

Analysis and sequencing of h6hmRNA, last enzyme in the tropane alkaloids pathway from anthers and hairy root cultures of *Brugmansia candida* (Solanaceae)

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Abbreviations: B5: Gamborg medium

H6H : Hyoscyamine 6- β hydroxylase

HR: hairy roots

NaClO: sodium hypochlorite

PCR: polymerase chain reaction

RT-PCR: reverse-transcription polymerase chain reaction

Brugmansia candida (Solanaceae) is a native tree distributed across South-American and produces the pharmacologically- important group of tropane alkaloids including scopolamine. This biocompound is synthesised from hyoscyamine by action of Hyoscyamine 6- β hydroxylase (H6H, EC 1.14.11.11) at the end of the tropane alkaloid pathway. Here are reported the tissue and organ-specific expression of h6hmRNA by RT-PCR analyses and the isolation, cloning and sequencing of the cDNA obtained from *B. candida* anthers and hairy root transformed cultures. Bioinformatic analysis of the nucleotide sequence revealed an uninterrupted ORF of 1038 bp and the predicted aminoacid sequence could be 344 aminoacid long. A database search showed that this sequence has

high homology (97% identity) to *Hyoscyamus niger* H6H protein (Genbank accession number AAA33387.1).

Brugmansia candida is a South-American native tree that belongs to the *Solanaceae* family. Previous reports found that this plant is a high tropane alkaloid producer. Hyoscyamine and scopolamine are the most relevant tropane alkaloids widely used due to their effects on parasymphatic nervous system. These alkaloids can not be substituted by any other class of compounds and therefore their demand continues. Hyoscyamine 6- β hydroxylase (H6H, EC 1.14.11.11) catalyses hydroxylation of hyoscyamine leading to 6,7- β -epoxide of hyoscyamine (scopolamine) at the end of the tropane alkaloid pathway. This enzyme has been isolated and the corresponding gene

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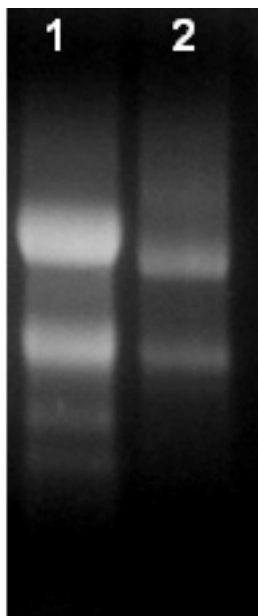


Figure 1. Total RNA isolated by the RNeasy Plant Kit and compared to the extraction with Trizol-Reagent, in *B. candida* microspore mother cells samples. Lane 1 RNeasy Plant Kit; lane 2 Trizol-Reagent extraction.

cloned from *Hyoscyamus niger* and *Atropa belladonna* plants (Rocha et al. 2002; Hashimoto and Yamada, 2003). Also, Hashimoto and Yamada (2003) localized the H6H protein at the pericycle of the root. In a previous work, Matsuda et al. (1991) demonstrated that h6h mRNA is abundant in cultured roots (hairy root), plant roots; but it is absent in stems, leaves and cultured cells.

The present work reports the tissue and organ-specific expression of h6hmRNA by RT-PCR analyses and the isolation, cloning and sequencing of the messenger obtained from *B. candida* anthers and hairy root cultures.

MATERIALS AND METHODS

Plant material

Different organs: root tips; apical stem and leaves and seeds were harvested from plants that grew at the “Jardín Botánico” of Buenos Aires (Argentina). Although, anthers with pollen at the mother cells stage of immature close flowers were analysed from the same plants.

Hairy root cultures

Seeds were surface sterilized by immersion in NaClO (4%) for 30 min, and rinsed three times with sterile distilled water. Thereafter, seeds were placed on B5/2 medium (hormone-free, half-strength Gamborg B5 medium) supplemented with sucrose 15 g/l and agar 8 g/l. Incubation was carried out at 24°C ± 2°C with a 16 hrs photoperiod.

Hairy root (HR) cultures of *B. candida* were obtained from 3-4 weeks old seedlings after infection with *Agrobacterium rhizogenes* strain LBA 9402. The HR growth at the infection sites were excised and cultured individually on B5/2 liquid medium supplemented with sucrose 15 g/l, ampicillin 2 g/l and agar 8 g/l. The resulting HR were incubated on a gyratory shaker at 100 rpm in the same conditions described above. The HR were routinely sub-cultured every 2 weeks reducing 1:10 the concentration of antibiotics until the elimination of *Agrobacterium*. Transformation event was confirmed according to methods described previously (Pitta Alvarez et al. 2003).

RT-PCR and PCR analysis

Total RNA was isolated from organs and HR mentioned above with Trizol-Reagent and compared to the extraction with RNeasy Plant Kit (Qiagen). Integrity and size distribution of purified total RNA were checked by gel electrophoresis on denaturing conditions. The cDNA synthesis was performed using Superscript II reverse Transcriptase (Life Technologies).

Primer design

Specific primers were designed based on the sequence of the h6h gene from *Hyoscyamus niger* (Genbank, M62719). The resulting primers were: 5’ATGGCTACTTTTGTGTCG3’ and 5’CACTCTAGACATATGAGT3’.

PCR cycle parameters

Melting step: 94°C for 4 min, annealing step: 40°C for 1 min, elongation step: 72°C for 3 min. At termination of 30

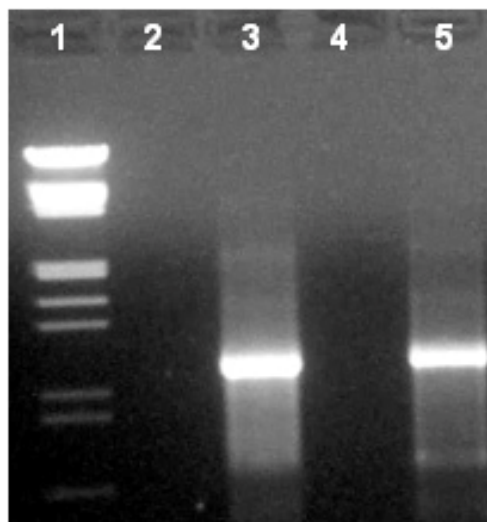


Figure 2. RT-PCR and PCR using specific primers in *B. candida* microspore mother cells samples. Lane 1 Molecular weight marker; lanes 2 and 4, water controls subjected to both RT and PCR reactions; lanes 3 and 5 PCR products from microspore mother cells samples.

cycles, there was a further, final elongation step of 72°C for 7 min.

Cloning in the pCR2.1-TOPO vector

Agarose gel electrophoresis was performed as described by Sambrook et al. (1989). The amplified fragments of the expected size were isolated from agarose gel and purified using the GFX columns (Amersham). They were cloned in the TOPO vector according to the manufacturer instructions (Invitrogen).

Escherichia coli strain DH5α was transformed with the construction obtained by chemical transformation. Positive clones obtained from screening were analyzed by restriction mapping and confirmed by sequencing.

Automated sequencing

The samples were sequenced by the DNA ABI 373A automated sequencer, based on the Sanger method. The results were analysed by bioinformatics tools.

RESULTS AND DISCUSSION

The induction of HR from *B. candida* plants using de *A. rhizogenes* LBA9402 was successfully obtained with a frequency of transformation around 80%. This is according to previous experiments done with this plant material (Pitta Alvarez et al. 2003). The transformation process was checked by PCR reaction (data not shown).

The root tips were isolated from roots growing into de soil. Shoot, leaves, anther were obtained from the same plants.

Different preparations of total RNA were obtained from 3

weeks-old HR cultures and from different tissues and organs of flowering plants.

Concerning to RNA isolation, the RNeasy Plant Mini Kit allowed us to obtain higher total RNA levels comparing to Trizol procedure. The results are shown with anthers due to the stronger specific signal obtained with this organ. Also, the integrity and size distribution of total RNA were checked by denaturing agarose gel electrophoresis as described in Materials and Methods. The ribosomal RNA appeared as sharp bands when Total RNA was extracted using the RNeasy Kit (Figure 1). Also, the 28S ribosomal RNA band presented an intensity of approximately twice that of the 18S RNA band (Figure 1).

In contrast, the ribosomal bands in Trizol methodology were not sharp and appeared smaller sized RNA bands. In this case, the RNA sample suffered major degradation during preparation (Figure 1).

The relative abundance of h6hmRNA was determined by RT-PCR in root tips, stem, leaves, anthers and HR cultures. No signal was detected in apical shoot and leave samples from flowering plants. HR and root tips samples showed a weakly signal. However, anthers showed the strongest signal.

Reverse transcription was carried out using an oligo-dT primer which allowed detection of multiple species of cDNAs from the total RNA. The RT reaction was amplified by PCR and appropriate specific primers to evaluate the h6hmRNA presence. The primers were designed using the known sequence of the *Hyoscyamus niger h6h* gene due to the homology founded among the members of the Solanaceae family (Genbank, M62719). In Figure 2 are

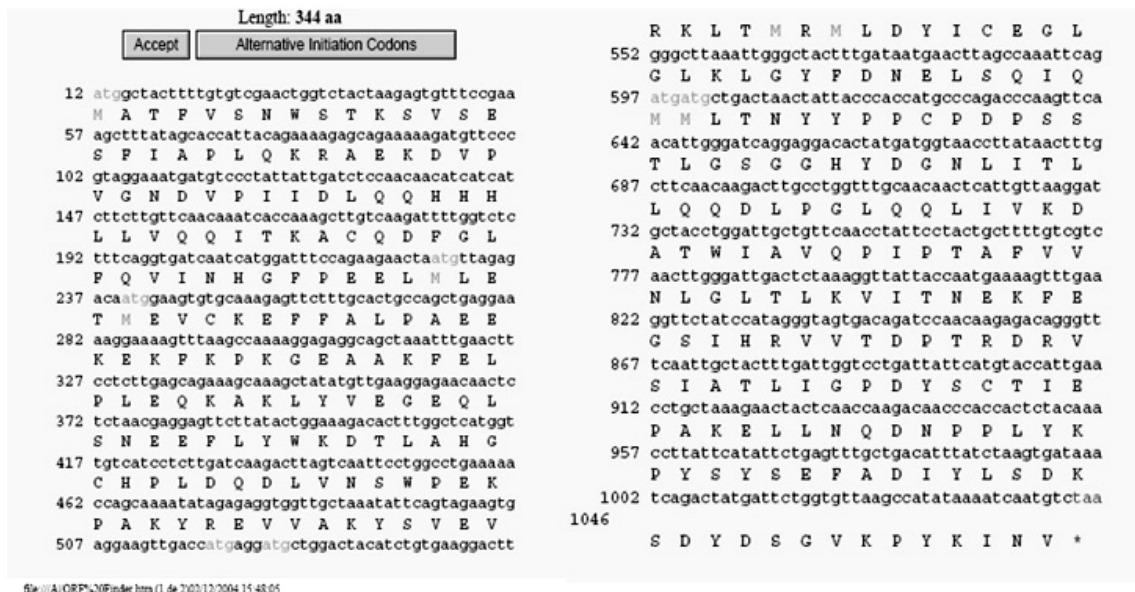


Figure 3. Nucleotide and the deduced amino acid sequence of *B. candida* H6H cDNA analyzed by the Open Reading Frame Finder (NCBI).

shown the PCR amplification products that have an estimated molecular mass of 1 Kb.

Amplified PCR product was cloned using the TOPOVector Technology. EcoRI restriction mapping performed on positive clones showed the release of 1 Kb fragment as expected (data not shown).

The 1Kb PCR product was sequenced (Figure 3). Furthermore, using the sequence information, internal primers were designed in order to confirm H6H sequence.

The bioinformatic analysis revealed an uninterrupted ORF of 1038 bp. The predicted aminoacid sequence is 344 aminoacids long. A database search showed that this sequence has high homology (97% identity) to *H. niger* H6H protein (Genbank accession number AAA33387.1) (Figure 3). These results are coincident to previous reports about the H6H from other related species (Matsuda et al. 1991). Also, the 2OG-Fe(II) oxygenase superfamily domain is conserved in the h6h gene from *B. candida*. In addition, the analysis shows that the h6h sequence has similarity to other hydroxylases including those involved in the formation of ethylene (locus AAA85365 *Picea glauca*, NP_914944 and XP_476309 *Oryza sativa*; AAM65315 *Arabidopsis thaliana*), and anthocyanins (locus BAD29052 *Oryza sativa*).

Using RT-PCR methodology combined with specific primer design has led to obtain a rapid cloning method. The expression of h6h gene was investigated by RT-PCR in different organs and tissues from flowering plants and HR cultures. Total RNA extracted was analyzed but the different degrees of variation observed in the expression of H6H among samples could not be explained (Brunner et al. 2004). It should also be remembered that *B. candida* is a self-fertile natural hybrid which will presumably increase the potential range for variation among the population (Giulietti et al. 1993). The highest concentration of h6hmRNA was identified in anther samples but these aspects need to be investigated in further experiments.

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