Improvement of tropane alkaloids production in hairy root cultures of *Atropa belladonna* by overexpressing *pmt* and *h6h* genes

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Abstract

Atropa belladonna L., the commercial plant to produce tropane alkaloids including hyoscyamine and scopolamine, which are widely used as anticholinergic agents. In the present study, the genes encoding the key enzymes including putrescine N-methyltransferase (PMT) and hyoscyamine 6β-hydroxylase (H6H) were simultaneously overexpressed in transgenic hairy root cultures of *A. belladonna*. All of the nine transgenic hairy root lines overexpressing both *pmt* and *h6h* produced tropane alkaloids at higher levels. The best line T3 produced 2.2 mg/g dry weight (DW) hyoscyamine, which was about 11 times more than that in non-transgenic hairy root cultures and 24 times more than that in the wild type (intact *A. belladonna*). Simultaneously, T3 produced 1.0 mg/g DW scopolamine, which was five times more than that in untransgenic hairy root cultures and four times more than that in the wild type. This is the first report of engineering the tropane alkaloids biosynthetic pathway via overexpressing both *pmt* and *h6h* in *A. belladonna* and the result demonstrated that the metabolic engineering strategy of BREAKING can facilitate accumulation of tropane alkaloids in *A. belladonna*. Further, it is also proved that transgenic hairy root cultures of *A. belladonna* can be used as bioreactor to produce pharmaceutical tropane alkaloids.

Keywords: *Atropa belladonna*, H6H, Hairy root, Metabolic engineering, PMT, Tropane alkaloids.

Abbreviations: CaMV-cauliflower mosaic virus; DW-dry weight; FW-fresh weight; H6H-hyoscyamine 6β-hydroxylase; HPLC-high performance liquid chromatography; Min-minute; MS-Murashige and Skoog; NPTII-neomycin phosphotransferase; PCR-Polymerase chain reaction; PMT-putrescine N-methyltransferase; Ri-root inducing; SDS- Sodium dodecyl sulfate; TAs-tropane alkaloids.

Introduction

Atropa belladonna L., commonly known as belladonna or deadly nightshade, is a perennial herbaceous plant and most important commercial source of pharmaceutical tropane alkaloids (TAs) in the family of Solanaceae (Zhang et al., 2005). This plant species (*A. belladonna*) is of interest due to its production of pharmaceutical tropane alkaloids, including hyoscyamine and scopolamine, which are widely used as anticholinergic agents that act on the parasympathetic nervous system (Guggisberg and Hesse, 1983) However, the commercial supply of *A. belladonna* is limited because of the low abundance of TAs in natural plants. For example, the content of scopolamine in *A. belladonna* is less than 0.1% DW. Hairy root cultures are viewed as an efficient means of biomass production due to the fast growing hairy roots are unique in their genetic and biosynthetic stability (Carvalho and Curtis, 1998). Furthermore, the root is the main organ of secondary metabolites biosynthesis in *Panax ginseng* (Li et al., 2005), *Astragalus species* (Motomura et al., 2009), *A. belladonna* (Hashimoto et al., 1991) and etc. Thus, hairy root cultures offer promise for production of valuable secondary metabolites in many plants, and large-scale cultures of hairy roots of several medicinal plant species have been reported so far (Giri and Narasu, 2000). Molecular biology and biochemistry of TAs biosynthetic pathway have been well studied in TAs-producing plant species of Solanaceae (Zhang et al., 2005). Five functional genes involved in TAs biosynthesis respectively encode putrescine N-methyltransferase (PMT), tropinone reductase I (TR I) and II (TR II), CYP80F1 and hyoscyamine 6β-hydroxylase (H6H). This makes it possible to engineer the biosynthetic pathway of TAs in related plant species. Some of these genes have been employed to genetically modify the TAs biosynthetic pathway in different plant species such as *Hyoscyamus niger* (Zhang et al., 2004), *Datura* species (Moyano et al., 2003), and *Atropa* species (Rothe et al., 2003). Among the five genes, *pmt* and *h6h* are generally considered to be the rate-limiting-enzyme genes. PMT is the first alkaloid-specific enzyme committed for the biosynthesis of TAs, which catalyzes the N-methylation to form N-methylputrescine (Hibi et al., 1992). Regulation of the expression of *pmt* gene...
has been shown to be crucial for increasing alkaloid production in hairy root cultures of *Datura metel* and *Hyoscyamus muticus* (Moyano et al., 2003). However, in some cases, overexpression of the same exogenous *pmt* in the same constructs can not improve alkaloid production as expected. It was found that increasing *pmt* expression alone was not sufficient to increase alkaloid production respectively in *A. belladonna* plants (Rothe et al., 2003) or *Duboisia* hybrid root cultures (Moyano et al., 2002). Unlike the effect of PMT overexpression on increasing production of TAs, it can be concluded that overexpression of H6H increases scopoamine production without exception in the reported transformed TAs-producing plant species. In transgenic *H. muticus* hairy root cultures with *h6h* overexpression, the best clone produced 17 mg/l scopoamine, which is over 100 times more than the control clones (Jouhikainen et al., 1999); in *h6h*-overexpressing transgenic *A. belladonna* plants, the alkaloid contents in the leaf and stem were almost exclusively scopoamine (Yun et al., 1992); and in *h6h*-transgenic hairy root cultures of *Datura innoxia* and *Duboisia* hybrid, the production of scopoamine was increased over three times (Dechaux and Boitel-Conti, 2005; Palazón et al., 2003). Unfortunately, there are no reports on transformation of both *pmt* and *h6h* in *A. belladonna*. In the present study, both *pmt* and *h6h* were overexpressed in hairy root cultures of *A. belladonna*.

**Materials and methods**

**Strains and plasmids**

The bivalent plant expression plasmid pXI containing both *pmt* and *h6h* genes, which constructed by Zhang et al. (2004), was used in the study. The pXI contained two separate expression cassettes for *pmt* and *h6h*, both driven by the CaMV 3SS promoter and the nptII cassette for conferring kanamycin resistance. Plasmid pXI was isolated from *Escherichia coli* strain DH5α and transformed into disarmed *Agrobacterium tumefaciens* strain C58C1 containing *A. rhizogenes* Ri plasmid pRIaA. A positive clone, after confirmed by PCR and enzymatic digestion analysis for the presence of both *pmt* and *h6h* genes, was used to transform *A. belladonna* tissues for simultaneous expression of *pmt* and *h6h*. *A. rhizogenes* strain A4 was used to transform *A. belladonna* to generate hairy roots which were used as control (non-transgenic hairy root cultures)

**Establishment of Hairy root cultures with/without overexpression of *pmt* and *h6h***

*A. belladonna* seeds were obtained from Southwest University (Chongqing, China) and germinated to plantlets. Sterile leaf discs of *A. belladonna* were inoculated with *A. tumefaciens* strain C58C1 (pRIA4, pXI) carrying *pmt* gene and *h6h* gene. In the meantime, plant materials were also transformed with *A. rhizogenes* strain A4 as a control to generate non-transgenic hairy root cultures. Roots generated at cutting edges were excised and cultured on hormone-free half strength solid MS medium, supplemented with 30 g/l sucrose as the carbon source. All of the culture media was added with 250 mg/l carbencillin to eliminate excess *Agrobacterium* and 50 mg/l kanamycin as selective pressure. Root culture clones were maintained at 25°C in dark and routinely subcultured every 28 days. Rapidly growing root clones that showed kanamycin resistance with no bacterial contamination were used to establish hairy root lines. About 100 mg fresh roots with 3 cm in length were inoculated into

**Results**

**Establishment of Hairy root cultures with/without overexpression of *pmt* and *h6h***

The young leaves of *A. belladonna* plantlets with 4-5 leaves (Fig. 1A) showed high sensitivity to C58C1 (pRIA4 and pXI) and A4, because transformed roots could emerged from all the infected leaves at the wounded sites 10 days after infection with *Agrobacterium* strains (Fig. 1B and 1C). The transformed roots obtained from C58C1 (pRIA4 and pXI) or A4 showed no morphological difference. The transformed
roots, which had lots of lateral branching, could grow very rapidly on hormone-free MS medium (Fig. 1D), just like another report on TAs-producing Przewalskia tangutica (Lan and Quan, 2010). Based on the typical morphologies of hairy root, it might be concluded that the subcultured roots were the hairy roots genetically transformed by Ri plasmid, but that should have to be confirmed at the molecular level. Finally, nine independently-transformed (with overexpression of pmt and h6h) hairy root clones and two clones of non-transgenic hairy roots were confirmed by genomic PCR, and then respectively cultured in half-strength liquid MS medium without plant growth regulators (Fig. 1E-H). All the cultured hairy roots in half-strength liquid MS medium reached maximum biomass 35 days after inoculation in flask.

**PCR analysis of rolC, pmt and h6h genes**

To confirm the root cultures successfully transformed by the target plasmids, genomic PCR was employed to detect integration of rolC, pmt and h6h genes into genome of A. belladonna. An rolC-specific amplified product could be obtained from A4, C58C1 and all hairy roots, which was 626 bp in length, while the 626-bp PCR product could not be amplified from the wild type plants of A. belladonna. At the same time, the kanamycin-resistant hairy root cultures, which were transgenic hairy roots with overexpression of pmt and h6h, were detected the integration of pmt and h6h into genome. The transgenic hairy root clones with overexpression of pmt and h6h yielded 462-bp pmt-specific PCR products and 1177-bp h6h-specific PCR products, both of which could be amplified from the positive control, pXI, while neither pmt nor h6h could be detected in the wild type plants of A. belladonna (Fig. 2). Based on the genomic PCR analysis, it could be concluded that transgenic hairy roots with/without overexpression of pmt and h6h were obtained in the present study.
HPLC Analysis of Tropane Alkaloids

The contents of hyoscyamine and scopolamine were detected in the roots of wild type plants, non-transgenic hairy roots (without overexpression of pmt and h6h) and transgenic hairy roots (with overexpression of pmt and h6h). TAs including hyoscyamine and scopolamine could be detected in all the tested samples. In analysis of HPLC, the retention time of authentic hyoscyamine was about 9.1 min (Fig. 3A) and that of authentic scopolamine about 7.6 min (Fig. 3B). So hyoscyamine and scopolamine could be respectively separated and then monitored. The retention time of hyoscyamine and scopolamine in the sample was consistent with authentic alkaloids (Fig. 3C). The total TAs levels in all the detected transgenic/non-transgenic hairy root cultures were increased. The best transgenic hairy root clone (T3) produced 3.21mg/g DW TAs, which was nearly 10 times compared with that in the roots of wild type plants (0.09mg/g DW hyoscyamine, 0.24 mg/g DW scopolamine and 0.33 mg/g DW TAs) and 8 times compared with that in non-transgenic hairy root cultures (H52) (0.195mg/g DW hyoscyamine, 0.198mg/g DW scopolamine and 0.39mg/g DW TAs). T3 produced 2.20mg/g DW hyoscyamine at the highest level, which was respectively 24 times compared with that in wild type roots (0.09mg/g DW hyoscyamine) and 11 times compared with that in non-transgenic hairy root line H52; and 1.00mg/g DW scopolamine at the second highest level among transgenic hairy roots. The different transgenic hairy root lines demonstrated different TAs-producing ability, in which the ration of hyoscyamine and scopolamine was also varied. However, all the detected transgenic hairy root lines produced more TAs than the non-transgenic hairy root line, and this proved that overexpression of pmt and h6h could promote biosynthesis of TAs in A. belladonna.

Discussion

Pathway-engineering hairy root cultures are not only the ideal systems of identity gene functions but the powerful bioreactors for producing natural products. In the previous reports, transgenic hairy root cultures of A. belladonna had been established by overexpressing different genes involved in the biosynthetic pathway of TAs. Overexpression of tobacco pmt alone in hairy roots of A. belladonna did not improve production of TAs including hyoscyamine and scopolamine (Rothe et al., 2003); while in the present study, overexpression of the same pmt gene and h6h gene in hairy roots of A. belladonna improved production of hyoscyamine and scopolamine. In 2004, Zhang et al. reported that overexpression of the same construct (pXI) in hairy roots of H. niger greatly increased production of hyoscyamine and scopolamine, and found that that transgenic hairy roots harboring both pmt and h6h were more efficient than those harboring only one of the two genes (Zhang et al., 2004). So, it might be concluded that there existed synergism between PMT and H6H. When h6h gene was overexpressed alone in transgenic plants of A. belladonna, the alkaloid contents of the leaf and stem were almost exclusively scopolamine (Yun et al., 1992). Unfortunately, the transgenic A. belladonna can not meet the standards of Pharmacopoeia of People's Republic of China, which makes it a rule of A. belladonna producing both hyoscyamine and scopolamine (Zheng, 2005). As a result, transgenic A. belladonna with engineered TAs pathway should have to produce both hyoscyamine and scopolamine. Technically, overexpression of both pmt and h6h is the alternative method, which has been proved in the present study. For example, transgenic hairy root line T3 and

![Fig 4. Contents of TAs in different hairy root lines and in roots of wild type A. belladonna plants. T: transgenic hairy root lines with overexpression of pmt and h6h; H: non-transgenic hairy root lines without expression of pmt and h6h; WT: roots of wild type A. belladonna plants]

T3 could produce both hyoscyamine and scopolamine at higher levels. So, the double-gene transformation strategy will be the preferable strategy of metabolic engineering the TAs biosynthetic pathway in A. belladonna to meet the markets of China. Actually, this kind of strategy has been applied to develop transgenic A. belladonna plants with higher-level TAs in our laboratory.

Conclusion

Transgenic hairy root cultures are the ideal systems for research on metabolic engineering. In the present study, transgenic hairy root cultures of A. belladonna with overexpression of pmt and h6h were established. Transgenic hairy root cultures demonstrated increased production of TAs. In the present study, the double-gene transformation strategy of overexpressing pmt and h6h was successfully employed to genetically modify the TAs biosynthetic pathway in A. belladonna for the first time, and that will facilitate development of transgenic A. belladonna plants based on this kind of strategy. Finally, transgenic hairy root cultures of A. belladonna obtained in the present study can be used as bioreactor to produce hyoscyamine and scopolamine.

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