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Comparison of two hyoscyamine 6β-hydroxylases in engineering scopolamine biosynthesis in root cultures of *Scopolia lurida*

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ABSTRACT

Scopolia lurida, a medicinal plant native to the Tibetan Plateau, is among the most effective producers of pharmaceutical tropane alkaloids (TAs). The hyoscyamine 6β-hydroxylase genes of *Hyoscyamus niger* (*HnH6H*) and *S. lurida* (*SlH6H*) were cloned and respectively overexpressed in hairy root cultures of *S. lurida*, to compare their effects on promoting the production of TAs, especially the high-value scopolamine. Root cultures with *SlH6H*/*HnH6H* overexpression were confirmed by PCR and real-time quantitative PCR, suggesting that the enzymatic steps defined by H6H were strongly elevated at the transcriptional level. Tropane alkaloids, including hyoscyamine, anisodamine and scopolamine, were analyzed by HPLC. Scopolamine and anisodamine contents were remarkably elevated in the root cultures overexpressing *SlH6H*/*HnH6H*, whereas that of hyoscyamine was more or less reduced, when compared with those of the control. These results also indicated that SlH6H and HnH6H promoted anisodamine production at similar levels in *S. lurida* root cultures. More importantly, *HnH6H*-overexpressing root cultures had more scopolamine in them that did *SlH6H*-overexpressing root cultures. This study not only provides a feasible way of overexpressing H6H to produce high-value scopolamine in engineered root cultures of *S. lurida* but also found that HnH6H was better than SlH6H for engineering scopolamine production.

1. Introduction

A great number of widely used pharmaceuticals are extracted from plants. Tropane alkaloids (TAs), such as hyoscyamine, anisodamine, and scopolamine, are synthesized in the roots of Solanaceae medicinal plants and clinically used as anticholinergic agents [1]. For example, TAs may be administered for anesthesia before operation, motion sickness, control of Parkinson's disease, improving microcirculation, abandoning drug habits, and pesticide poisoning, among others [2]. Of the three TAs, scopolamine is the best for treating patients because of its fewer adverse effects, stronger ability for crossing the blood-brain barrier, and higher

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physiological activity [3]. Generally, these TAs are produced in the medicinal species of the Solanaceae family, namely *Atropa belladonna*, *Hyoscyamus niger*, *Datura* spp., *Scopolia lurida*, etc. However, TA production in these plants is generally very low, with that of the high-value scopolamine even far lower than that of hyoscyamine. Not surprisingly, the plant yield of scopolamine has not met the huge market demand for it over the long-term, driving its price to 10000 USD per kilogram [2]. Hence, it is important that we find alternative ways to produce scopolamine. Due to the high costs involved, the chemical synthesis of scopolamine is not commercially viable. Traditional breeding methods—genetic, polyploidy, and radiation breeding—have failed to develop new, high-yield scopolamine varieties [4]. The only promising approach left appears to be metabolic engineering.

TA types and their concentrations vary among different plants. *Scopolia lurida*, also called Himalayan Scopolia or *Anisodus luridus*, is a perennial herb that grows on the Tibetan Plateau [5]. Unlike other TA-producing plants, *S. lurida* is among the most effective TA producers because of its large biomass and high contents of TAs, especially of hyoscyamine (1.5% dry weight in aerial parts) [6]. Furthermore, the scopolamine content of *S. lurida* is greater than that of *A. belladonna*, a widely cultivated TA-producing plant [5]. Its high-yield of hyoscyamine makes *S. lurida* an interesting plant for producing high-value scopolamine by converting hyoscyamine to scopolamine via metabolic engineering. The key enzyme in this process is hyoscyamine 6β-hydroxylase (H6H) [7], which catalyzes the 6β-hydroxylation of hyoscyamine to form anisodamine, followed by the epoxidation of anisodamine into scopolamine (Supplementary Fig. 1). Overexpression of H6H is thus often used to engineer scopolamine production in TA-producing plants, but this has never been reported for *S. lurida*. The expression level of the *S. lurida hyoscyamine 6β-hydroxylase* (*SlH6H*) gene was markedly upregulated, and scopolamine production significantly promoted, when acetylsalicylic acid or UV-B was applied to *S. lurida* root cultures; this suggested the scopolamine production was enhanced by upregulating *H6H* [8].

Hyoscyamus niger is rich in scopolamine, so its *H6H* gene (*HnH6H*) is often used to engineer scopolamine production [9]. Considering that *S. lurida* produces scopolamine at a relatively higher level than does *A. belladonna* and some other TA-producing plant species, we cloned the *H6H* gene of *S. lurida* (*SlH6H*). Then, we developed hairy root cultures of *S. lurida*, in which the *SlH6H* and *HnH6H* genes were respectively overexpressed, to compare their effects on the production of TAs, namely hyoscyamine, anisodamine, and scopolamine.

2. Materials and methods

2.1. Plant materials

Scopolia lurida mature seeds were harvested from cultivated plants in the medicinal plant garden of Xizang Agricultural and Animal Husbandry College (Nyingchi, Tibet, China) in September 2014.

2.2. Gene cloning and vector construction

Total RNAs were extracted from the secondary roots of *H. niger* and *S. lurida* with a RNA isolation kit (TianGen, Beijing, China) and used as initial templates for cDNA synthesis by using a kit made by TaKaRa (Dalian, China). The coding sequence of *HnH6H* [9] was isolated by a pair of primers—with *Bam*HI and *Sac*I restriction sites, respectively, F-BamHI-hnh6h (5′-CGCGGATCCATGGCTACTTTTGTGTCGAACT-3′) and R-SacI-hnh6h (5′-CGAGCTCGCTTAGACATTGATTTTATATGGC-3′)—and then inserted into the binary expression vector pBI121 by using *Bam*HI and *Sac*I enzymes to generate the plant expression vector pBI121-HnH6H. After its confirmation by sequencing, pBI121-HnH6H was introduced into the disarmed *Agrobacterium* strain C58C1 (pRiA4) to generate the engineered bacteria [10]. Based on the sequenced transcriptomes of *S. lurida* (unpublished data), we isolated the coding region for SlH6H by using two primers with restriction sites: F-BamHI-slh6h (5′-CGCGGATCCATGGCTACTCTTGTCTCCAACTGG-3′) and R-SacI-slh6h (5′-CGAGCTCTTAGGCATTGATTTTATAAGGCTTAACACCAGC-3′). The *SlH6H* gene was also inserted into pBI121, by the restriction enzymes Ba*m*HI and *Sac*I, to generate the plant expression vector pBI121-*SlH6H*. The *SlH6H* sequence was confirmed, and then pBI121-*SlH6H* was introduced into the *Agrobacterium* strain C58C1 (pRiA4) to obtain the engineered bacteria.

2.3. Bioinformatics analysis

BLASTP analysis was performed online, at https://blast.ncbi.nlm.nih.gov/Blast.cgi [11]. To compare the amino acid sequences of SlH6H and HnH6H we used Vector NTI 8 software. Based on the sequenced transcriptomes of the secondary roots, primary roots, stems, and leaves (unpublished data), heat maps were generated in MutiExperiment Viewer v4.2 software [12] to show the corresponding tissue profiles for the TA biosynthesis genes of *S. lurida*.

2.4. Establishment of hairy root cultures

Seeds were germinated on MS medium [13] after disinfection at 25 °C under 16-h light and 8-h dark. When the plants had four true leaves, they were used for inducing hairy roots according the reported methods. Approximately 50 mg of fresh root tips (3–4 cm in length) were inoculated into 250-ml flasks containing 100 ml liquid MS medium and grown at 110 rpm and 25 °C in the dark [8]. After 30 days, these hairy root cultures were harvested for analysis.

2.5. Molecular analysis of hairy root cultures

To confirm the authority of transgenes, we detected the marker gene *NPTII*, the rooting genes including *rolB* and *rol* C, *35S::SlH6H* and *35S::HnH6H* in root cultures, respectively using *Agrobacterium* strain C58C1 (pRiA4 and pBI121-*HnH6H*), genomic DNA of root cultures and roots of wild-type *S. lurida* as templates. The expression levels of *SlH6H* and *HnH6H* were analyzed by real-time quantitative PCR as described in our prior publications [8,10]. The primers used for molecular detection are listed in Tables 1 and 2.

2.6. Alkaloid analysis

The hairy root cultures were dried to a constant weight at 40 °C, and, TAs were extracted from 200-mg samples by using a method derived from an earlier report [14]. The content analysis was performed by HPLC (Shimadzu LC-20AD). The detection wavelength was 226 nm. The temperature of the column (150 mm × 4.6 mm) was 40 °C. The mobile phase was composed of 11% acetonitrile and an 89% buffer solution (20 mM ammonium acetate and 0.1% formic acid, pH 4.0). The flow speed was 1 ml per min. The sample solution per injection was 20 μl. Authentic samples of hyoscyamine, anisodamine, and scopolamine were bought from Sigma-Aldrich (St. Louis, USA).

3. Results

3.1. Cloning and analysis of SlH6H and HnH6H

The coding sequence of *SlH6H* was 1035 bp and encoded a

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