Plant Physiology and Biochemistry 70 (2013) 188–194

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

An atypical pattern of accumulation of scopolamine and other tropane alkaloids and expression of alkaloid pathway genes in *Hyoscyamus senecionis*

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ARTICLE INFO

Article history:
Received 19 April 2013
Accepted 9 May 2013
Available online 18 May 2013

Keywords:
Hyoscyamus senecionis
Solanaceae
Hyoscyamine 6β-hydroxylase (H6H)
Expression profile
Tropane alkaloids

ABSTRACT

A cDNA encoding hyoscyamine 6β-hydroxylase (H6H, EC 1.14.11.11), a bifunctional enzyme catalyzing the last two steps in the scopolamine biosynthetic pathway, was isolated from *Hyoscyamus senecionis*, a medicinal plant endemic to the Iranian plateau. Expression analysis indicates that Hsh6h is expressed in all tested organs of *H. senecionis* including roots, rhizomes, leaves, stems and flowers unlike the other tropane alkaloid producing species. In parallel to this, in leaves, levels of scopolamine, the product of H6H, were higher than the substrate hyoscyamine. These data suggest that not only does the conversion of hyoscyamine to scopolamine take place in the root, followed by translocation to aerial parts, but also accumulated hyoscyamine in the aerial parts may be converted to scopolamine by activity of HsH6H. Analysis of expression profiles of putrescine N-methyltransferase and tropinone reductase I and II genes also indicates the organ-independent expression of these genes. Here we also introduce *H. senecionis* as an important tropane alkaloid producing species with its thick underground parts as a source of hyoscyamine, while its leaves can be considered as a source of scopolamine.

1. Introduction

Tropane alkaloids such as hyoscyamine and scopolamine are among the oldest drugs in medicine with wide pharmaceutical applications for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties [1,2]. Chemical synthesis of tropane alkaloids is not economically feasible, so commercial production of these compounds is entirely depended on their isolation from various members of the Solanaceae including the genera *Hyoscyamus*, *Atropa*, *Duboisia*, *Datura* and *Anisodus* [3–6]. Hyoscyamine is usually the main alkaloid in most tropane alkaloid producing plants such as *Hyoscyamus muticus* and *Atropa belladonna*, whereas scopolamine is only produced in small amounts [3,4,7]. Scopolamine is a more valuable drug due to its higher pharmacological activities, fewer side-effects and has commonly lower yields [8]. The worldwide demand for scopolamine is about 10 times greater than hyoscyamine and atropine combined [4,9]. Accordingly, there has been a long-standing interest in raising the scopolamine content of plants and their *in vitro* cultures [10] by conventional and biotechnological approaches.

Hyoscyamine 6β-hydroxylase (EC 1.14.11.11, H6H) is a key enzyme which catalyzes the last two steps in the scopolamine biosynthetic pathway (Fig. 1). This enzyme belongs to the 2-oxoglutarate-depedent dioxygenase family and catalyzes two consecutive oxidation reactions including the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine and the epoxidation of 6β-hydroxyhyoscyamine to yield scopolamine [9,11,12]. The encoding gene of hyoscyamine 6β-hydroxylase (h6h) has been cloned and characterized from various Solanaceous species such as *Hyoscyamus niger* [11], *A. belladonna* [13], *Anisodus tanguticus* [14], *Anisodus acutangulus* [15] and *Atropa baetica* [16]. It has been reported that overexpression of

Abbreviations: BSTFA, bistrimethylsilyltrifluoroacetamide; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside; MS, Murashige and Skoog; PMT, putrescine N-methyltransferase; ORF, open reading frame; TMCS, trimethylchlorosilane; TRI, tropinone reductase I; TRII, tropinone reductase II; X-Gal, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside.

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http://dx.doi.org/10.1016/j.plaphy.2013.05.007

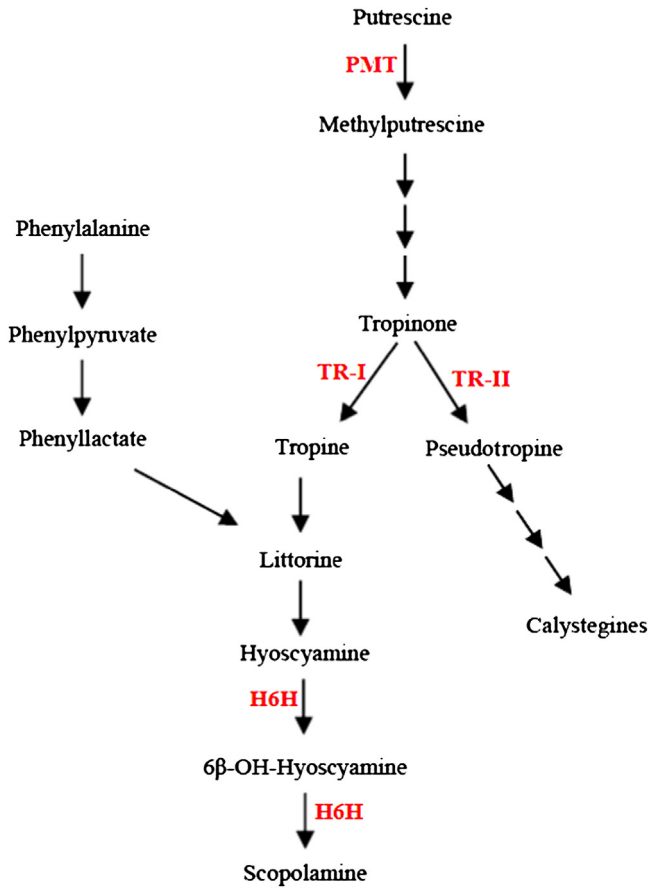

Fig. 1. Biosynthesis pathway of tropane alkaloids. PMT putrescine *N*-methyltransferase, TR tropinone reductase, H6H hyoscyamine 6 beta-hyroxylase.

the h6h gene, alone or in combination with other genes, is a feasible approach to enhance the production of scopolamine in different plants or *in vitro* cultures such as *H. muticus* [17], *H. niger* [18], *Duboisia* hybrid [10], *A. baetica* [19] and *A. belladonna* [20,21].

With increasing demand for herbal medicine, new resources for secondary metabolites extraction needed to be investigated. *Hyoscyamus senecionis* var. bipinnatisectus, is a rare herbaceous perennial medicinal plant belonging to the family Solanaceae, which grows at high altitudes in the Iranian plateau. This plant produces thick rhizomes and grows as a series of creeping stems up to 120 cm in height [22]. Cloning and expression analysis of scopolamine biosynthetic genes and study of levels of scopolamine, its precursor hyoscyamine and other tropane alkaloids in various organs of the producing plants, are important for improving our understanding of the basic mechanisms which regulate biosynthesis and transport of these compounds and enhancing tropane alkaloid production by manipulation of the biosynthetic pathway. To our knowledge, there is no report about the pattern of tropane alkaloid accumulation and also the sequences and expression profiles of tropane alkaloid pathway genes in *H. senecionis*. In order to obtain a better view of alkaloid production and accumulation at the whole plant level, the amounts of some tropane alkaloid compounds such as littorine, 3′-hydroxylittorine, tropine, hyoscyamine and scopolamine were investigated in *H. senecionis*. This led our group to clone the h6h cDNA and establish its sequence. Furthermore we also determined the expression level and pattern of this gene and other important genes of the tropane alkaloids pathway in the root, rhizome, leaf, stem and flower of *H. senecionis*.

2. Results

2.1. Tropane alkaloid profile of *H. senecionis* and its comparison with *H. muticus*

The amounts of hyoscyamine, scopolamine, tropine, littorine and 3′-hydroxylittorine in leaf and root tissues of *H. senecionis* were estimated by GC and GC–MS analysis (Table 2). The results showed that the ratio of scopolamine to hyoscyamine in the aerial parts of the plant is significantly higher than that in the roots. The scopolamine contents (mg g^{-1} dry weight) of leaf, root and rhizome organs were 0.52, 0.13 and 0.08 mg/g DW, respectively. Compared to scopolamine, hyoscyamine content was low in leaf at about 0.31 mg/g DW.

As it is shown (Table 2) these two *Hyoscyamus* species have completely different alkaloid levels and profiles in the leaves. While hyoscyamine is the main tropane alkaloid of the leaf and root of *H. muticus*, as well as underground parts (root and rhizome) of *H. senecionis*, scopolamine is the main tropane alkaloid compound in *H. senecionis* leaves. In both species, higher amounts of littorine (as an intermediate compound in the pathway) and 3′-hydroxylittorine are accumulated in roots than other organs. Overall, the most interesting result is the very high scopolamine/hyoscyamine ratio in the leaves of *H. senecionis*, an observation that, to our knowledge, has not been observed in the genus *Hyoscyamus* until now. This raised questions about the role of H6H in the production of scopolamine in various tissues.

2.2. Cloning, sequencing and bioinformatics analysis of *Hsh6h*

A full length cDNA corresponding to Hsh6h was obtained by RT-PCR and introduced into the pTZ57R/T vector. The sequencing result showed an open reading frame (ORF) of 1035 bp, which encodes a protein with 344 amino acid residues. This ORF was deposited to NCBI as a new cDNA, encoding Hsh6h with accession number of JX258921. Sequence analysis at the nucleotide level indicated that this ORF has high sequence similarity homology with other *h6h* genes from other plant species, such as *H. niger* (96%), *Brugmansia candida* (95%), *Scopolia parviflora* (91%), *Anisodus tanguticus* and *A. acutangulus* (90%) and *Atropa baetica* (89%).

The deduced protein has a theoretical isoelectric point (pI) of 5.08 and a calculated molecular mass of about 39.015 kDa. The estimated half-life is 30 h and the instability index (II) is computed to be 36.66, so this classifies the protein as stable one. The predicted protein shows sequence identity on the amino acid level with other H6Hs such as *H. niger* H6H (97%), *S. parviflora* and *A. baetica* H6Hs (89%), *A. tanguticus*, *A. acutangulus* and *A. belladonna* H6Hs (88%) and *Datura metel* H6H with 83% identity. This polypeptide also showed lower identities with other dioxygenases such as a putative hyoscyamine 6-dioxygenase from *Solanum demissum* (52%), a 2-oxoglutarate-dependent dioxygenase from *Solanum chacoense* (44%) and flavonol synthase/flavanone 3-hydroxylase from *Medicago truncatula* with 37% identity. This protein shows typical motifs and similar active site residues for dioxygenase activity, related to 2-oxoglutarate-dependent dioxygenase, including two iron-binding regions corresponding to amino acid residues Gly_{59}–Gly_{67} and Val_{253}–Val_{276} and 2-oxoglutarate co-substrate binding region relating to his_{217}–Asp_{229} [11,15] (Fig. 2). It can be noted that three strictly conserved amino acid residues that already mentioned as invariant residues among structurally non-heme oxygenases residues (His_{217}, Asp_{219}, His_{274}) [11,16] are also present in the deduced HsH6H. Comparison of *H. senecionis* and *H. niger* H6H amino acid sequences showed that these two hyoscyamine 6β-hydroxylases have 10 different amino acid residues. One of the differences is in the iron binding site, where Thr_{265} in HnH6H was