 5, AtLHY is indeed binding to the *PcISPS* promoter fragments. The 200-fold molar excess of unlabeled DNA used in the binding reactions loaded in lines 3 and 6 proved that the observed shift (due to the binding of

Figure 7. *PcISPS* promoter sequence. The two circadian elements are shaded black. Core promoter sequences (TATA and CAAT boxes) are bold. Fragments 1 (top) and 2 (bottom) amplified for EMSA studies (see Fig. 8) are shaded light gray.

TAATACAACAAAATTTTATATGAAAAGGTTGAAAAACACTTAAATATATAAGCAAACTATACATACTATACAAAA
ATATCAATAAATTAATCTAAAATATAAATAAATAGGTTTGTTTACTTCATATAGTGAAAATTAAAAAAAAAGAAT
AAAAATAATGACGACACTATTAATTGTTGAGGTGATGGAATTTATAAAAATATATAACTCTAGATTTATGCTAAT
ATAGAAGGGGGAAGGGTGAATTTTTTGCATTTAAAGAAAAAAAAAGGAAGAAGAAGAAAAAGGGAGAAGCGGTCG
TTGTTAGTAATAATATTTTTAAATTTATTAATGGAATTTTCAACAAAAACTAGTGAACAAATATATTTTTTTTAT
TTTATCAATAAATAATAACGTGTCCCGTGCACTATCAATAAAATCACAAACAAAATATTTTTTAATTATTTTTAT
TCTATTGACAAATTCATCAAAGACAGAACATGTTCTGTCATTATTGTTATCAAACATTACAAATAGAATATTTTT
TTTTGCCAATAATTCCTTACTATTTAATAATTTTATAGCAGTGAGAAAGTATTTCCAATTGAGCAAATAAGGGGT
GGGGACCCATAAGCACGAAGTGTCACGTTTGAGACGTGGAAGGAAGAAAGGATGGATGCATTGTTCCATAATGGT
TTGTCATCATGCATGCATGTTCCCTTTGTTTTTTTTTCCTGGCTCGAAATTTGGCAGGCGTCAGTGTAAAGGAGC
AAAAGACATTGAGAAAATACACATCCATAAAAAAAAAAAAAAAAAAAAAAAAAGGAGGAAAAAAACAATGGAAAAT
ATACGACCATGAAAACCCTTTATTATATAACAATATTATGATTTACGTACAGACACTCGACCACCGTAAACCTTG
CTAATTGCTATAAAAAATTTTAGTCCACGTATTGATGATAACATTAGCACTTGGCCATCACCACAATCTTCAAAC
CAAAGGCAACGCTACCTTTTCTTTTCAGAACCTGTAATATCTTTTGAAAAAATTTCTGGTGCTGAATGCATCAAA
GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAATCAAGATTCCAGAAGCATCAGTGAATATGG
AAAAATCTGCAACAAAGACGTCTGAAATTGTCACCTTGCATGCCTCTCATGGAAAATATAGCTTCAACTGAACAT
TCCAGGTCTAATGTTGATTTCTGACGATTTTGACAACAGAGTAACACTTCACCATTTACTACAATCTATGGTGAG
CATGATGCCATGTAATAAACAGAAAAAAAATCTAACCAATCTTTTTATCCTATTAGAACAAGACCTGGAACTGTA
GGTTCTATAAATGAGTTTACGTTTCTTGAATTGGCAATTTGAAACTTTGAGAAGCAAAGAGAGAGAGAGAAAATC
CTGCTGCAGTTTCCATTACTAGAGGC

AtLHY proteins onto *PcISPS* promoter fragments) is specific.

DISCUSSION

Gene expression of *PcISPS*, *PcDXR*, and *PcPSY* in Grey poplar leaves follows diurnal variations under day-night conditions, being indeed enhanced by light and reduced during night and under DD. This indicates a primary role of light as a trigger of the expression of these isoprenoid biosynthesis-related genes. Formation of the carbon-rich compounds, such as isoprenoids, requires carbon pools. Because carbon fixation occurs during daylight, the observed diurnal variations in isoprenoid biosynthesis-related genes appear logical. It had even been shown that 75% of the carbon used to produce isoprene in poplar leaves come directly from photosynthesis (Schnitzler et al., 2004).

The role of isoprene is still controversial, but, being either a valve to evacuate carbon or energy overflow (Rosenstiel et al., 2004; Magel et al., 2006) or protection against high temperature or oxidative stress (Sharkey and Singsaas, 1995; Loreto and Velikova, 2001; Affek and Yakir, 2002), it is produced during the day when these functions can be exerted. To anticipate the necessity for isoprene, the *PcISPS* gene expression level seems to start increasing before dawn and peaks early after it. If light is necessary for the expression of the three studied genes, it is not the only regulating factor for *PcPSY* and *PcISPS* whose approximately 24-h period-length variations go on, even if shifted in time, under LL. This feature testifies to a circadian clock element of regulation because, in a recent review, McClung (2006) has characterized circadian rhythms as endogenously generated features persisting under constant environmental conditions, typically under LL and/or DD. In our data, mRNA undergoes rapid damping after plants were transferred to darkness. A similar feature was shown on mustard (*Sinapsis alba*) germin-like protein (SaGLP; Heintzen et al., 1994), in which mRNA could no longer be detected after 1 d in DD, even if its expression is endogenously clock controlled. Like SaGLP, *PcISPS* and *PcPSY* are under circadian control, but light positively influences their expression.

The presence of circadian regulatory elements and putative light elements in the promoter sequence of *PcISPS* could explain the observed regulation of this gene by the circadian clock and by light. The presence of two morning elements known to be recognized in Arabidopsis by the self clock-regulated factors LHY and CCA1 could be responsible for the early peak of expression of *PcISPS*. Recently, Wilkinson et al. (2006) showed that isoprene emission in oil palm (*Elaeis guineensis*) is under strong circadian control, but it was not determined at which levels circadian regulation influences isoprene emission, even if the authors seem to link it with the daily fluctuations of *LHY* and *CCA1* transcript levels. LHY/CCA1 binding sites were observed previously to be conserved in light-harvesting complex gene promoters (Wang and Tobin, 1998) and also in several circadian-regulated gene promoters (McClung, 2000; Nozue and Maloof, 2006). Our experimental data, showing binding of AtLHY proteins to the two *PcISPS* promoter fragments containing a morning element, demonstrate that, in poplar, expression of *PcISPS* is probably controlled by the circadian clock at the transcriptional level.

TUB is a common housekeeping gene used as an internal control for data normalization in real-time PCR measurements. However, a recent analysis of common housekeeping genes in poplar, over the

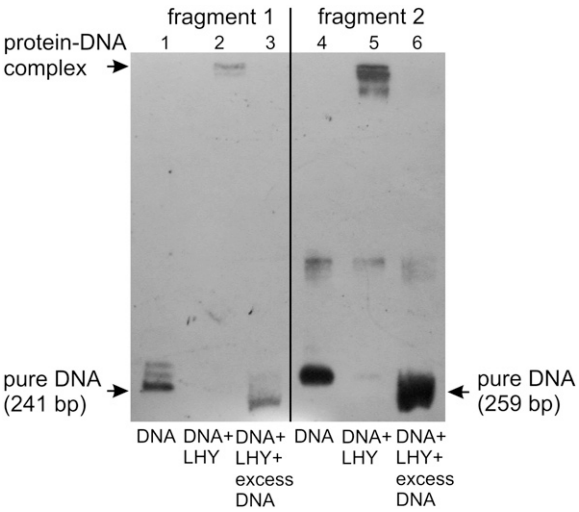

Figure 8. EMSA. Identification of two regions in the *PcISPS* gene promoter that contain target sequences for the LHY protein. Line 1, Biotin-labeled fragment 1; line 2, biotin-labeled fragment 1 and LHY protein; line 3, biotin-labeled fragment 1, LHY protein, and 200-fold molar excess of unlabeled fragment 1; line 4, biotin-labeled fragment 2; line 5, biotin-labeled fragment 2 and LHY protein; line 6, biotin-labeled fragment 2, LHY protein, and 200-fold molar excess of unlabeled fragment 2.

development, showed that TUB may not be the most appropriate housekeeping gene in this species under the tested conditions (Brunner et al., 2004). Concerning potential daily fluctuations of TUB transcript abundance, they had already been observed in developing tomato (*Lycopersicon esculentum*) fruit (Piechulla and Gruissem, 1987), but were not characterized in poplar. In this study, TUB transcript levels in poplar displayed low, but obvious, diurnal fluctuations with the tendency to increase during the night. Although the span of transcript-level variation of *PcTUB* was approximately 25% of the span of isoprene biosynthesis-related genes and therefore does not much influence the last ones when used for normalization, TUB as a housekeeping gene may not be the most appropriate, in particular when different time points during the day or different light regimes are to be studied.

Absence of a correlation between *PcISPS* gene expression level and PcISPS protein/activity detected at the daily level was also observed at the seasonal level (Mayrhofer et al., 2005). The discrepancy between protein quantity and activity indicates that PcISPS concentration does not only determine overall PcISPS activity. As already proposed in Mayrhofer et al. (2005) at the level of seasonal variation, posttranslational regulation of the PcISPS protein may exist that would present at least two forms with different activities. However, the factors involved in this third level of regulation are still to be discovered in future experiments.

In field conditions, isoprene emission from poplars was known to present daily variations linked to temperature and light intensity (Mayrhofer et al., 2005). Our results on poplar shoot cultures grown under controlled conditions confirm this by showing that, during the night, isoprene emission of the shoots is indeed dramatically reduced, but not zero. Moreover, if we consider the emission observed under DD (wrapped culture glasses in an enlightened chamber), it appears to be low, but not as low as during a real night (whole chamber put in darkness). An explanation for this higher emission in the shoot cultures could be the higher temperature maintained in the wrapped pot in comparison to pots in dark chambers (2°C–3°C more). Even if the effect of temperature was not the scope of our study, it should not be forgotten as an important factor influencing isoprene emission. Besides, as in darkness, no new carbon is fixed; this continuous emission of isoprene testifies for a carbon source other than photosynthesis. Plants were grown on Murashige and Skoog medium, which contains sugar. These artificial growing conditions (compared to soil) could therefore explain this unusual nightly emission of isoprene, the saccharose being a potential carbon source. However, an experiment with ^{13}C-labeled carbon would be necessary to confirm or refute this hypothesis. The low, but clear, variations of emission rate under LL present circadian periodicity and therefore testify to a clock element controlling isoprene emission. It is surprising that the first level of regulation of isoprene emission, namely, the expression rate of its synthesizing enzyme gene, and the emission itself present circadian rhythms, when neither the PcISPS protein level nor its activity display significant diurnal variations. Therefore, the observed fluctuations of emission do not seem to be due to PcISPS level variations. It is well documented that numerous genes and proteins involved in photosynthesis are clock regulated, as is fixed carbon allocation itself (Harmer et al., 2000). Because recently fixed carbon is the major pool used to produce isoprene in Grey poplar (Schnitzler et al., 2004), isoprene emission variations under LL may simply be the result of circadian fluxes of fixed carbon into the DOXP pathway. This would explain the lower amplitude of the fluctuations in comparison to LD cycles under which the instant PcISPS activity may vary.

DOXP pathway genes are regulated by development (Kuzma and Fall, 1993; Guevara-Garcia et al., 2005). *DXR* levels are shown to be at their highest values in young plants and during inflorescence development (Carretero-Paulet et al., 2002; Guevara-Garcia et al., 2005). In peppermint (*Mentha* × *piperita*), overexpression of *DXR* leads to higher accumulation of essential oil and cosuppression of this gene limits growth and leads to abnormal pigmentation (Mahmoud and Croteau, 2001), indicating a limiting and nonreplaceable role of *DXR* in the DOXP pathway for this species. Moreover, being the first committed step of the DOXP pathway, *DXR* is proposed to be one of the rate-limiting steps of isoprenoid biosynthesis (Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2002). However, because *PcDXR* levels appear in our study to be neither synchronized with *PcISPS* nor with *PcPSY*

fluctuations and, because they do not show circadian regulation, *PcDXR* may not be a key in daily regulation of the DOXP pathway and subsequent plastidic isoprenoid biosynthesis. Supporting our results, it is known that fluctuations of carotenoid biosynthesis in tomato do not require similar fluctuations of *DXR* (Rodríguez-Concepcíon et al., 2001). In addition, many studies suggest *DXS* as a regulating element of the DOXP pathway and carotenoid biosynthesis (Lois et al., 2000; Estévez et al., 2001; Guevara-Garcia et al., 2005). Therefore, it is possible that in Grey poplar no direct regulation of isoprene biosynthesis by *PcDXR* occurs. Knowing that *DXR* is not circadian regulated and that, in addition to its diurnal changes, its levels fluctuate strongly according to plant development (Carretero-Paulet et al., 2002), a possible significant role of *DXR* in the DOXP pathway more likely takes place in special stages of plant development.

PSY is the first dedicated and regulating enzyme of the carotenoid pathway (Von Lintig et al., 1997). Carotenoid functions are really diverse, ranging from primary metabolites involved in photosynthesis to secondary ones stored in chromoplasts (attracting pollinators and seed dispersers) or to vitamin and hormone precursors. Consistent with its role in synthesizing carotenoids for photosynthesis, this gene is highly expressed under light and repressed in the dark. Moreover, the main peak of *PcPSY* expression observed under LD early in the morning could be related to the need for the plant of these photosynthesis-related carotenoids. However, only further analysis of the downstream genes involved in this pathway could confirm this hypothesis. The circadian pattern observed under light conditions testifies to a role of the circadian clock in the regulation of *PcPSY* and may reflect the essential role of carotenoids in photosynthesis.

Gene expression of *PcDXR* and *PcISPS* is not synchronized, which raises the question of the pool of dimethylallyl diphosphate, a substrate of ISPS. Interestingly, dimethylallyl diphosphate pools have been shown to fluctuate diurnally in different species (Fisher et al., 2001; Brüggemann and Schnitzler, 2002), including poplar (Magel et al., 2006). Assuming that formation of photosynthates undergoes to a certain extent a circadian change, it might be hypothesized that the circadian change of isoprene emission is due to slightly enhanced metabolic flux within the DOXP pathway. To test this assumption, future experiments with stable $^{13}CO_2$ feeding may help to clarify whether there might be changes in the labeling rate of isoprene as an indicator for fluctuations in carbon supply for isoprene biosynthesis under continuous conditions.

MATERIALS AND METHODS

Plant Material, Growth, and Experimental Designs

Six to seven wild-type Grey poplar (*Populus* × *canescens*) shoots were grown on one-half-strength concentrated Murashige and Skoog (1962) medium (approximately 200 mL), in 1-L glass containers, for 7 to 8 weeks under standard conditions (27°C day/24°C night), using a 16-h light/8-h dark photoperiod (from 6 AM–10 PM) and 65 μmol m^{-2} s^{-1} photosynthetic photon flux density during the light period (for details of the cultivation procedure, see Leplé et al., 1992). DD was achieved by wrapping the jars in aluminum foil after which the temperature stayed at 25°C.

All experiments were repeated twice. Each sample consisted of all the leaves of one shoot. Nine containers (3/d over 3 d) were necessary per condition (LD, DD, and LL) for the circadian experiment. For all experiments, plants were placed in tested conditions at 10 PM the day before the first sampling.

For all experiments, samplings were done at 5 AM (1 h before the end of the standard night), 7 AM, 2 PM, 10 PM (just before start of the standard night), and midnight. For the darkness condition, containers were opened and samples were taken under red light. All samples, consisting of all leaves of one shoot, were immediately frozen in liquid nitrogen and stored at −80°C.

DNA Sequencing

Cycle-sequencing dideoxy chain termination reactions with Big Dye terminators (PE Applied Biosystems) were performed for both DNA strands of all DNA segments investigated, using universal forward and backward primers (Invitrogen) or sequence-specific oligonucleotides. Sequences were analyzed by using an ABI PRISM system 310 (PE Applied Biosystems).

To verify the specificity of primers on experimental cDNA, sequencing of purified products of real-time PCR was done by MWG Biotech AG using the same specific forward and backward primers as for quantitative reverse transcription (RT)-PCR.

Isolation of the *PcISPS* Promoter Sequence

Because the Grey poplar *ISPS* gene sequence (accession no. AJ294819) displays only 56 bases identified in front of the start codon, it was not possible to use it directly to find indications on the putative promoter sequence within the genome sequence of *Populus trichocarpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Therefore, the *PcISPS* sequence was BLASTed on the National Center for Biotechnology Information server to GenBank, revealing homologies with different terpene synthases, including two sequences of ISPS, one from *Populus alba* (accession no. AB198180) without a promoter part and one from *Populus tremuloides* (accession no. AY341431) displaying around 1 kb at the 5′ end of the start codon. This sequence (5′ end until the end of the first exon) was then used on the draft genome of *P. trichocarpa*. Sequences with the higher similarity were selected and aligned (using ClustalW; ClustalW WWW service at the European Bioinformatics Institute [http://www.ebi.ac.uk/clustalw]). The sequence with highest similarity to the *P. tremuloides* sequence was chosen and used for the second BLAST run on the genome. The same procedure was repeated three more times, until around 2.1 kb of the putative promoter region were identified. At different positions on the 5′ end of this sequence, 10 forward primers were randomly designed, each used combined to a reverse primer annealing in the exon 1 of the *PcISPS* gene (2325R primer designed in the first exon: CGTAATTGGCAGAGCGTCTG) with the following PCR conditions on a Biometra thermocycler, using polymerase platinum Taq (Invitrogen): 3-min denaturation at 94°C, 35 cycles at 94°C for 15 s, 50°C for 30 s, 72°C for 2 min, and 72°C for 4 min. The largest fragment (around 1.5 kb) was obtained with the primer 711F (CAAATAAACCTTAACATACAAATCATATTG). Two clones of this fragment were sequenced in both directions twice (nested sequencing, also repeated twice on both clones, had to be performed with internal primers because of the length of the sequence). Alignment of the obtained sequences leads to one sequence (published under accession no. AM084344) as the putative promoter region for the *PcISPS* gene, displaying 1,434 bp in front of the start codon.

RNA Isolation and cDNA Synthesis

Total RNA from frozen poplar leaves was isolated with the Qiagen RNeasy mini kit (Qiagen) following the Qiagen standard protocol. Amount and purity of isolated RNA were determined with NanoDrop ND-1000, a full-spectrum spectrophotometer having very high accuracy. The absorbance ratio 260/280 nm testified for very pure RNA, the mean of the samples ±SD being 2.116 ± 0.037.

For first-strand cDNA synthesis, 3 μg of total RNA were reverse transcribed using oligo(dT) primers and SuperScript II reverse transcriptase

(Invitrogen) in a total volume of 20 μL according to the manufacturer's protocol. cDNA was stored at −20°C prior to analysis.

Quantitative RT-PCR

For quantitative PCR measurements of transcript levels, two to three primers pairs were designed for each gene of interest with PrimerExpress software (version 2.0.0; ABI-Prism). Their efficiency was tested by RT-PCR on the reference plasmid containing a sequenced fragment of the gene of interest and on experimental cDNA. The primer pairs used for further measurements were selected as being the most efficient ones, giving similar amplicons (checked on agarose gel) and dissociation curve patterns from plasmid and cDNA templates. Their sequences are as follows: for *PcISPS*, forward (5′-tttgcctactttgccgtggttcaaaac-3′) and reverse (5′-tcctcagaaatgccttttgtacgcatg-3′); for *PcDXR*, forward (5′-gcatatgtcttttccagcttctattgc-3′) and reverse (5′-ggaatagtaggttgcgcaggc-3′); for *PcPSY*, forward (5′-atgcatcacatatcacacccaaa-3′) and reverse (5′-ctcctagcatcttctctccaacatctc-3′); for *PcTUB* (accession no. AY353093), forward (5′-gatttgtccctcgcgctgt-3′) and reverse (5′-tcggtataatgacccttggcc-3′). The resulting PCR segment lengths were 197 bp (*PcISPS*), 66 bp (*PcDXR*), 379 bp (*PcPSY*), and 151 bp (*PcTUB*), respectively. As a fluorescent marker for the increasing amount of double-stranded DNA, SYBR Green was used. The assays contained 12.5 μL 2× SYBR Green PCR master mix (Applied Biosystems), 300 nM of each primer, and 5 μL of total cDNA (diluted five times) in a final volume of 25 μL. After a hot start (10 min, 95°C), 45 PCR cycles were performed with a 15-s melting step at 95°C and a 1-min annealing/extension step at 60°C on a GeneAmp 5700 sequence detection system (Applied Biosystems). Transcript levels were related to the quantity of RNA used for RT. Transcript levels were calculated by the threshold cycle method with a standard curve under the GeneAmp 5700 sequence detection system software. To verify the amplification of the correct genes from poplar cDNA, all amplicons were purified from 0.8% agarose gels and sequenced.

Determination of PcISPS Activity

ISPS activity was assayed as previously described by Mayrhofer et al. (2005). A poplar-adapted plant extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 100 mM $CaCl_2$, 5% [v/v] glycerol, 0.1% [v/v] Tween 80, 20 mM dithiothreitol), with 250 mg polyvinylpolypyrrolidone was added prior to use and stirred for 15 min. Protein concentrations were determined by Bradford assay with bovine serum albumin as a standard.

Quantification of PcISPS Protein with ELISA

Quantification of ISPS protein was performed according to Schnitzler et al. (2005) using purified polyclonal anti-PcISPS IgG generated against N-terminal 6x-His-tagged PcISPS (Miller et al., 2001). For use as second antibody in ELISA, anti-PcISPS IgG was conjugated with horseradish peroxidase by BioGenes.

Heterologous Expression of LHY Protein from Arabidopsis and EMSA with *PcISPS* Promoter Fragments

Expression and purification of the recombinant Arabidopsis (*Arabidopsis thaliana*) LHY protein was performed using the AtLHY coding region fused to a C-terminal hexahistidine tag in pQE60 vector (Kim et al., 2003). Experiments were done according to QIAexpress protocols (Qiagen). Protocol 5 was used for the expression procedures, with the exception that *Escherichia coli* cells were disrupted by a French pressure cell press (SLM Instruments) two times at 1,000 psi and 0°C to 4°C. His-tagged AtLHY was purified under native conditions by affinity chromatography on a nickel-nitrilotriacetic acid column following QIAexpress protocol 11.

The following PCR fragments of the *PcISPS* gene promoter region were used in the experiment: the 241-bp-long fragment 1 amplified using the forward primer (5′-TAGTCCACGTATTGATGATAACA-3′) and the reverse primer (5′-AGGTGACAATTTCAGACGTC-3′), and the 259-bp long fragment 2 amplified using the forward primer (5′-CTGAACATTCCAGGTCTAATG-3′) and the reverse primer (5′-CCTCTAGTAATGGAAACTGCA-3′). As determined by in silico analysis, each fragment contains one putative binding box for the LHY protein. Biotin end labeling of the DNA fragments was carried out using the biotin 3′-end DNA-labeling kit (Pierce). The LightShift Chemiluminescent EMSA kit (Pierce) was used to detect whether the AtLHY protein binds to the *PcISPS* promoter. Experimental procedures (binding reaction and revealing the biotin-labeled DNA on the membrane) were performed according to the manufacturer's instructions. In binding reactions, 10 ng of biotin-labeled *PcISPS* promoter fragment DNA (control) plus 2 ng of AtLHY protein (binding reaction), and, finally, about 2,000 ng of unlabeled *PcISPS* promoter fragment DNA (to check the binding specificity) were used. Samples were loaded on a 6% DNA retardation gel (Invitrogen). DNA and protein were then semidried and transferred after half an hour at 300 mA to a nylon membrane (Qiabrane nylon plus; Qiagen).

Analysis of Isoprene Emission with Proton Transfer Reaction-Mass Spectrometry

Measurements of isoprene emission from shoot culture containers were performed with an adapted head-space analysis system using online proton transfer reaction (PTR)-mass spectrometry (MS), a combination of a PTR reaction drift tube and quadrupole MS. The instrument allows fast detection of most VOCs in combination with low detection limits (10–100 pptv; for details, see Lindinger et al., 1998; Schnitzler et al., 2004; Tholl et al., 2006). Measurements were performed on two gas-tight culture containers in parallel, each containing six to seven cell-cultured shoots, aged 6 to 8 weeks, partially with a developed root system. Clean air adjusted to a dew point of 28°C was flushed at 500 mL min^{-1} into the containers and from the outlet air 100 mL min^{-1} was pumped into the PTR-MS to analyze VOCs (mass 69 for isoprene). Measurements were performed on each container alternatively (automatically switched each 3 min with 60-s stabilization time). To avoid drying of the plants used in a confined environment, water was added carefully on the surface of the agarose, neither touching the leaves nor interfering with gas exchange (low changes of the pressure in the containers) at the beginning (50 mL) of and once during (30 mL) the experiment. Temperature was measured continuously inside containers with thermocouples.

The first 24 to 48 h of the experiments were used to flush excess isoprene previously accumulated in the containers during the development of the plants and to let the plants adapt to the constant gas stream. After the isoprene level appears stable, isoprene emission was measured over a LD day-night cycle. At 10 PM of the following day, one container was placed in LL and the other in DD (covered with aluminum foil) and emissions were measured during three virtual day-night cycles.

For calibration of PTR-MS, a gas standard (Apel-Riemer) with a continuous flow (20 mL min^{-1}) of a mixture of VOCs, including isoprene at 1.05 ppmv, was diluted into the gas stream of 500 mL min^{-1} and flushed through an empty container for one-half hour at the beginning and end of the experiments. Because the sensitivity of the PTR-MS went slightly down during the measuring period, the standard curve for isoprene through the experiment was corrected according to the declining signal of the primary ion (mass 21, deuterium isotope of H_3O^+). At the end of the experiments, isoprene emission from agar and roots (green material removed) was measured and these background values were subtracted from previously measured global isoprene emissions.

Statistical Analysis

Statistical and correlational analysis was performed with SPSS for Windows NT (release 8.0.0) and SigmaPlot for Windows (version 9.0), both programs from SPSS.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AM084344.

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