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Full Length Research Paper

Promoting scopolamine accumulation in transgenic plants of *Atropa belladonna* generated from hairy roots with over expression of *pmt* and *h6h* gene

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***Atropa belladonna* is the most important commercial source for obtaining pharmaceutical tropane alkaloids such as scopolamine and hyoscyamine. In the present study, two rate-limiting enzyme genes including putrescine *N*-methyltransferase and hyoscyamine 6*β*-hydroxylase were introduced into *A. belladonna*, and integration of the *pmt* and *h6h* genes into the genomic DNA of transgenic plants were confirmed by genomic polymerase chain reaction (PCR) analysis. The scopolamine content of transgenic lines was increased to 1.3 - 2.5 folds than that in wild type, which was caused by over expression of the *pmt* and *h6h* genes in the transgenic plant lines of *A. belladonna*. The current study provides a more effective approach for commercially large-scale production of scopolamine by cultivating *A. belladonna* plants in large fields but not using the hairy root systems as bioreactors.**

Key words: *Atropa belladonna*, *pmt*, *h6h*, scopolamine, transformation, regeneration.

INTRODUCTION

Atropa belladonna, commonly known as belladonna or deadly nightshades, is a perennial herbaceous plant and most importantly commercial source of pharmaceutical tropane alkaloids in the family of Solanaceae. The plant is of interest due to its production of bioactive tropane alkaloids, including scopolamine and hyoscyamine, which are wildly used as antagonists of acetylcholine in both the autonomic and central nervous system (Guggisberg and Hesse, 1983). For medicinal purposes, scopolamine is much more useful and valuable because of its higher physiological activity and fewer side-effects (Evans, 1996). However, because of the low abundance of tropane alkaloids especially scopolamine in natural plants, there has been a long-standing interest in increasing the content of alkaloids, especially the much more valuable scopolamine in cultivated medicinal plants.

Over the past years, extracting these secondary metabolites from whole plants was viewed as the only economical method for the production of these pharmaceuticals. However, in natural plants, the low endogenous content of these chemicals remains as the limitation for mass production. Although, other options are available for tropane alkaloid production, they are not currently available. For example, chemical synthesis remains extremely expensive due to the existence of multiple chiral centers. Also, incomplete characterization of the pathways required for tropane alkaloid production makes it impossible to acquire them by means of alternative biosystems such as microorganisms (Verpoorte, 2000). Nowadays, with the development in plant cell cultures,

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tissue cultures and genetic engineering, it becomes feasible to manipulate the biosynthetic pathways of plant secondary metabolisms such as tropane alkaloids at the level of metabolic and enzyme level to increase the pharmaceutical tropane alkaloids content in the cultivated *A. belladonna* (Oksman-Caldentey, 2004; Trethewey, 2004). Putrescine is a common precursor of both polyamines such as spermidine and spermine and tropane/pyridine alkaloids (Guggisberg and Hesse, 1983; Hashimoto et al., 1989; Hibi et al., 1994).

Putrescine *N*-methyltransferase (PMT) is the first alkaloid-specific enzyme committed for the biosynthesis of tropane alkaloids, because it involves in the removal of putrescine from the polyamine pool and then catalyzes the *N*-methylation of this diamine to form *N*-methylputrescine (Hibi et al., 1992), the product *N*-methylputrescine is the first specific metabolite on the route to nicotine, tropane and nortropane alkaloids (Biastoff et al., 2009). The *pmt* cDNAs were already cloned from tobacco (Hibi et al., 1994) and *Hyoscyamus niger* (Suzuki et al., 1999). Regulation of the expression of *pmt* gene has been shown to be crucial for alkaloid production in several cases. After introducing the tobacco *pmt* gene into the genome of *Datura meteland* and *Hyoscyamus muticus* respectively, it was found that both hyoscyamine and scopolamine production were improved in hairy root cultures of *D. meteland*, whereas in *H. muticus* only hyoscyamine contents were increased (Moyano et al., 2002).

However, in many cases, the over expression of exogenous *pmt* can not improve alkaloid production as expected. For example, over expression of *pmt* in *A. belladonna* did not affect tropane alkaloid levels either in transgenic plants or in hairy roots (Sato et al., 2001); Moyano et al. (2002) inserted the tobacco *pmt* gene into the hairy roots of a hybrid of *Duboisia* and improved the *N*-methylputrescine level of the transgenic hairy roots, but there was no significant increase in either tropane or pyridine-type alkaloids. Rothe et al. (2003) also found that increased *pmt* expression alone was not sufficient to increase alkaloid production in *A. belladonna* plants and in root cultures. All of these reports indicated that the regulation of tropane alkaloids synthetic pathways varied with different plant species, and the effect of over expression of a single enzyme may be inadequate.

Although, genetic manipulation on the upstream pathway of tropane alkaloid in some plant species was unsuccessful, efforts closer to the final products have been effective. Hyoscyamine 6*β*-hydroxylase (H6H; EC 1.14.11.11) is a bifunctional enzyme, which catalyzes the last two oxidative reactions of tropane alkaloids biosynthetic pathway, converting hyoscyamine to the more pharmaceutically valuable tropane alkaloid scopolamine (Hashimoto et al., 1993a; Matsuda et al., 1991; Jaber-Vazdekis et al., 2009). In the case of *h6h* expression, metabolic engineering at a single step resulted in improved alkaloid productivity and profiles. Researchers were able to elevate scopolamine levels by over expressing *h6h*. Yun et al. (1992) reported that *h6h* from *H. niger* was constitutively expressed in *A. Belladonna* plants, including stems and leaves where endogenous *h6h* is not expressed. Hashimoto et al. (1993b) reported a five fold increase in the scopolamine content of transgenic *A. Belladonna* hairy roots over expressing the same *h6h* gene. Over expression of *h6h* gene in *H. muticus* hairy roots produced over 100-fold higher level of scopolamine and hyoscyamine compared with controls (Jouhikainen et al., 1999).

Over expressing multiple key-enzyme genes or manipulating regulatory genes in the target bioengineering pathway is a promising way to manipulate the biosynthetic pathways of tropane alkaloids since the single rate-limiting step of most biosynthetic pathways for secondary metabolites may not exist (Sato et al., 2001). A good example is simultaneous introduction and over expression of genes encoding the rate-limiting upstream enzyme PMT and the downstream key enzyme H6H of scopolamine biosynthesis in transgenic *H. niger* hairy root cultures (Zhang et al., 2004). Transgenic hairy root lines expressing both *pmt* and *h6h* produced significantly higher levels of scopolamine compared with both the wild type and the transgenic lines harboring a single gene (*pmt* or *h6h*). The best line produced more than nine folds scopolamine than that in the wild type and more than twice the amount in the highest scopolamine-producing *h6h* single-gene transgenic line. It indicated that the expression of *h6h* played an important role in scopolamine biosynthesis, while the contribution to the increase of scopolamine production result from the activity of *pmt* is limited.

In order to investigate the secondary metabolism response to *pmt* and *h6h* over expression in *A. belladonna*, the *Nicotiana tabacum pmt* gene and the *H. niger h6h* gene were introduced into *A. belladonna* in the present work. The regenerated plants from transgenic hairy roots were obtained, which is different from previous studies, the phenotype and scopolamine production of transgenic plant lines were investigated after planted in the field, with the blank-transformed plant lines and the wild-type plants used as controls respectively.

MATERIALS AND METHODS

Strains and plasmids

The bivalent plant expression plasmid pXI containing both *pmt* and *h6h* genes, constructed by Zhang et al. (2004), were 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 *Agrobacterium rhizogenes* Ri plasmid pRiA4. A positive clone, after confirmed by polymerase chain reaction (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*.

Regeneration of plants from hairy roots

A. Belladonna seeds were obtained from Southwest University (Chongqing, China) and germinated into plants. Sterile leaf disks of *A. Belladonna* were inoculated with *A. tumefaciens* strain C58C1 (pRiA4, pXI) carrying *pmt* gene and *h6h* gene, with the simultaneous transformation with strain A4 of *A. rhizogenes* as control. Wild-type plants were grown in the same growth chamber. The hairy root clones were obtained from leaf discs as described (Yang et al., 2006). We selected the strong hairy root clones from the conical flasks containing the liquid media and then inoculated on the plates with half-strength MS media and hormone free for shoots growing. We picked out some monoclones from the plates and after 10-day cultivation, moved the roots to the half-strength MS supplemented with NAA (0.2 mg/L), BA (2.0 mg/L) continue cultivating in light at 25°C. After cultivation for 60 days, buds grown from the hairy roots and the medium for inducing the root was 1/2 MS supplemented with IBA(0.1 mg/L) or 1/2 MS. Finally, the regenerated plants were transferred to field soil with control and wild type plants.

Polymerase chain reaction analysis

Genomic DNA was isolated from the putative engineered regenerated plants, control plants and wild-type plants using the SDS method (Sambrook and Russell, 2002). The DNA was then used for PCR analysis to detect the presence of *Agrobacterium rol* C, *pmt* and *h6h* genes in transgenic regenerated plants. PCR primes for *rol* C detection were *frol*C (5'-TAACATGGCTGAAGACGACC-3'), *rrol*C (5'-AAACTTGCACTCGCCATGCC-3'); primes for *pmt* detection were *fpmt* (5'-GCCATTCCCATGAACGGCC- 3'), *rpmt* (5'-CCTCCGCCGATGATCAAAACC- 3'); primes for *h6h* detection were *fh6h* (5'- GGATCCCAACGTATAGATTCTTC-3') and *rh6h* (5'-CGGGAATTCGGA TCCCAAACCATCACTGCAAT-3'). PCR was carried out in total volumes of 25 µl reaction mixtures, containing 10 × PCR buffer 2.5 µl, 25 mmol/l $MgCl_2$ 1.5 µl, 10 mmol/l dNTP mix 0.5 µl, 10 mmol/l primerf 0.5 µl, 10 mmol/l primerr 0.5 µl and 1.25 U *Taq* DNA polymerase (Fermentas, Canada) with 100 ng of genomic DNA as template.

For detection of the *rol* C gene, the template was denatured at 94°C for 5 min followed by 35 cycles of amplification (45 s at 94°C, 45 s at 55°C, 1 min at 72°C) and by 8 min at 72°C. Amplified procedure for *pmt* gene was: 5 min of predenaturation at 94°C , followed by 35 cycles of amplification (40 min at 94°C, 40 min at 60°C, 45 s at 72°C) and by 5 min at 72°C. For the detection of the *h6h* gene, the template was denatured at 94°C for 5 min followed by 35 cycles of amplification (1 min at 94°C, 1 min at 54°C, 1.5 min at 72°C) and by 5 min at 72°C. The mixture of all the above amplified products were used for electrophoresis detection of *pmt, h6h* and *rolC* from plants' samples derived from the different monoclones on 1% agarose gel.

Alkaloid extraction and analysis

The scopolamine in the transgenic plants, non-transgenic plants and wild plants was extracted as described by Hashimoto et al. (1993b) and analyzed by HPLC: mobile phase was consist of methanol, acetate (methanol : 0.05 mol/l ammonium acetate pH 4.6 = 58 : 42) and 0.0025 mol/l SDS. The speed of flow is 1 ml per minute. The detecting wavelength is 226 nm. The temperature of CTP ODS stainless steel column (150 × 4.6 mm) is 40°C. The sample solution of injection is 20 µl each time. The standard sample of scopolamine (Sigma, USA) was prepared in methanol at a final concentration of 1000 µg/ml and diluted into 500, 250, 100, 50, 25, 10 and 5 µg/ml.

RESULTS AND DISCUSSION

Plants regeneration derived from the hairy root system

A. belladonna plants regenerated directly from transgenic hairy roots were designated as the T series (T3, T5 and T13).There were significantly morphological changes associated with plants transformed by *A. tumefaciens* strain C58C1 containing *A. rhizogenes* Ri plasmid pRiA4. The transgenic plants had short internodes, wrinkly, narrow and small leaves, and multiple branches (Figure 1). The consenescence period of transgenic plants with earlier flower becomes shorter than the wild type. These morphological changes were also observed in other reports (Saito et al., 1992; Chaudhuri et al., 2006; Aoki et al., 1997). Some studies had been performed to explain the changes of transgenic plants and the results indicated that during plant-microbe interaction, the *rol*-gene may interfere with general plant defense pathways or perturb secondary metabolism by an unusual way, which is not common for plants (Bulgakov et al., 2004). Constitutive *rol* B expression suppressed the growth of tobacco cells, and the *rol* C gene was able to attenuate this growth inhibition (Schmülling et al., 1988).

Polymerase chain reaction analysis of regenerated plants

All of the regenerated plants were revealed containing the *rol* C gene, and at the same time the *pmt* and *h6h* genes were confirmed integrated into the genomic DNA of transformed plants by PCR analysis (Figure 2). We confirmed that the *pmt* and *h6h* genes were introduced in three of the transformed plants named T3, T5 and T13. Only *rol* C was detected in the regenerated plant lines derived from *A. belladonna* hairy roots through blank transformation with *A. rhizogenes* strain A4. Because the primers for *pmt*, *h6h* covered the target sequences of genes and vectors, none of the target DNA band was amplified from the wild type.

Analysis for scopolamine

The content of scopolamine in transgenic *A. belladonna* lines T3, T5 and T13 with *pmt* and *h6h* were detected by HPLC, the same to non-transgenic plants A4, A5 and A9 regenerated from hairy roots and wild type *A. belladonna*. The results of analysis for scopolamine by HPLC were shown in Figure 3. The scopolamine content of T-series lines were 1.3 - 2.5 folds than wild type while the content

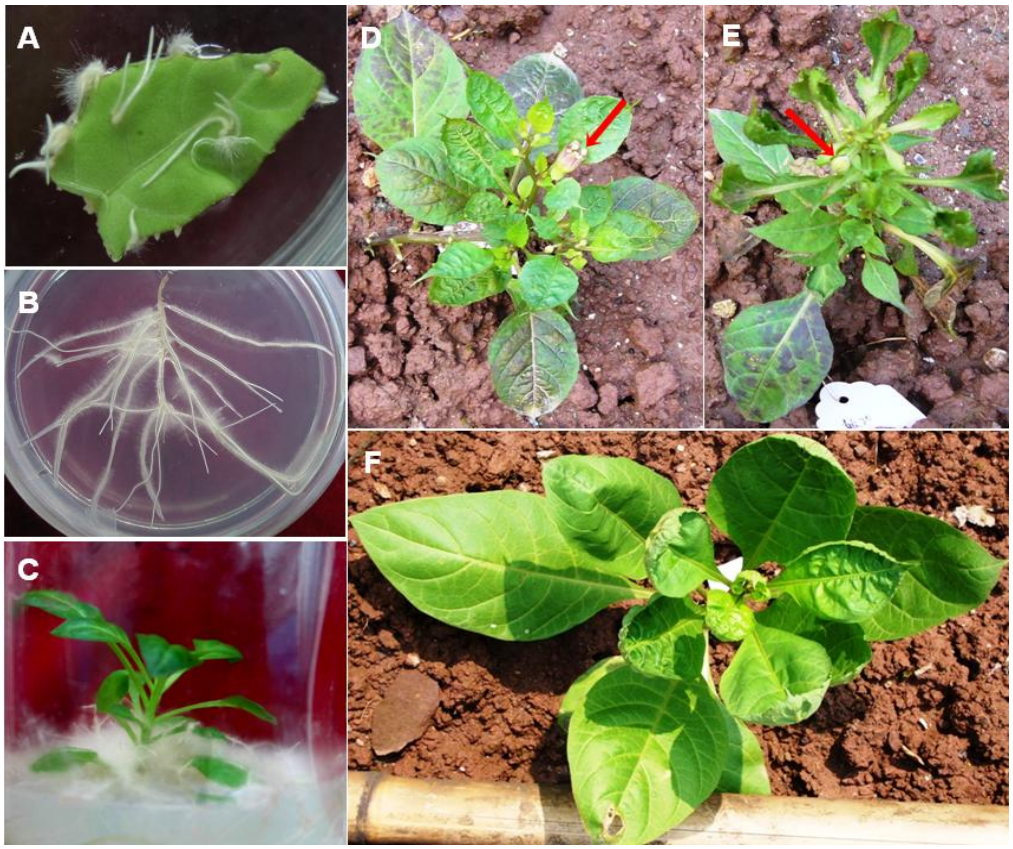

Figure 1. The plants regeneration of the *A. belladonna* derived from hairy root clone and wild type plant. (A) the hairy roots induced from the leaf disks (B) the cultivation of hairy roots on the 1/2 MS (C) the regenerated plants derived from hairy root clone (D) the transgenic plant with over expression of *pmt* and *h6h* (flower was indicated by red arrow) (E) the plant derived from hairy root but without over expression of *pmt* and *h6h* (flower was indicated by red arrow) (F) wild type plant.

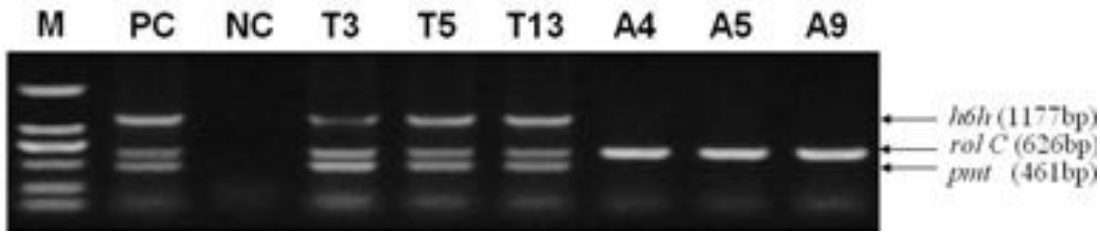

Figure 2. Representative PCR analysis of *rolC*, *pmt* and *h6h* genes in transgenic plant lines. M, DL-2000 Marker (100-2,000 bp); PC, positive control: the *A. tumefaciens* strain C58C1 with the plasmid of pXI carrying *pmt* and *h6h*; NC, negative control: the wild type *A. belladonna*; T, transgenic *A. belladonna* lines containing *pmt* and *h6h* genes induced by *A. tumefaciens* C58C1strain (pRiA4, pXI); A, regenerated plants derived from the hairy roots through blank transformation with *A. rhizogenes* strain A4.

in A series was 0.4 - 0.6 times lower than that in wild type. Content variation in different independently-transformed plants suggested the different levels of *pmt* and *h6h* transcription in different lines. These results were consistent with the results in the hairy roots of *H. niger* (Zhang et al., 2004). The data indicated that the metabolic flux was changed in transgenic plant lines. Previous studies had described that merely increasing of *pmt* expression was not sufficient for increasing alkaloid production in *A. belladonna* plants, cultures and *H. niger* hairy roots (Rothe et al., 2003; Zhang et al., 2004). Moyano et al. (2002) found that tropane alkaloid contents

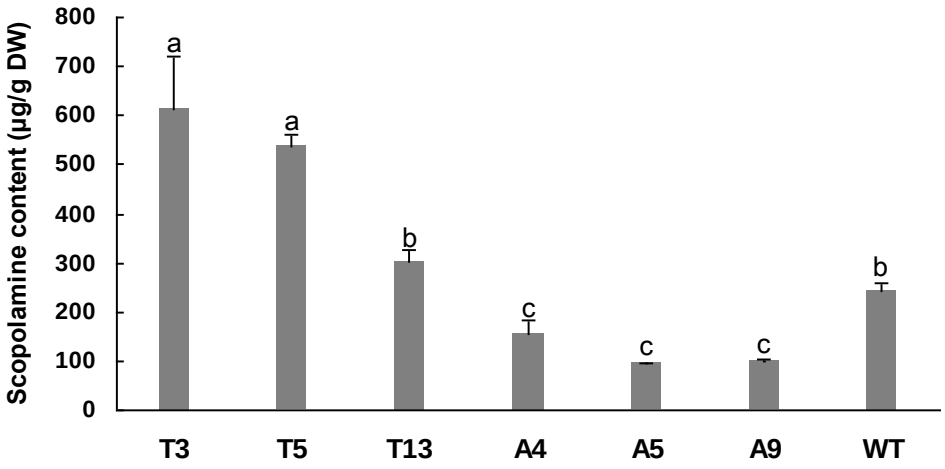

Figure 3. Analyses for the scopolamine contents in transgenic *A. belladonna* plant lines. T, transgenic *A. belladonna* lines containing *pmt* and *h6h* genes induced by *A. tumefaciens* C58C1strain (pRiA4, pXI); A, regenerated plants derived from the hairy roots through blank transformation with *A. rhizogenes* strain A4; WT, wild type *A. belladonna* plant. Each data was presented as the means ± SD from three replicates, the different small letters above the columns were significantly different at 0.05 level according to LSD, n = 3.

of transformed roots in *H. muticus* with tobacco *pmt* gene were enhanced up to 5-fold but the enhancement concerned only hyoscyamine, not scopolamine. According to Zhang's reports, the *h6h* played a more important role in stimulating scopolamine accumulation coordinating with upstream *pmt*. A transgenic plant that constitutively and strongly expressed the transgene *h6h* was selected by Yun et al. (1992) and they found that the alkaloid contents in leaf and stem were almost exclusively scopolamine. They confirmed that it was *h6h* converting hyoscyamine to scopolamine, which means that it was the *h6h* that stimulated the accumulation of scopolamine in transgenic plants. In our present work, the same results were obtained to the transgenic *pmt* and *h6h* plant lines T3, T5 and T13. Scopolamine in A4, A5 and A9 was 0.4 - 0.6 times lower than in wild type, which might caused the effects of *rol* genes in regenerated lines derived from hairy roots. Palazón et al. (1998) found that roots of tobacco lines transformed with *rol* A, B and C together or with *rol* C alone showed more growing capacity and production of nicotine, alkaloid synthesized in the roots, compared with controls. Lower scopolamine content in the non-transgenic *A. belladonna* lines derived from hairy roots may caused by the precursors were transferred to nicotine or other tropane alkaloids through transcriptional activation of the *rol* genes.

Over expression of multiple biosynthetic genes in plants that control the expression of genes in the target bioengineering pathway is a promising and practical strategy to alter the accumulation of certain secondary metabolic products. In the present work, we obtained the regenerated transgenic *A. belladonna* plant lines, in which the significant enhancement of scopolamine accumulation was caused by simultaneously over expressing the *pmt* and *h6h*. The present study provides a more effective approach for commercially large-scale production of scopolamine by cultivating transgenic *A. belladonna* plants in large fields instead of using the hairy root systems as bioreactors.

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