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Overexpression of *Populus* × *canescens* isoprene synthase gene in *Camelina sativa* leads to alterations in its growth and metabolism

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ABSTRACT

Isoprene (2-methyl-1,3-butadiene) is a hemiterpene molecule. It has been estimated that the plant kingdom emits 500–750 million tons of isoprene in the environment, half of which results from tropical broadleaf trees and the remainder from shrubs. Camelina (*Camelina sativa* (L.) Crantz) is an emerging bioenergy plant for biodiesel. In this study, we characterized isoprene formation following a diurnal/nocturnal cycle in wild-type Camelina plants. To understand the potential effects of isoprene emission on this herbaceous plant, a gray poplar *Populus* × *canescens* isoprene synthase gene (*PcISPS)* was overexpressed in Camelina. Transgenic plants showed increased isoprene production, and the emissions were characterized by a diurnal/nocturnal cycle. Measurements of the expression of six genes of the plastidial 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway revealed that the expression patterns of three key genes were associated with isoprene formation dynamics in the three genotypic plants. Conversely, dissimilar gene expression levels existed in different genotypes, indicating that dynamics and variations occurred among plants. Moreover, transgenic plants grew shorter and developed smaller leaves than the wild-type and empty vector control transgenic plants. Photosynthetic analysis showed that the CO_2 assimilation rate, intracellular CO_2 concentration, mesophyll conductance and contents of chlorophylls *a* and *b* were similar among *PcISPS* transgenic, empty-vector control transgenic, and wild-type plants, indicating that the transgene did not negatively affect photosynthesis. Based on these results, we suggest that the reduced biomass was likely a trade-off consequence of the increased isoprene emission.

1. Introduction

Since 1957, when the first plant was reported to produce isoprene (Sanadze, 1957), numerous different plant species have been described as isoprene emitters (Kesselmeier and Staudt, 1999; Sharkey et al., 2013). Particularly, many woody plants, such as those in the genus of *Quercus, Populus*, and *Salix,* have been identified as important isoprene producers (Loreto and Fineschi, 2015; Sharkey et al., 2013). Interestingly, it has been estimated that the plant kingdom emits 500–750 million tons of isoprene, approximately half of which results from the tropical broadleaf trees, and the other half is produced by shrubs (Guenther et al., 2006).

Isoprene (2-methyl-1,3-butadiene) is a hemiterpene, which is the simplest among terpenes. The biosynthetic pathway of isoprene is well understood in plants. Isoprene is synthesized in the plastids through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Fig. 1) (Phillips et al., 2008; Rohmer et al., 1993; Vickers et al., 2011; Wolfertz et al., 2004). The MEP pathway produces both the isopentenyl diphosphate (IDP) and the dimethylallyl diphosphate (DMADP). DMADP is the direct precursor of isoprene catalyzed by isoprene synthase (Fig. 1). The MEP pathway genes have been cloned from multiple plants. These genes encode *1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methylerythritol synthase/2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase (CMS/MCT), 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK), 2-C-methyl-Derythritol-2,4-cyclodiphosphate synthase (MCS), 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (HDS),* and *4-hydroxy-3-methylbut-2-enyl-diphosphate reductase (HDR)*. (Banerjee and Sharkey, 2014; Phillips et al., 2008) (Fig. 1). Moreover, DMADP is the primary starter C5 precursor to which the building block IDP is added to form mono- and diterpenes, carotenoids, plastoquinones, and the prenyl side chains of chlorophyll in plastids (Lange and Turner, 2013; Ma et al., 2015).

Numerous advances have been made in understanding the role of isoprene biosynthesis in plants. Evidence shows that the isoprene emission is associated with stress conditions and different temperatures

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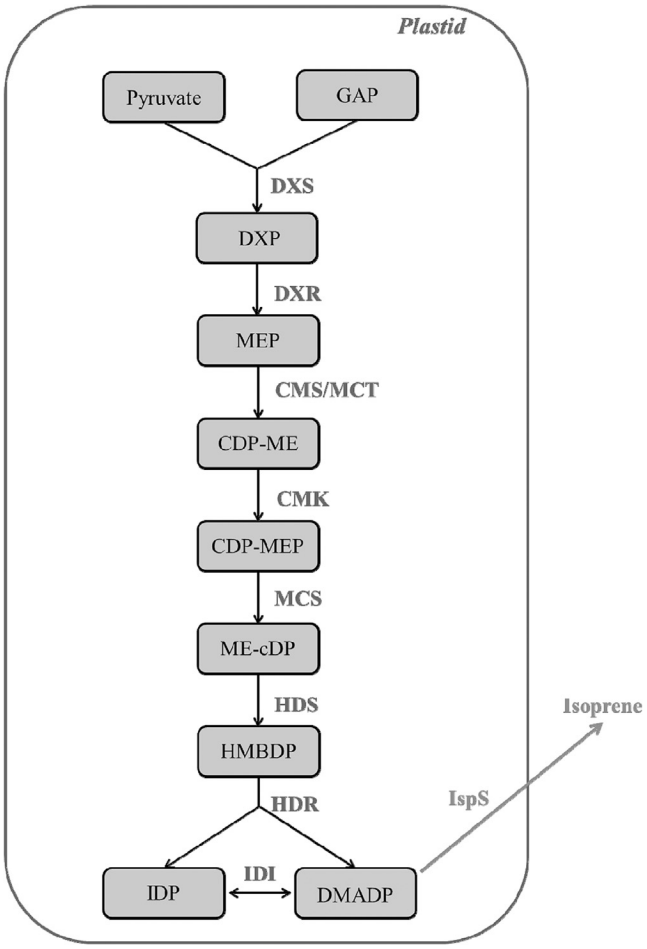

Fig. 1. Schematic representation showing the plastidial 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway toward the formation of isoprene in plastids. Gene abbreviation: *1-deoxy-D-xylulose-5-phosphate synthase (DXS); 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR); 4-diphosphocytidyl-2-C-methylerythritol synthase/2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase (CMS/MCT); 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK); 2-C-methyl-Derythritol-2,4-cyclodiphosphate synthase (MCS); 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS); 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR).*

(Mutanda et al., 2016a,b). Studies indicate that isoprene can enhance the thermo-tolerance and quench oxidative stress of woody plants, such as aspen (Sharkey et al., 2008). Moreover, the formation of isoprene can prevent leaf metabolic processes from damages associated with thermal (Sharkey and Singsaas, 1995; Singsaas et al., 1997) and oxidative stress conditions (Loreto and Fineschi, 2015; Loreto and Velikova, 2001). Other investigations suggest that isoprene emission serves as an overflow mechanism for excess carbon intermediates or photosynthetic energy (Logan and Monson, 1999; Logan et al., 2000; Rosenstiel et al., 2004).

Camelina sativa (L.) Crantz (Camelina) is a species of the Brassicaceae family. This annual plant has been traditionally cultivated as an oilseed crop for nutritional vegetable oil products in Europe and North America (Zubr, 1997). Camelina has emerged as a new biofuel crop that can grow in marginal lands; it does not need a high dose of fertilizer and produces a high yield of seed oil (Berhow et al., 2014; Borghi and Xie, 2016). In the past few years, its seed oil has been converted efficiently to jet fuel and has become a sustainable biofuel source (Allen et al., 2013; Berti et al., 2016; Mupondwa et al., 2016; Sainger et al., 2017). In a recent study (Xi et al., 2016), the overexpression of a synthetic plant-insect geranyl pyrophosphate synthase gene was shown to alter isoprene formation in Camelina grown in growth chambers significantly. Although no *ISPS (isoprene synthase)* gene has been elucidated in Camelina, the over-expression of *ISPS* gene from other plants allows the plants to produce and emit isoprene from their own metabolism.

The role of isoprene emission on herbaceous plant growth and biomass has not been determined so far. Moreover, Camelina is a newly emerging biofuel crop, and increasing its biomass is important for accelerate its application as a biofuel crop. Therefore, the objective of this study was to understand effects of increased isoprene formation on Camelina growth performance and predict the potential biotechnological application of isoprene emission reduction in increasing its biomass. In this study, we synthesized a *Populus* × *canescens* isoprene synthase cDNA and overexpressed it in Camelina. Multiple homozygous progeny lines were obtained from genetic transformation and selection. Isoprene formation was characterized for wild-type vs. transgenic plants in the greenhouse using natural light. Transgenic plants produced higher isoprene than control plants. Compared to control plants, transgenic plants grew smaller and developed smaller leaves. Moreover, we analyzed other physiological features and diurnal expression profiles of six MEP pathway genes. Our data indicate the potential for the future development of Camelina as a biofuel crop.

2. Materials and methods

2.1. Plant growth and sampling

Seeds of Camelina (*Camelina sativa* (L.) Crantz) var. Calena were stratified with water in a refrigerator (4 °C) for two days and were cultivated in plastic pots (8- × 9- × 12-cm) filled with potting mix soil (2P soil mix, Fafard, Inc., Anderson, SC, USA) in a greenhouse. The day/night temperature and relative humidity were 26/20 °C and 55/75%, respectively. Then, potting soil was watered once a day using tap water. After seed germination, the plants were supplied with 1 g L^{-1} of Osmocote fertilizer (The Scotts Miracle-Gro Company, Marysville, OH, USA) bi-weekly and watered daily. Both wild-type and transgenic plants were grown in these conditions for the duration of the experiment. By the end of the experiment, all plants were carefully divided into root, stem, and leaves, and separately weighted. Plant materials used for biochemical and molecular analyses was quickly frozen in liquid nitrogen and stored at −80 °C, or weighed for biomass.

2.2. Synthesis of an isoprene synthase gene and construction of the vector

A full length (2062 bp) of an *isoprene synthase* (*ISPS*) cDNA sequence from gray poplar (*Populus* × *canescens*, a hybrid of *Populus alba* and *Populus tremula*), was obtained from GenBank (sequence AJ294819). This specific *ISPS* was selected as it was previously shown to promote isoprene production in heterologous organisms (Loivamäki et al., 2007; Mayrhofer et al., 2005). The sequence fragment from position 39 to position 1868 was chosen from the full-length cDNA to obtain a fragment consisting of 1821 nucleotides, which encoded a full-length of amino acid sequence including the 5-end plastid transit peptide (PTP). Then, the attL and attR GATEWAYÔ sequences were added to the N- and C-terminals of this fragment, respectively. The resulting new sequence was synthesized by GenScript (Piscataway, NJ, USA) and cloned to the pUC57 entry vector to obtain a new plasmid, namely, pUC57-ISPS. The plasmid p7FWG2 (Karimi et al., 2002), which bears the constitutive p35S promoter and GFP reporter, was used to construct a vector to express *ISPS* in Camelina. To complete this, pUC57-ISPS and p7FWG2 plasmids were mixed and digested by attL and attR clonase to develop a new vector plasmid, namely, p7FWG2-ISPS, in which the synthetic *ISPS* was driven by a 35S promoter (Fig. 2A). This vector plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101::mp90(RK) using electroporation (Eppendorf, Hamburg, Germany). Positive colonies were selected on agar-solidified LB containing gentamicin (100 mg mL^{-1}), rifampicin (50 mg mL^{-1}), and spectinomycin (50 mg mL^{-1}). Moreover, the p7FWG2 plasmid alone, which we

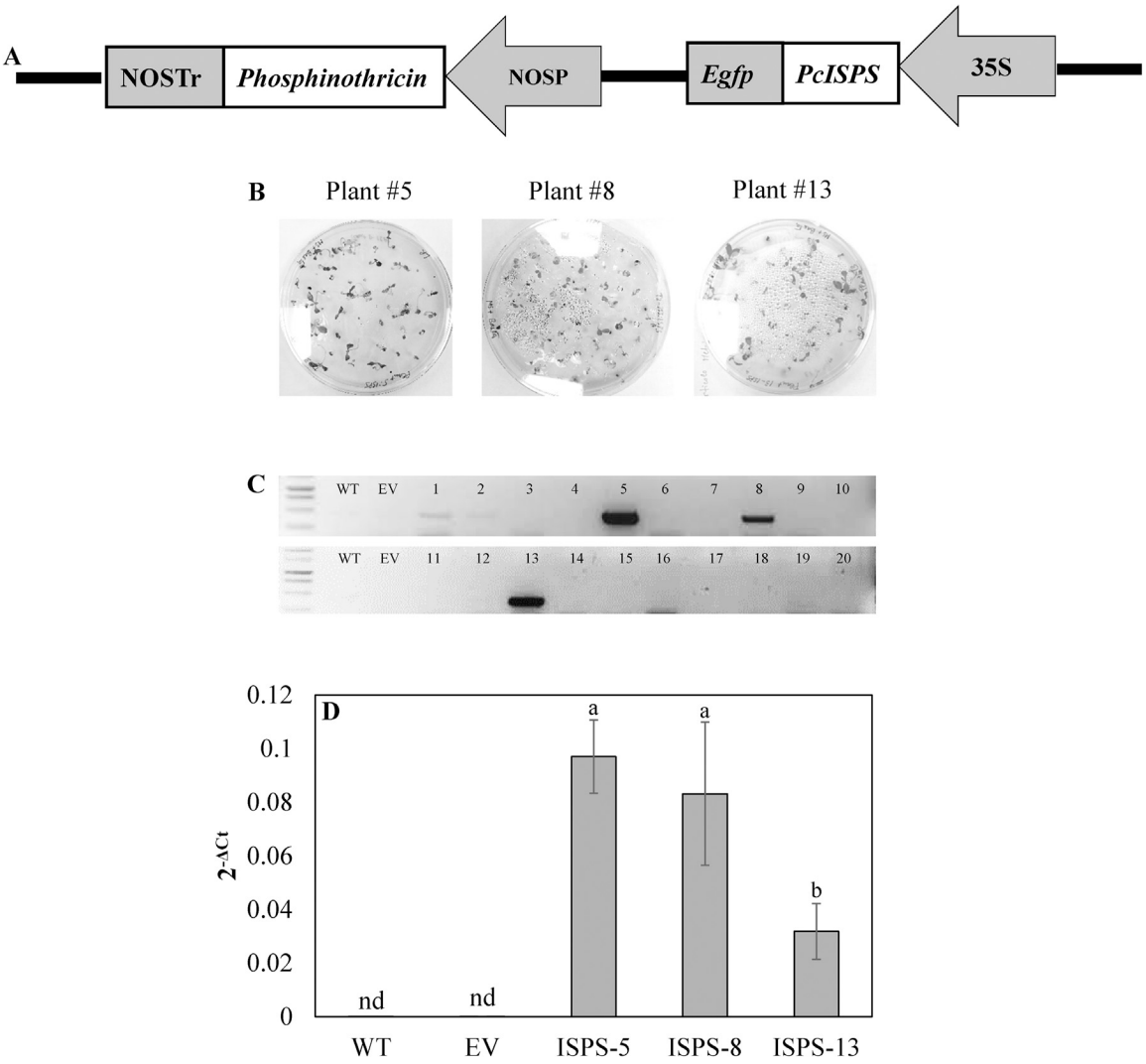

Fig. 2. Selection of transgenic plants. (A) A simplified cassette containing *PcISPS* driven by 35S promoter was constructed for genetic transformation of Camelina. (B) phosphinothricin-resistant seedlings. (C) PCR-based genotyping. (D) qRT-PCR showing expression of the *PcISPS* transgene expression in T1 plants. Level of *ISPS* mRNA measured in leaves of transgenic and wild-type plants. Data are presented as $2^{-\Delta Ct}$ and are calculated from three independent biological replicates per genotype. *CsaACT* was used for the normalization across the samples. Error bars represent the standard deviation.

used as an empty vector control, was also introduced into GV3101::mp90(RK) for genetic transformation as described below.

2.3. Plant transformation, selection, and identification of transgenic plants

In this study, we used a vacuum infiltration transformation method that was successfully developed for Camelina (Lu and Kang, 2008). Briefly, wild-type plants were grown in the greenhouse. After 40 days of growth, plants that started to flower were used for vacuum infiltration, as was reported recently (Borghi and Xie, 2016). *Agrobacterium tumefaciens* strain GV3101::mp90(RK) harboring the constructed binary vector was used for plant transformation. We dipped plants into activated *Agrobacterium tumefaciens* strain GV3101::mp90(RK) under vacuum twice in one week with an interval of 7 days between two dips. After infection, the plants were continuously grown to produce seeds in the greenhouse for further screening of transgenic plants.

The screening of transgenic plants germinated from seeds was performed on one half-strength basal MS medium (Murashige and Skoog, 1962). Briefly, basal MS medium was purchased from Caisson Laboratories (Smithfield, UT, USA), dissolved in double distilled water, adjusted to pH 5.5 and then autoclaved. The selection medium used was ½ strength MS medium solidified with 15 g L^{-1} agar and supplemented with phosphinothricin (Goldbio Technologies, St. Louis, MO, USA) with a final concentration of 80 mg L^{-1}. T1 seeds were sterilized with 70% ethanol for 2 min, followed by 35% Clorox for 10 min, and then thoroughly rinsed with autoclaved distilled water. The sterilized seeds were placed on the selection medium contained in Petri dishes. Seedlings that survived on the selection medium and were healthy (Fig. 2B) were planted in the potting soil for qRT-PCR genotyping analysis (described below). T2 seeds from each individual T1 plants were germinated on this selection medium contained in petri dishes. During selection, the survival ratio of seeds was calculated. Plant lines with a segregation ratio of 3:1 were selected for qRT-PCR analysis and a further selection of homozygous plants with a single copy of the transgene.

Young leaves were collected from 30-day-old T1 and T2 transgenic candidates vs. control plants grown in a greenhouse. Total RNA was isolated from young leaves with the Qiagen RNeasy Minikit (Qiagen, Redwood City, CA, USA) following the manufacturer's protocol. The concentration of isolated RNA was determined on a spectrophotometer (Thermo Scientific NanoDrop Products, Wilmington, DE, USA). For the first-strand cDNA synthesis, 3 mg of total RNA was reversely transcribed using oligo(dT) primers and SuperScript® II Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a total volume of 20 ml according to the manufacturer's protocol. The

resulting cDNA product was stored at −20 °C until use for RT-PCR.

Both semi-quantitative RT-PCR (Fig. 2C) and qRT-PCR (Fig. 2D) analyses were conducted to perform genotyping and determining the *PcISPS* transgene expression levels in the transgenic lines. One pair of primers was designed for the semi-quantitative PCR, DYX657F (5′-atgaacttcagggtcagcttg-3′) and DYX927R (5′-ttccgtatcctgctacatgcgtac-3′). The other pair of primers, DYX934F (5′-tccttatgtcactctcgaaccgg-3′) and DYX706R (5′-actatccttcgcaagaccc-3′), were designed for the qRT-PCR analysis described below.

2.4. Analysis of MEP pathway gene expression in both wild-type and transgenic plants

To analyze the expression profiles of the MEP pathway genes, young leaves from 30-day-old T2 plants were collected at 9:00 A.M., 12:00 noon, 5:00 P.M., and 9:00 P.M. from different genotype plants, which were grown side by side in the greenhouse. Leaves from the same node positions of the *ISPS* transgenic, empty-vector control, and wild-type plants were collected to extract total RNA. The collected leaf samples were immediately placed in liquid nitrogen and stored in a −80 °C freezer until use.

Frozen leaves were ground into fine powder in liquid nitrogen. One hundred milligrams of the powdered sample was extracted with Trizol Plus RNA Isolation kit (ThermoFisher Scientific, Waltham, MA, USA) by following the manufacturer's protocol. One hundred nanograms of total RNA was used for the synthesis of the first-strand cDNA with SuperScript Vilo cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. qRT-PCR reactions were performed in a StepOne Plus qRT-PCR machine (Applied Biosystem, Foster City, CA, USA) using 1.5 ng of cDNA mixture as a template. Genes selected for expression analysis included the *PcISPS* transgene in transgenic plants and six MEP pathway genes, *DXS, DXR, CMS/MCT, CMK, HDS,* and *HDR* (Fig. 1). Actin (*CsaACT*) was used as the reference gene for normalization across all samples. Gene-specific primers (Supplemental Table 1) were designed for each gene based on the DNA or RNA sequences of these genes in the GenBank using the software Primer Express (version 3.0.1, Applied Biosystem, Foster City, CA, USA). The thermal cycle for PCR consisted of stage I, 50 °C per 2 min; stage II, 95 °C per 10 min; stage III, 95 °C per 15 s, 60 °C per 1 min (repeated 40 times). Denaturation curves were run at the end of each PCR cycle to verify the specificity of amplicons. Expression values were obtained from measurements performed on three biological replicates.

2.5. Measurement of chlorophyll contents and photosynthetic parameters

Foliar disks, 7 mm in diameter, were excised from the fully expanded leaves of T2 plants. Four disks were used as one biological replicate to extract chlorophylls with 2.0 ml N,N-dimethylformamide (1:20, w:v) for 48 h at 4 °C in the dark. Then, 1.5 ml of the extract was used to measure chlorophylls *a* at 664 nm and *b* at 647 nm on a Helios-c spectrophotometer (Thermo scientific, USA). The resulting absorbance values were used to calculate chlorophyll contents according to the classic method developed by (Moran, 1982).

CO_2 assimilation rate, intracellular CO_2 concentration, and mesophyll conductance of 20-day-old T2 *versus* wild-type plants were measured using the LI-6400*xt* Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) connected to a 6 cm^2 Infra-Red chamber (LI-COR Biosciences, Lincoln, NE, USA).

2.6. Measurement of isoprene emissions

T2 *PcISPS* transgenic plants, vector control transgenic plants, and wild-type plants were grown in the greenhouse side by side as described above. Fast Isoprene Sensor (FIS, Hills Scientific, Inc., Boulder, CO, USA) (Guenther and Hills, 1998) was connected to a chamber (Fig. S1A). Before the measurement of isoprene from plants, the instrument was calibrated using an isoprene standard. Based on the instrumental manufacturer's protocol, a series of isoprene concentrations were tested to develop a standard curve. After calibration, 30-day-old plants were used for isoprene analysis. Two types of measurement periods were performed to understand isoprene formation from plants of three different genotypes. The first one was a two-hour period. This short time period measurement allowed the comparison of different plant genotypes during daytime from mid-morning to early afternoon, when the fluctuations of light intensity and temperatures were similar. The second time period spanned a longer duration of 24 h, enabling the comparison of the daily production of isoprene and characterization of diurnal dynamics. This isoprene measurement was carried out in April, the late spring in North Carolina, which is the period of the first cropping season of Camelina.

For the short period of measurements, the plants were measured for two hours. For the long period of measurement, the plants were continuously measured for 24 h. Light intensity (300–400 mmol m^{-2} s^{-1}) was examined daily at noon to ensure consistency during each measurement. Photons emitted every 5 s were counted and recorded using the FIS software. At the end of the experiment, all plants were carefully weighed and sampled for analyses. The measurements were repeated for several days with different biological replicates (n = 3).

Photon data were exported from the FIS software and elaborated using an excel spreadsheet following the method described in our previous report (Xi et al., 2016). Briefly, the concentration measured by the FIS was volumetric, ppbv (part per billion in volume). The initial isoprene concentration measured before starting the experiment was 160 ppbv in the chamber. The final results were based on the following assumptions: (1) leak rate into the chamber is 1.1 slpm (standard liters per minute), matching extraction flow rate into FIS; (2) the 160 ppbv isoprene level can be maintained continuously; (3) the leaks air entering the chamber contains 0 ppbv isoprene.

Based on the above parameters, the flux of isoprene from plants was calculated by:

Concentration × Flow

$$Flux = \left(\frac{ppbv\ of\ isoprene}{1\ L\ air}\right)\left(\frac{1\ mole\ isoprene}{22.4\ L\ isoprene}\right)\left(\frac{1.1\ L\ air}{1\ minute}\right)\left(\frac{60\ minute}{1\ hour}\right)\left(\frac{24\ hour}{1\ day}\right)$$

$$Flux = \left(\frac{mole\ isoprene}{1\ day}\right)$$

$$Flux = \left(\frac{mole\ isoprene}{1\ day}\right)\left(\frac{68.1\,g\ isoprene}{1\ mole\ isoprene}\right)$$

$$Flux = \left(\frac{g\ isoprene}{1\ day}\right)$$

$$Flux\ isoprene\ per\ gram\ of\ fresh\ plant\ material = \left(\frac{7.7\times10^{-4} g\ isoprene}{1\ day}\right)\left(\frac{1}{g\ wet\ plant\ material}\right)$$

Flux of isoprene per gram of fresh plant material per day

$$= \left(\frac{g\ isoprene}{g\ wet\ plant\ material \times day}\right)$$

2.7. Statistical analysis

The experiment was set up in a completely randomized design with three biological replicates (n = 3) for each treatment. One-way analysis of variance (ANOVA) and Tukey's pairwise comparison were used as post hoc tests ($P \leq 0.05$) after ANOVA. Statistical analyses were conducted using the Minitab 17 Statistical Software (Minitab Inc., State

College, PA).

3. Results

3.1. Overexpression of Populus × canescens isoprene synthase gene (PcISPS) in camelina

Multiple T1 transgenic lines were obtained from selection after putative transgenic seeds were germinated on ½ strength MS medium supplemented with the herbicide phosphinothricin (Fig. 2B). Semi-quantitative RT-PCR analysis showed that the resistant plants expressed the *PcISPS* transgene, which was not expressed in the wild-type and vector control transgenic plants (Fig. 2C). Moreover, this result was supported by qRT-PCR analysis (Fig. 2D). These plants were then grown on potting soil for obtaining seeds.

Multiple T2 seeds were obtained for the selection of progeny. The seeds were germinated on the MS medium supplemented with phosphinothricin, and multiple resistant T2 plants were obtained for further analysis. Semi-quantitative RT-PCR identified numerous T2 plants that highly expressed the *PcISPS* transgene (Fig. S2). These positive T2 seedlings were labeled and grown on the potting soil for isoprene estimation, and other physiological analyses (Fig. S1B). Moreover, T2 empty vector transgenic plants were grown as the control.

3.2. Comparison of growth and development between transgenic and wild-type plants

PcISPS transgenic T2 plants, empty-vector control transgenic T2 plants, and wild-type plants were grown side by side in the greenhouse. The *PcISPC* transgenic plants appeared shorter (Fig. 3A) and developed smaller leaves (Fig. 3B) compared with the wild-type and the empty-vector transgenic plants. The total fresh weight of the *PcISPS* transgenic plants was decreased by 40% (Fig. 3C) compared with the wild-type and the empty-vector transgenic plants. Moreover, the height of *PcISPS* transgenic plants was 10% shorter than the wild-type and the vector control transgenic plants (Fig. 3D). The P-value was higher than 0.05, indicating no differences in height (cm).

3.3. Photosynthesis analyses of transgenic versus wild-type plants

To understand whether the overexpression of *PcISPS* affected the other physiological processes, we used the LI-6400*xt* Portable Photosynthesis System to measure the CO_2 assimilation rate (Fig. 4A), the intracellular CO_2 concentration (Fig. 4B), and the mesophyll conductance (Fig. 4C). No significant differences were observed among the *PcISPS* transgenic, wild-type and vector control transgenic plants. Moreover, chlorophyll *a* and *b* contents were similar in the leaves of the three genotypic plants (Fig. 4D).

3.4. Isoprene formation increase from ISPS transgenic plants

Isoprene is synthesized from DMADP in plastids by isoprene synthase (*ISPS*) (Silver and Fall, 1991) (Fig. 1). FIS was used to measure daily isoprene emission from the WT plants grown in a greenhouse (Fig. S1A). In the presence of ozone, FIS specifically converts isoprene into photons, which are counted by a special photon counter associated with the instrument. To characterize isoprene emission, we completed the measurements of two different types of periods: two- and 24-h. FIS measurements were not initiated until the calibration of the instrument with an isoprene standard provided an ideal curve with a nearly perfect regressive efficiency value (Fig. 5A). The isoprene emitted by plants was recorded every 5 s. Multiple recordings in each measurement were performed to characterize the isoprene formation by Camelina. Moreover, during the measuring periods, we recorded the sunshine, cloudy periods and temperature (approximately 28 °C). The photon counts recorded from 2-h measurements showed a non-linear curve (Fig. 5B), indicating a dynamic profile. The recording for the 24-h period showed a dynamic isoprene emission profile that was characterized by a diurnal trend (Fig. 5C). The recorded curves showed that peak values increased starting from 6–7:00 A.M. in the morning through 4–6:00 P.M. in the early evening during daytime, while the values were relatively steady and low in the dark period from evening to early morning before 5:00 A.M. (Fig. 5C). These results showed that isoprene formation mainly occurred during daytime from the morning to early evening. The same method was used to measure isoprene emission from *ISPS* and empty vector transgenic plants. The 2-h measurement was carried out in the same time frame from late morning to early afternoon as used for the wild-type plants, but for different days. The resulting emission curves showed that the transgenic plants consistently emitted higher levels of isoprene than the wild-type and the vector control transgenic plants (Fig. 5B). The resulting emission curves showed that the transgenic plants consistently emitted higher levels of isoprene than wild-

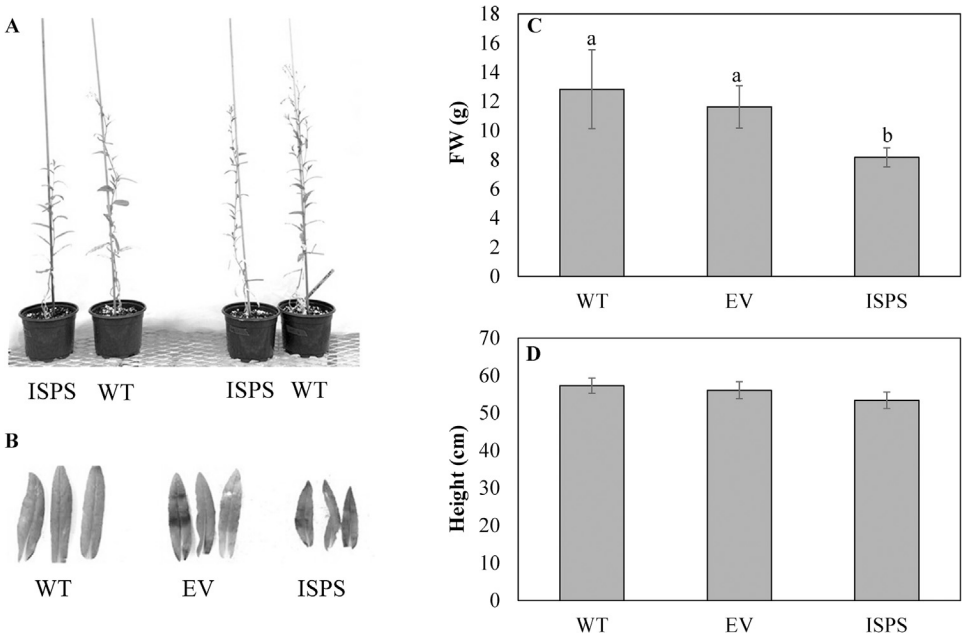

Fig. 3. Effects of *PcISPS* overexpression on plant growth. (A) Picture shows the height of *PcISPS* transgenic vs. wild-type plants. (B) Picture shows leaf sizes of *PcISPS* transgenic vs. wild-type plants. (C) The average fresh weight was calculated to compare biomass of three genotypic plants. (D) The average height value was calculated to compare three genotypic plants. WT: wild-type; EV: empty vector; ISPS: transgenic ISPS plants. Statistical differences calculated by ANOVA followed by Tukey's post-hoc test ($P < 0.05$) are indicated by letters, data are means of 3 replicates ± SD.

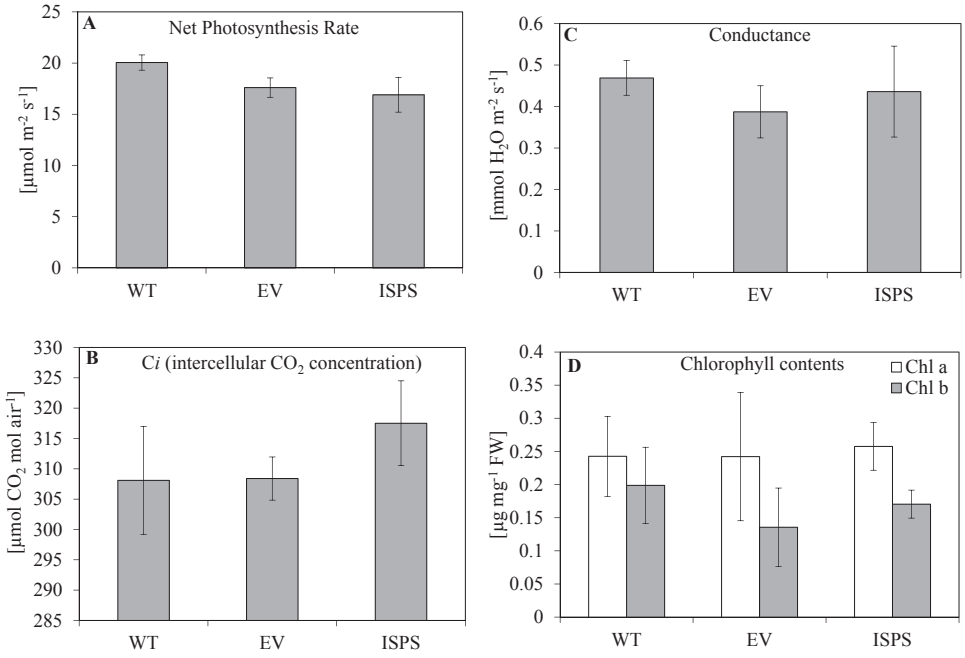

Fig. 4. Comparison of photosynthetic parameters measured using a LI-6400-XT Portable Photosynthesis System and Chlorophylls *a* and *b* contents. (A) CO_2 assimilation rate, (B) intracellular CO_2 concentration, (C) mesophyll conductance, (D) contents of chlorophylls *a* and *b*. Chlorophylls were extracted and measured according to Moran (1982). Statistical differences were evaluated by ANOVA followed by Tukey post-hoc test ($P < 0.05$) but data was found non-statically significant. Data were means of 3 replicates ± SD.

type and vector control transgenic plants (Fig. 5B). For the 24-period measurement, the transgenic plants could not be measured together with the wild-type and vector control transgenic plants because we only had one FIS. However, this did not affect the comparison of the *PcISPS* transgenic, empty vector control transgenic, and wild-type plants. Multiple repetitive measurements were performed for statistical evaluation. The resulting emission curves showed that *PcISPS* transgenic plants produced higher levels of isoprene during the daytime, approximately, from 7:00 A.M. to 5:30 P.M. (Fig. 5C). During the dark period from evening to the following early morning, the recorded curve from *PcISPS* transgenic plants was similar to those from wild-type and vector control transgenic plants. The living recording curves showed certain overlaid signals among the *PcISPS* transgenic, vector control transgenic, transgenic and wild-type plants (Fig. 5C). Based on our sunny and cloudy weather records, these overlaid signals were likely associated with sunny and cloudy moments or the sun rising and setting time due to the 24-h period difference between the first and the second measurements and the 48-h difference between the first and the third measurements. Although overlaid signals existed, the calculation of a total amount of isoprene emitted showed that transgenic plants significantly produced more isoprene (+40%) than wild-type and vector control transgenic plants during a 24-h period (Fig. 5D).

3.5. Diurnal expression profiles of the MEP pathway genes in the wild-type, PcISPS transgenic, and vector control transgenic plants

Expression patterns of six MEP pathway genes were analyzed using qRT-PCR. These included *DXS*, *DXR*, *CMS/MCT*, *CMK, HDS,* and *HDR* (Fig. 1). Based on isoprene formation patterns, four time points, 9:00 A.M., 12:00 noon, 5:00 P.M., and 9:00 P.M., were selected to collect samples for analyzing the gene expression profiles. These six genes were expressed with different patterns in the three genotypic plants at the four time points (Fig. 6).

DXS is the first committed gene of the MEP pathway (Fig. 1). Its expression level was the lowest at 9:00 P.M. in all three genotypes (Fig. 6A). Its peak expression level was observed at 12:00 noon in wild-type plants, at 12:00 noon and 5:00 P.M. in vector control transgenic plants, and at 9:00 A.M. and 12:00 noon in *PcISPS* transgenic plants. This expression pattern was closely associated with the isoprene emission dynamics (Fig. 5C). The *DXR* expression patterns were similar in all three genotypes (Fig. 6B). *DXR* expression reached a peak value at 5:00 P.M., followed by 12:00 P.M.; however, its expression levels at 9:00 A.M. and 9:00 P.M. were lower. The expression pattern was relatively associated with isoprene formation during day time. The expression of *CMS/MCT* in the three genotypes was the lowest at 9:00 P.M. (Fig. 6C). This expression pattern suggested its close association with isoprene formation during daytime (Fig. 5C). The *CMK* expression level was the lowest in all three genotypes at 9:00 P.M. (Fig. 6D). This suggested that its expression was associated with isoprene emission during the daytime. Interestingly, the *CMK* expression trend at the three daytime points in the *PcISPS* transgenic plants was different from those in the wild-type and vector control transgenic plants. Its expression peak time was at 12:00 noon in wild-type plants and vector control transgenic plants but was at 5:00 P.M. in *PcISPS* transgenic plants. The expression pattern of *HDS* was different in the three genotypes (Fig. 6E); it was different in the *PcISPS* transgenic plants from those of the wild-type and vector control transgenic plants (Fig. 6F). The expression levels of *HDR* in the wild-type and vector control transgenic plants were higher at three daytime points than at 9:00 P.M. However, in the *PcISPS* transgenic plants, its expression level reached a peak value at 12:00 noon. Overall, the data obtained revealed that the peak expression patterns of three key genes (*DXS*, *CMS/MCT* and *CMK*) were associated with isoprene formation dynamics in the three plant genotypes, although the lack of similarity between the results obtained in the two control lines (wild-type and empty-vector) indicated variations in gene expression levels.

4. Discussion

The overexpression of *ISPS* including our synthetic *PcISPS* reported here showed that isoprene emission from herbaceous plants could be increased. Camelina is a non-tropical herbaceous plant that was previously not reported to produce isoprene. Our recent study reported that Camelina emitted isoprene during winter (Xi et al., 2016) the period of which is the second cropping season of Camelina in North Carolina. Interestingly, our present transgenic study, conducted in late spring, further supported our previous research. Particularly, the overexpression of the synthetic *PcISPS* increased isoprene formation. These data suggested that the *in planta* isoprene formation is controlled by a Camelina *ISPS* homolog. *Arabidopsis thaliana* has been

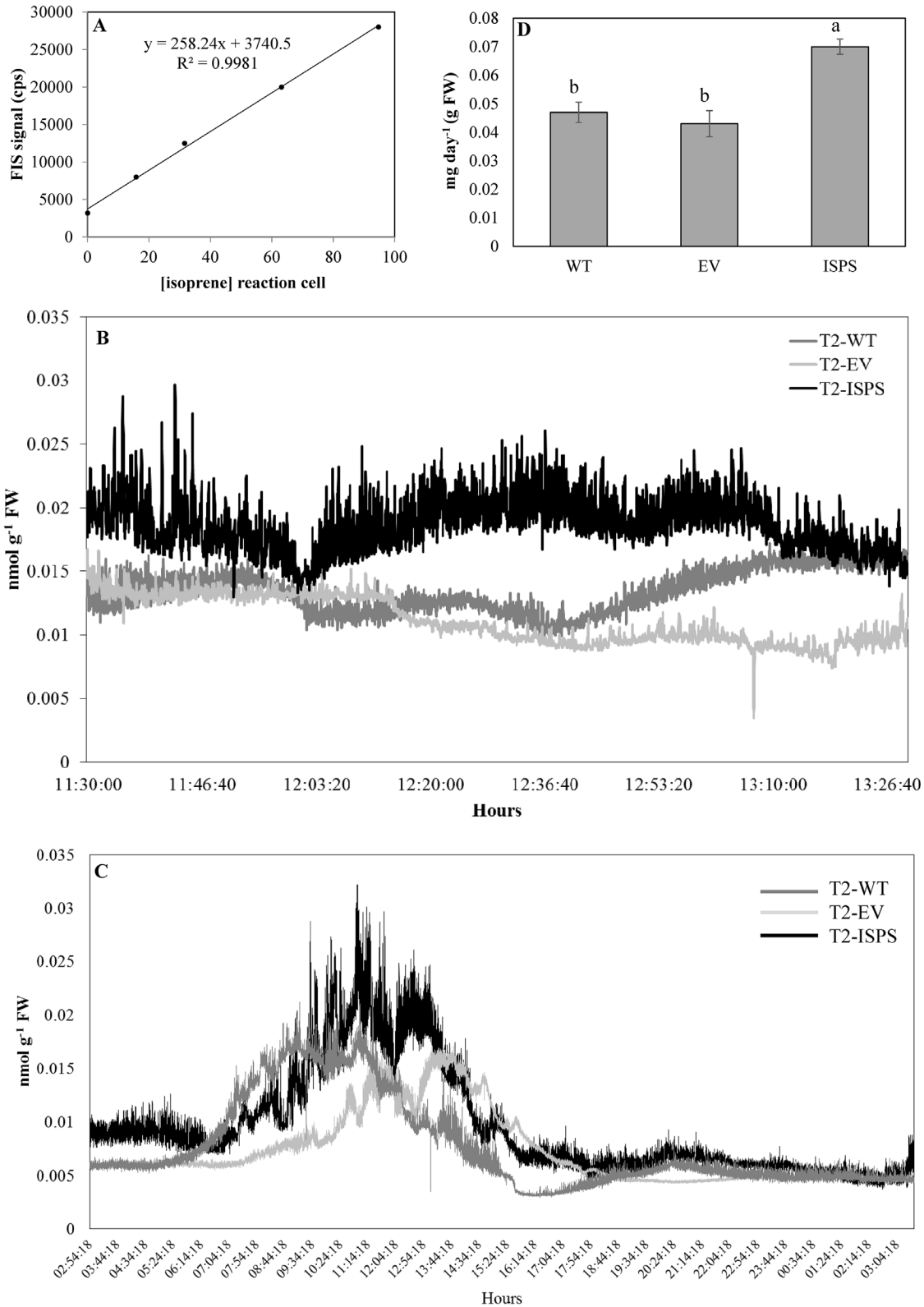

Fig. 5. Fast Isoprene Sensor measurements: (A) Standard curve obtained using standard isoprene for calibration. (B) Dynamics of isoprene emission from Camelina was recorded for approximately 2 h in the greenhouse under sunny and cloudy conditions. (C) Dynamics of isoprene emission from *PcISPS* (T2 progeny), EV control transgenic and WT plants continuously recorded for 24 h in greenhouse. (D) Comparison of daily isoprene emission from the *PcISPS*, EV control transgenic and WT plants. Data were exported from the FIS software and elaborated. Statistical differences calculated by ANOVA followed by Tukey's post-hoc test ($P < 0.05$) are indicated by letters, data are means of 3 replicates ± SD.

demonstrated to be a non-isoprene emitting plant, but transgenic Arabidopsis overexpressing a Kudzu *ISPS*, the 5′-end nucleotide sequences encoding the N-end with PTP could emit isoprene (Sharkey et al., 2005). A poplar *ISPS*, the 5′-end nucleotide sequences encoding PTP of which were removed, was overexpressed in tobacco plants. The resulting transgenic tobacco plants were shown to emit isoprene (Vickers et al., 2011), increasing their tolerance to ozone damage (Velikova et al., 2005), and reduce herbivore damages (Laothawornkitkul et al.,

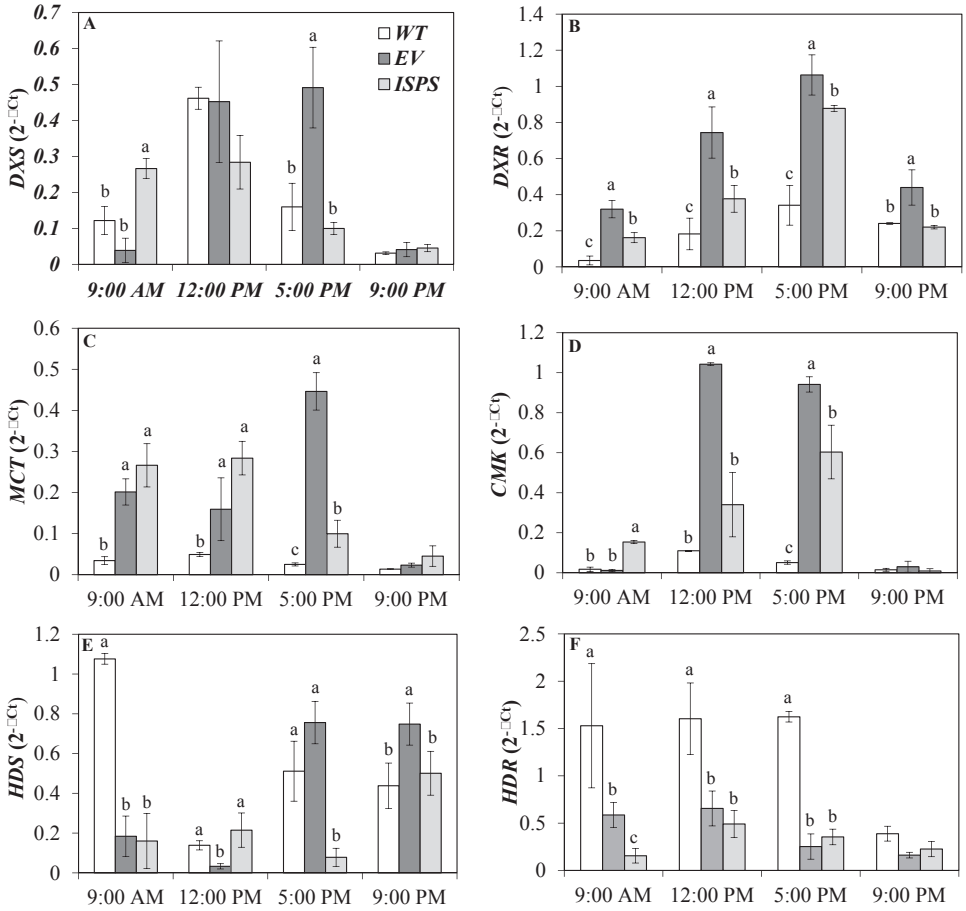

Fig. 6. qRT-PCR analysis showing expression profiles of six plastidial 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway genes at four time points during 24 h. (A) *DXS*; (B) *DXR*; (C) *CMS/MCT*; (D) *CMK*; (E) *HDS*; (F) *HDR*. Expression is represented as $2^{-(\Delta ct)}$. Error bars show standard deviation values. Statistical differences calculated by ANOVA followed by Tukey's post-hoc test ($P < 0.05$) are indicated by letters, data are means of 3 replicates ± SD.

2008). In our study, the synthesized *PcISPS* cDNA was composed of the full-length of its open reading frame sequence, which encodes amino acid sequence including the 5-end PTP. Based on our recent plastidial targeted protein localization in *C. sativa* (Borghi and Xie, 2016; Xi et al., 2016), we predicted that *PcISPS* was most likely localized in the plastids to convert DMAPP to isoprene. Moreover, we used a 35S promoter to overexpress the transgene; therefore, it was also possible that the residues of *PcISPS* could exist in the cytosol to associate with the formation of isoprene. Altogether, these studies, including the present study, provide evidence that the overexpression of ISPS could produce isoprene in herbaceous plants. These studies also imply that isoprene may be produced by more annual herbaceous plants.

Our study using three plant genotypes elucidated that the emission of isoprene from Camelina followed a diurnal cycle regardless of the overexpression of the *PcISPS* transgene. The formation of isoprene mainly occurred during the daytime in the greenhouse. All dynamic curves showed that isoprene emission was impacted by both sunny and cloudy times in a greenhouse. These observations provide evidence that isoprene formation in this annual herbaceous plant, as in woody plants (Wildermuth and Fall, 1996), is light-dependent. These results showed that the formation of isoprene is likely controlled by DMADP (Fig. 1), which is synthesized from the MEP pathway in plastids. Our data also supports the previous observation that the content of DMADP controls isoprene production in the *ISPS* transgenic tobacco plants (Vickers et al., 2011).

We observed that the T1 *PcISPS* transgenic plants grew shorter and developed smaller leaves than the wild-type and vector control transgenic plants. The total plant biomass of T1 *PcISPS* transgenic plants was significantly decreased. These results supported our previous observation regarding an association between isoprene emission reduction and an increase in plant growth. We overexpressed a synthetic plant-insect GPPS in the cytosol of Camelina, wherein this enzyme utilized DMADP and IDP to increase triterpenes and GA-like metabolites but led to significant reduction of isoprene emission (Xi et al., 2016). Thus, similar to these phenotypes, *GPPS* transgenic plants grew faster and developed bigger leaves than the wild-type and vector control transgenic plants. Our GPPS data and *PcISPS* data presented here suggest that the formation of isoprene competes with GPPS or farnesyl pyrophosphate synthase for the substrates IDP and DMADP. The overexpression of *PcISPS* led to the enhancement of metabolic flux from DMADP to isoprene, thus, decreasing this substrate content that is essential for all downstream metabolites, such as steroids and gibberellic acid. As a metabolic competition result, *PcISPS* transgenic plants showed morphologic alterations. Our observations can also be supported by other experiments that have attempted to overexpress the isoprenoid pathway. Many reports showed altered phenotypes of transgenic plants being restricted in growth due to the depletion of precursors (Aharoni et al., 2003, 2006; Fray et al., 1995). For example, the transformation of (*S*)-linalool and linalool derivatives synthase-encoding genes into *Arabidopsis* (Aharoni et al., 2003) or into potato (*Solanum tuberosum*) (Aharoni et al., 2006) led to altered phenotypes when higher linalool levels were detected. According to those authors, the change in phenotype might result from a reduction in precursor availability. Other isoprene-emitting plants were also reported to grow slower than the wild type (Aharoni et al., 2003, 2006; Fray et al., 1995). However, our experiments did not show that these developmental alterations resulted from a negative effect of the transgene overexpression on photosynthesis. The photosynthetic analysis did not show different CO_2 assimilation rates, intracellular CO_2 concentrations and mesophyll conductance between the transgenic and wild-type plants. Moreover, chlorophylls *a* and *b* concentration do not show any significant differences. Therefore, the enhancement of isoprene emission likely leads to a trade-off

consequence of biomass depletion.

Interestingly, our observation indicates a diurnal emission of isoprene in the three different plant genotypes compared with nocturnal. As observed in many woody species (Pier, 1995), Camelina produces more isoprene during the diurnal phase than during the nocturnal phase, regardless of the three genotypes. Many studies have demonstrated that this phenomenon is associated with the expression of the MEP pathway genes and isoprene synthesis gene (Bruggemann and Schnitzler, 2002). *DXS* converts the first step of the MEP pathway. Its activity is essentially associated with plant growth and metabolism. A disruption of *DXS* was reported to cause albino and other developmental defects in *A. thaliana* (Xing et al., 2010). In our study, we used qRT-PCR analysis to understand the expression patterns of the six MEP pathway genes. The resulting data showed that although the expression patterns of genes were associated with the diurnal dynamics of isoprene emission, the gene expression patterns were different in the two control lines (WT and EV), indicating variations in the expression levels of the genes (Fig. 6). The expression pattern of *DXS* showed a high diurnal activity, which was closely associated with the diurnal formation of isoprene. Moreover, our experiments revealed the highest expression levels of *DXR* and *CMK* in the diurnal phase. This phenomenon was also observed in the monoterpene biosynthesis in Arabidopsis. A different study in Arabidopsis (Aharoni et al., 2003) showed that when plants were transformed with a limonene synthase gene under regulation of a constitutive promoter, limonene emission followed a diurnal pattern. This characteristic could be related to the fact that intermediates are provided diurnally from the MEP pathway (Magel et al., 2006; Mayrhofer et al., 2005). In addition, other studies showed that not only is isoprene production in transgenic plants dependent on inserted gene expression and enzyme activity levels but is also regulated in a more complicated way. For example, *DXS* and *DXR* gene expression levels are similar in light-grown seedlings and the inflorescence (Carretero-Paulet et al., 2002).

In conclusion, Camelina transgenic plants showed that the overexpression of *PcISPS* increases isoprene emission but leads to a reduction in plant height and leaf biomass. Moreover, studies using three different plant genotypes showed that the formation of isoprene followed a dynamic diurnal/nocturnal trend. These results are informative for the metabolic engineering of Camelina for biofuel in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.06.005.

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