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Simultaneous over-expression and silencing of some benzylisoquinoline alkaloid biosynthetic genes in opium poppy

Seyyed Mohsen Sohrabi, Ahmad Ismaili*, Farhad Nazarian-Firouzabadi

Department of Agronomy and Plant Breeding, Lorestan University, PO Box 465, Khorramabad, Iran

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ABSTRACT

Opium poppy (*Papaver somniferum* L.) remains the only commercial source for several pharmaceutical alkaloids. In present study, opium poppy plants were genetically manipulated via VIGS technique using pTRV2-BBE (to silencing of *BBE1* gene), pTRV2-COM (to simultaneous silencing of *T6ODM* and *CODM* genes) and pTRV2-BBE-COM (to simultaneous silencing of *T6ODM*, *CODM* and *BBE1* genes) constructs. Also, via Agrobacterium-mediated transient expression technique, poppy plants were genetically manipulated using ACS (to simultaneous silencing of *T6ODM*, *CODM* and *BBE1*) and ACS-4'OMT2 (to simultaneous silencing of *T6ODM*, *CODM* and *BBE1* and over-expression of *4'OMT2*) constructs. HPLC analysis showed lower sanguinarine and noscapine levels and elevated levels of morphine in plants infiltrated with pTRV2-BBE construct. In plants infiltrated with pTRV2-COM construct, reduction in the morphine and codeine content and a substantial increase in accumulation of thebaine and papaverine were observed. A substantial increase in the accumulation of thebaine and papaverine and lower levels of morphine, codeine, sanguinarine and noscapine were observed in plants infiltrated with pTRV2-BBE-COM and ACS constructs. Plants infiltrated with ACS-4'OMT2 showed lower levels of codeine, sanguinarine and noscapine and elevated levels of morphine, thebaine and papaverine. Although, previous studies reported the role of our selected genes in benzylisoquinoline alkaloids (BIAs) biosynthetic pathway, but we simultaneously engineered these genes to alter the levels of specific BIAs and take advantage of all genes at the same time. For the first time, we showed different pattern in BIAs accumulation in simultaneous gene manipulation compared with single gene manipulation in previous reports, especially for higher levels of thebaine (about 1550%) and papaverine (about 155%). We also determined potential role of *BBE1* gene in noscapine biosynthesis. This is the first report of simultaneous silencing and over-expression of biosynthetic genes in opium poppy and our finding could establish vast potential for metabolic engineering in opium poppy.

1. Introduction

Plant secondary metabolites are a diverse group of natural product comprising over 200,000 known compounds (Neilson et al., 2013). Benzylisoquinoline alkaloids (BIAs) are a major group of these compounds, found in species of the *Papaveraceae*, *Berberidaceae*, *Ranunculaceae*, and *Menispermaceae* plant families (Beaudoin and Facchini, 2014; Liscombe et al., 2005; Winzer et al., 2012). Opium poppy (*Papaver somniferum* L.) is one of oldest medicinal plants and different therapeutic properties of this plant have been known since ancient times. Yet over the years, it remains the only commercial source of the morphine, codeine and semisynthetic derivatives such as hydrocodone, oxycodone, naltrexone, and buprenorphine. The opium poppy also produces a number of other BIAs including thebine, papaverine, noscapine and sanguinarine (Allen et al., 2008; Beaudoin and Facchini, 2014). Morphine is one of the most powerful analgesics and codeine is a mild painkiller and potent cough suppressant. Papaverine is used as a muscle relaxant. Noscapine and sanguinarine are potent anticancer and antimicrobial agents, respectively. Thebaine remains the source of medically useful derivatives such as oxycodone and buprenorphine (Lednicer, 2007). Chemical synthesis of most of these BIAs is not economically affordable (Rice, 1980). Metabolic engineering is an affordable way to produce BIAs and other plant secondary metabolites (Ehrenworth and Peralta-Yahya, 2017).

Metabolic engineering is one of the most valuable applications of plant biotechnology. In the metabolic engineering process, genetic and regulatory mechanisms within cells are altered to produce higher yields of a certain substance. Decreasing the yield of useless metabolites is also one of the aims of metabolic engineering (Glenn et al., 2013).

Many of the metabolic engineering studies have focused on the

* Corresponding author.
E-mail address: ismaili.a@lu.ac.ir (A. Ismaili).

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identification and manipulation of biosynthetic genes which catalyze the reaction in one or more steps in metabolic pathways. To date, several BIAs biosynthetic genes have been identified in opium poppy (Beaudoin and Facchini, 2014). Some of these genes are involved in two or more branches of BIAs biosynthetic pathways. For example, *4′OMT2* (3′-hydroxyl-*N*-methylcoclaurine 4′-O-methyltransferase 2), *TNMT* (tetrahydroprotoberberine N- methyltransferase), *DBOX* (dihydrosanguinarine oxidase), *CODM* (codeine O-demethylase), *T6ODM* (thebaine 6-O-demethylase) and *COR* (codeinone reductase) genes are involved in two branches of BIAs biosynthetic pathways. Furthermore, some other genes such as *BBE1* (berberine bridge enzyme), *CFS* (cheilanthifoline synthase), *SOMT1* (scoulerine 9-O-methyltransferase) and *SalSyn* (salutaridine synthase) are present in the critical points of noscapine, sanguinarine and morphinan alkaloids biosynthetic branches. So far, over-expression and silencing of these biosynthetic genes have led to altering the accumulation of some BIAs (Beaudoin and Facchini, 2014; Desgagné-Penix and Facchini, 2012; Wijekoon and Facchini, 2012; Winzer et al., 2012).

Previous studies have often focused on manipulation of single genes in BIAs metabolic pathways. In BIAs biosynthetic pathway, codeinone reductase (*COR*) reduces codeinone and morphinone to codeine and morphine, respectively (Beaudoin and Facchini, 2014; Unterlinner et al., 1999). Silencing of *COR* gene family in opium poppy resulted in the accumulation of reticuline and methylated derivatives with a corresponding reduction in the levels of codeine and morphine (Allen et al., 2004; Wijekoon and Facchini, 2012). In contrast, over-expression of *COR* resulted in a significant increase in thebaine, codeine and morphine content (Larkin et al., 2007). (S)-Reticuline is a very important intermediate in the biosynthetic pathway of most BIAs, including thebaine, codeine, morphine, sanguinarine, berberine and noscapine. (S)-Reticuline is formed from (S)-3′-hydroxy-*N*-methylcoclaurine through O-methylation by *4′OMT2* (Beaudoin and Facchini, 2014; Samanani et al., 2006). Knockdown of *4′OMT2* using virus-induced gene silencing (VIGS) reduced accumulation of the thebaine, codeine, papaverine and total alkaloid in opium poppy plants. On the other hand, over-expression of this gene increased the papaverine, noscapine and morphine amounts in the poppy tissues (Desgagné-Penix and Facchini, 2012; Gurkok et al., 2016).

Thebaine 6-O-demethylase (*T6ODM*) converts thebaine to neopinone, which undergoes rearrangement to codeinone. *T6ODM* also catalyzes the conversion of oripavine to morphinone (Beaudoin and Facchini, 2014; Hagel and Facchini, 2010). Systemic knock-down of *T6ODM* in opium poppy plants resulted in significant decrease in codeine and morphine content and increase in thebaine content (Hagel and Facchini, 2010; Wijekoon and Facchini, 2012). Codeine O-demethylase (*CODM*) catalyzes the O-demethylation of morphinan alkaloids and converts thebaine to oripavine and codeine to morphine. The VIGS-mediated suppression of *CODM* transcripts resulted in elevated levels of codeine and thebaine and also led to significant decrease in morphine content (Hagel and Facchini, 2010; Wijekoon and Facchini, 2012).

(S)-Scoulerine, the branch-point intermediate to protopine, sanguinarine, berberine and noscapine, is formed from (S)-reticuline by berberine bridge enzyme (*BBE*) (Dittrich and Kutchan, 1991; Facchini et al., 1996; Kutchan and Dittrich, 1995; Winkler et al., 2006). Suppression of *BBE1* resulted in elevated levels of reticuline, codeine, morphine and papaverine. On the other hand, suppression of *BBE1* reduced accumulation of sanguinarine and dihydrosanguinarine (Beaudoin and Facchini, 2014; Frick et al., 2004; Hagel et al., 2012).

Before developing of specific multi-gene engineering approaches, multiple transgenes were being transferred to transgenic plants using specific methods, such as successive transformation of transgenic plants with new transgenes and sequential crosses between different transgenic plants. Both methods are time-consuming, expensive and laborious (Farre et al., 2015; Naqvi et al., 2010). Multi-gene engineering do not have these limitations and allows researchers to achieve goals, such as the expression of entire protein complexes, the import of whole biosynthetic pathways and the development of simultaneously engineered transgenic plants (Naqvi et al., 2010). Metabolic pathways of valuable secondary metabolites are controlled by multiple enzymes. Therefore, to achieve the high production or reduction of specific metabolites, multiple genes must be simultaneously manipulated. Construction of simultaneous silencing and over-expression gene constructs is the crucial step in multi-gene engineering (Naqvi et al., 2010).

Most studies in *P. somniferum* so far have focused on single gene over-expression or silencing. In the present study, we silenced *BBE1* (to increase level of (S)-reticuline), *T6ODM* and *CODM* (to increase level of thebaine and decrease in codeine and morphine content) genes and over-expressed *4'OMT2* (to increase levels of (S)-reticuline and total alkaloid) gene in poppy BIAs biosynthetic pathway. Although these genes have been separately manipulated in the previous studies (Desgagné-Penix and Facchini, 2012; Dittrich and Kutchan, 1991; Facchini et al., 1996; Gurkok et al., 2016; Hagel et al., 2012; Kutchan and Dittrich, 1995; Wijekoon and Facchini, 2012), but we simultaneously manipulated these genes to take advantage of all genes at the same time and enhancing the levels of specific BIAs. To our knowledge, in the present study, for the first time we evaluated the effect of simultaneous silencing and over-expression of some genes on the changes in BIAs metabolic pathways in *P. somniferum*. We developed two groups of gene constructs to manipulation of important biosynthetic genes, including VIGS constructs (pTRV2-BBE, pTRV2-COM and pTRV2-BBE-COM) and Agrobacterium-mediated transient expression constructs (ACS and ACS-4'OMT2) (Figs. 1 and 2).

2. Materials and methods

2.1. Plant material and greenhouse growth condition

Seeds of *Papaver somniferum* (Iranian genotype) were potted in soil mixture consisting of peats and clay (60:40) and then transferred to a growth chamber. Pots were kept at 20/24 °C (light/dark) with a photoperiod of 16/8 h and fertilized once a week with a soluble NPK fertilizer (20:20:20). Leaves of young seedling were used for RNA extraction and subsequent uses. Leaves of one and two month-old plants were selected for virus-induced gene silencing and Agrobacterium-mediated transient expression experiments, respectively.

2.2. RNA isolation and cDNA synthesis

Harvested plant tissues were ground into a fine powder in liquid nitrogen. Total RNA was isolated using CinnaPure RNA extraction kit (SinaClon BioScience, Tehran, Iran) following the manufacturer's instructions. Quantity and integrity of the extracted total RNA was determined using spectrophotometer and agarose gel and then total RNA was treated with *DNase*I (Thermo Fisher Scientific, Lenexa, USA) to remove the genomic DNA contamination. First strand cDNA was synthesized from 1 µg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lenexa, USA). The synthesis of cDNA was confirmed by PCR using specific *Ef1* gene primers (Table S2).

2.3. Isolation of 4′OMT2 cDNA

To identify cDNA encoding *P. somniferum* 4′*OMT2* (for applying in over-expression experiment), the GenBank was searched for known 4′*OMT2* genes from this species. A single full-length sequence (AY217334.1) was identified and used to search the *P. somniferum* EST library. The BLASTn search against the *P. somniferum* EST library revealed 12 ESTs. The identified ESTs were assembled in Vector NTI 10.3 software (Invitrogen, Carlsbad, USA) and 2 contigs comprising full-length ORF were obtained. Multiple alignment showed 80–100% identity among identified sequences (100% between AY217334.1 and contig 1; 84% between AY217334.1 and contig 2; 84% between contig