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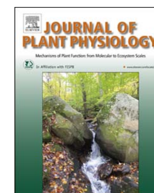

Physiology

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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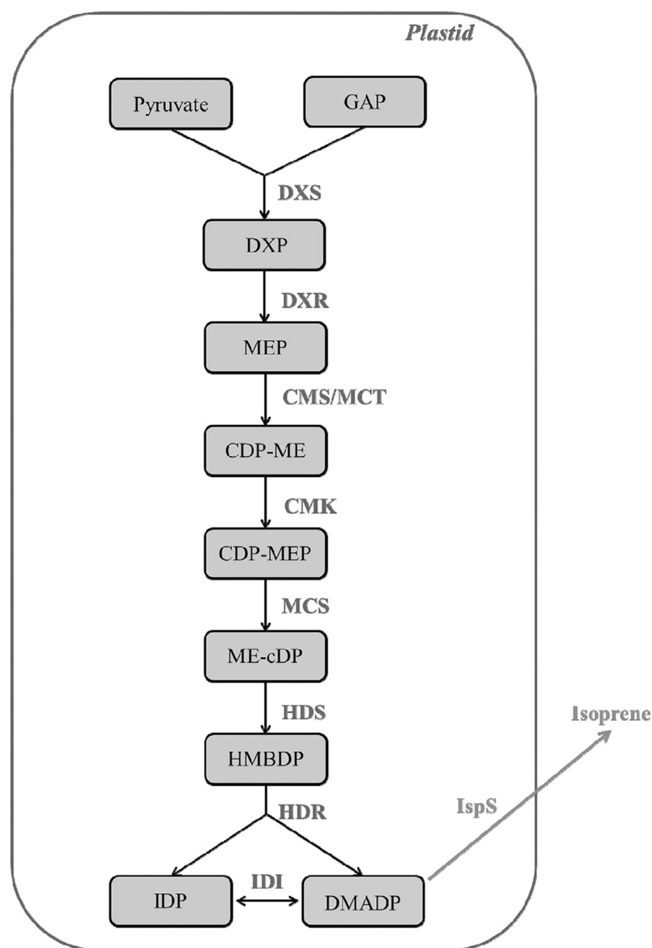

Fig. 1. Schematic representation showing the plastidial 2-C-methyl-D-erythriol-4-phosphate (MEP) pathway toward the formation of isoprene in plastids. Gene abbreviation: *1-deoxy-D-xylulose-5-phosphate synthase (DXS); 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR); 4-diphosphocytidyl-2-C-methylerythritol 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 over-expression 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

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