Plant Omics Journal POJ

POJ 4(1):29-33 (2011) ISSN:1836-3644

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

Chunxian Yang[1], Min Chen[2], Lingjiang Zeng[1], Lei Zhang[3], Xiaoqiang Liu[1], Xiaozhong Lan[4], Kexuan Tang[5] and Zhihua Liao[1,*]

[1]Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), Laboratory of Natural Products and Metabolic Engineering, School of Life Sciences, Southwest University, Chongqing 400715, People's Republic of China
[2]School of Pharmaceutical Sciences, Southwest University, Chongqing 400715, People's Republic of China
[3]School of Pharmacy, Second Military Medical University, Shanghai 200433, People's Republic of China
[4]Tibet Agricultural and Animal Husbandry College, Nyingchi of Tibet 860000, People's Republic of China
[5]Plant Biotechnology Research Center, School of Agriculture and Biology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, Shanghai Jiaotong University, Shanghai 200030, People's Republic of China

***Corresponding author: zhliao@swu.edu.cn or zhihualiao@163.com**

Abstract

Atropa belladonna L. is 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 ovexpressing 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 wildly 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 scopolamine 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 scopolamine, which is over 100 times more than the control clones (Jouhikainen *et al*.,1999); in *h6h*-overexpresing transgenic *A. belladonna* plants, the alkaloid contents in the leaf and stem were almost exclusively scopolamine (Yun *et al*.,1992); and in *h6h*-transgenic hairy root cultures of *Datura innoxia* and *Duboisia* hybrid, the production of scopolamine 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 35S promoter and the *npt*II 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 pRiA4. 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 carbenicillin to eliminate excess *Agrobacterium* and 50 mg/l kanamycin as selective pressure. Root culture clones were maintained at 25□ 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

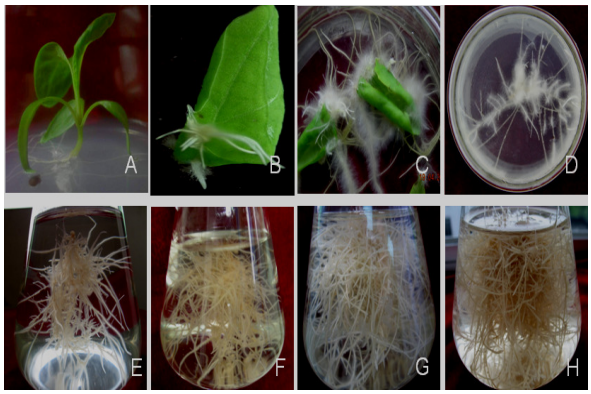

Fig 1. Establishment of hairy root cultures of *A. belladonna*. A: young *A. belladonna* plantlet free of bacteria; B: transformed roots emerged from wounded sites 10 days after infection with *Agrobacterium*; C: transformed roots growing well 20 days after infection with *Agrobacterium*; D: the monoclone of hairy root; E-H: hairy roots cultured in liquid half-strength MS medium without plant growth regulator (E: 10 day culturing; F: 20 day culturing; G: 30 day culturing; H: 35 day culturing)

150 ml conical flasks containing 50 ml of liquid half-strength MS medium and maintained on an orbital shaker at 100 rpm, 25 ℃ in dark. 35 days later, the hairy root cultures were harvested for analysis of the fresh, the dry weight and TAs contents.

PCR analysis of rolC, pmt and h6h genes

Genomic DNA was isolated from the putative engineered hairy root lines, control hairy root lines and wild-type roots using the SDS method (Sambrook and Russell, 2002). The PCR analysis was performed according to Zhang *et al* (Zhang *et al*., 2004).

HPLC Analysis of Tropane Alkaloids

Tropane alkaloids hyoscyamine and scopolamine in the transgenic hairy root lines, non-transgenic hairy root lines and wild type plant roots were extracted as described by Zhang *et al* (Zhang *et al*., 2004) and analyzed by HPLC: mobile phase was consisted of methanol and 0.05 mol/l ammonium acetate solution (added with 0.0025 mol/l SDS) at a ratio 58:42. The speed of flow is 1 ml per minute. The detecting wavelength is 226 nm. The temperature of column (150 mm × 4.6 mm) is 40□. The sample solution of injection is 20 μl each time. The standard samples of hyoscyamine and scopolamine (Sigma, USA) were prepared in methanol at a final concentration of 1000 μg/ml and diluted into 500, 250, 100, 50, 25, 10, 5 μg/ml.

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

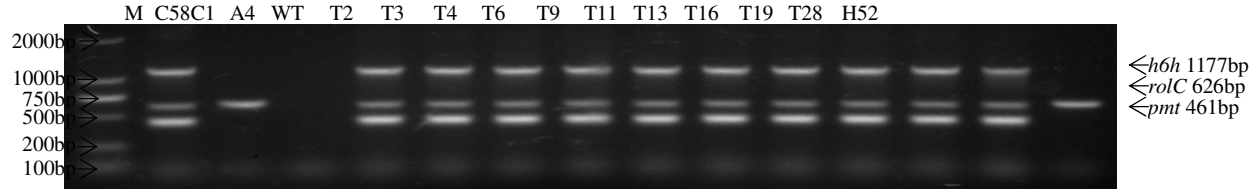

Fig 2. Detecting *rolC*, *pmt* and *h6h* genes by genomic PCR. M: DNA marker (DL2000); C58C1: disarmed *Agrobacterium tumefaciens* strain C58C1 with the plasmids of pRiA4 and pXI carrying *pmt* and *h6h*; *Agrobacterium rhizogenes* strain A4; WT: wild type plants of *A*. *belladonna*; T2-28: different independent-transgenic hairy root lines with overexpression of *pmt* and *h6h*; H52: non-transgenic hairy root line (without overexpression of *pmt* and *h6h*)

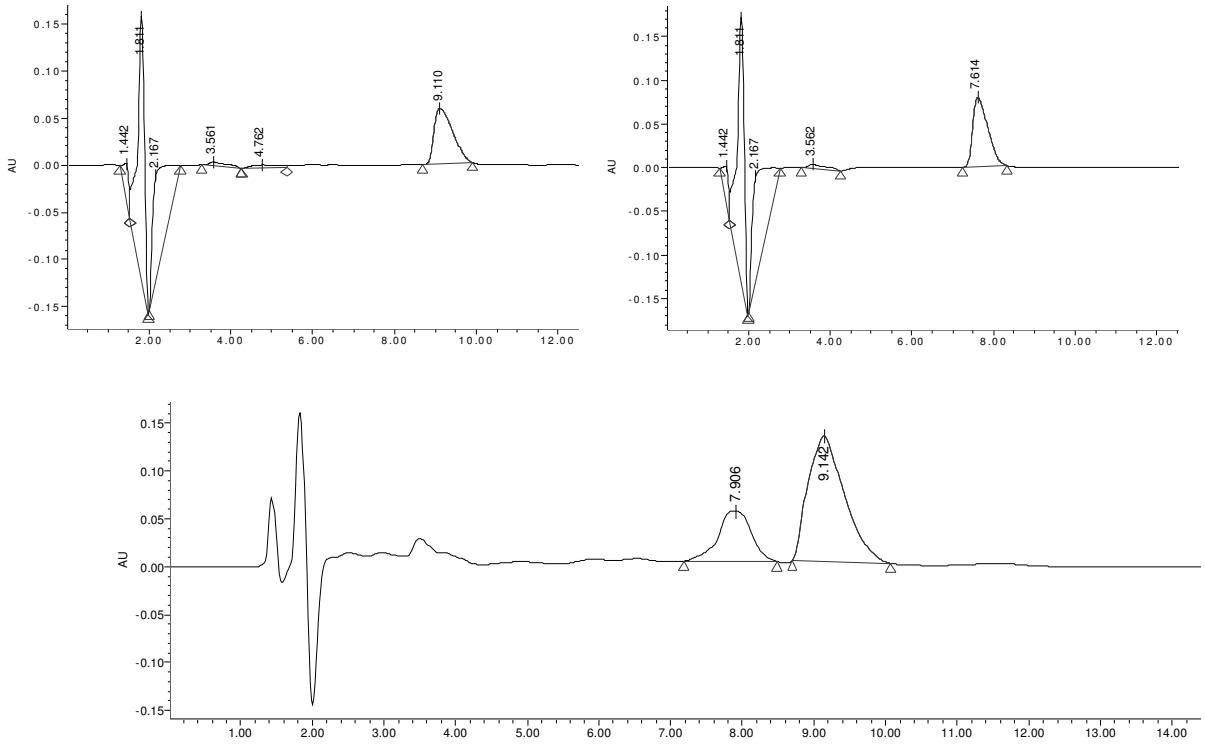

Fig 3. HPLC analysis of hyoscyamine and scopolamine. A: HPLC analysis of authentic hyoscyamine; B: HPLC analysis of authentic scopolamine; C: HPLC analysis of hyoscyamine and scopolamine in samples

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 been 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 (Fig. 3A) min, 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 identify 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

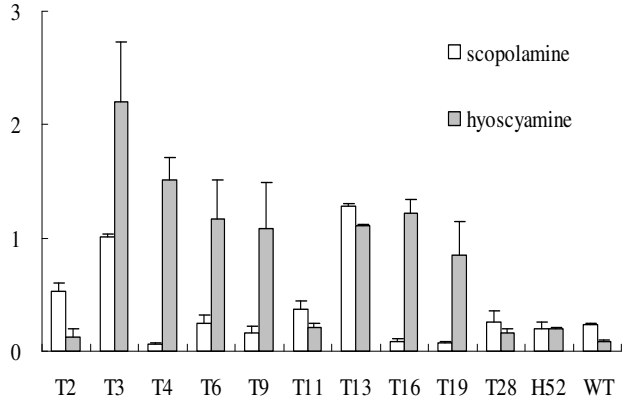

Fig 4. Contents of TAs in different hairy root lines and in roots of wild type *A. belladonna* plants. T: transgenic hairy root lines with overexpression of *pmt* and *h6h*; H: non-transgenic hairy root lines without expression of *pmt* and *h6h*; WT: roots of wild type *A. belladonna* plants

T13 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.

Acknowledgement

This research was financially supported by the China National "863" High-Tech program (Project No. 2010AA100503), the Natural Science Foundation of Chongqing Science & Technology Commission (2009-BB5309), the Fundamental Research Funds for the Central Universities (XDJK2010C087) and "Excellent Scientists of Universities in Chongqing City".

References

Carvalho EB, Curtis WR (1998) Characterization of fluid flow resistance in root cultures with a convective flow tubular bioreactor. Biotechnology and Bioengineering 60:375-384.

Dechaux C, Boitel-Conti M (2005) A strategy for overaccumulation of scopolamine in *Datura* innoxia hairy root culture. Acta Biologica Coviensia Series Botanica 47: 101-107

Giri A, Narasu ML (2000) Transgenic hairy roots: recent trends and applications. Biotechnology Advances 18:1-22

Guggisberg A, Hesse M (1983) Putrescine, spermidine, spermine, andrelated polyamine alkaloids. In: Brossi A (ed) The Alkaloids, Academic Press, New York

Hashimoto T, Hayashi A, Amano Y, Kohno J, Iwanari H, Usuda S, Yamada Y (1991) Hyoscyamine 6-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. Journal of Biological Chemistry 226: 4648-4653

Hibi N, Fujita T, Hatano M, Hashimoto T, Yamada Y (1992) Putrescine N-methyltransferase in cultured roots of Hyoscyamus albus. Plant Physiology 100:826–835

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey KM (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208: 545-551

Lan XZ, Quan H (2010) Hairy root culture of Przewalskia tangutica for enhanced production of pharmaceutical tropane alkaloids. Journal of Medicinal Plants Research 4: 1477-1481

Li W, Koike K, Asad Y, Yoshikaw T, Nikaido T (2005) Biotransformation of paeonol by Panax ginseng root and cell cultures. Journal of Molecular Catalysis B: Enzymatic 35:117-121

Motomura K, Fujiwara Y, Kiyota N, Tsurushima K, Takeya M, Nohara T, Nagai R, Ikeda T (2009) Astragalosides Isolated from the Root of Astragalus Radix Inhibit the Formation of Advanced Glycation End Products. Joural of Agricultural and Food Chemistry 57: 7666-7672

Moyano E, Fornalé S, Palazón J, Cusidó RM, Bagni B, Piñol MT (2002) Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the *pmt* gene. Phytochemistry 59: 697-702

Moyano E, Jouhikainen K, Tammela P, Palazón J, Cusidó RM, Piñol MT, Teeri TH, Oksman-Caldentey KM (2003) Effect of pmt gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. Journal of Experimental Botany 54: 203-211

Palazón J, Moyano E, Cusidó RM, Bonfill M, Oksman-Caldentey KM, Piñol MT (2003) Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the *h6h* gene. Plant Science 165: 1289-1295

Rao AQ, Bakhsh A, Kiani S, Shahzad K, Shahid AA, Husnain T, Riazuddin S (2009) The myth of plant transformation. Biotechnology Advances 27:753-763

Rothe G, Hachiya A, Yamada Y, Hashimoto T, Dräger B (2003) Alkaloids in plants and root cultures of *Atropa belladonna* overexpressing putrescine N□methyltransferase. Journal of Experimental Botany 54: 2065-2070

Rothe G, Hachiya A, Yamada Y, Hashimoto T, Dräger B (2003) Alkaloids in plants and root cultures of *Atropa belladonna* overexpressing putrescine N-methyltransferase. Journal of Experimental Botany 54: 2065-2070

Yun DJ, Hashimoto T, Yamada Y (1992) Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proceedings of the National Academy of Sciences 24:11799-803

Zhang L, Ding RX, Chai YR, Bonfill M, Moyano E, Oksman-Caldentey KM, Xu TF, Wang ZN Zhang HM, Kai GY, Liao ZH, Sun XF, Tang KX (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proceedings of the National Academy of Sciences 17:6786-6791

Zhang L, Kai GY, Lu BB , Zhang HM, Tang KX, Jiang JH, Chen WS (2005) Metabolic engineering of tropane alkaloid biosynthesis in plants. Journal of Integrative Plant Biology 47:136-143

Zheng XY (2005) Herba Belladonna. In: Zheng XY (ed) Pharmacopoeia of People's Republic of China, Beijing of China, 2005