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IDENTIFICATION AND AMPLIFICATION OF HYOSCYAMINE 6β-HYDROXYLASE GENE IN GENOMIC DNA OF IN VITRO CULTURED ROOTS OF *HYOSCYAMUS NIGER* L. BY POLYMRASE CHAIN REACTION

HASSAN EBRAHIMZADEH, AFSANEH TEIMOORI and TAHMINEH LOHRASEBI

Department of Biology, Faculty of Science, Tehran University

ABSTRACT

The H6H gene for hyoscyamine 6β-hydroxylase (H6H), which converts hyoscyamine to scopolamine, was isolated from *Hyoscyamus niger*. The roots of 14 days sterile seedlings were transferred to a modified liquid B_5 medium containing 1 μM indolebutyric acid, and after appearance of the lateral roots, subcultured in a free hormone medium. Following a week, its genomic DNA was extracted and PCR performed. By extraction an accurate DNA and using suitable primers, H6H gene can be isolated completely.

Key words: *Hyoscyamus niger*, Cultured roots, H6H gene

INTRODUCTION

Recent advances in molecular biology and plant transformation techniques include attempts to enhance the productivity of useful secondary products by metabolic engineering (1). Cultured roots were found to be especially active in alkaloid biosynthesis and have been widely used for biosynthetic studies of tropane alkaloids (2). Tropane alkaloids such as hyoscyamine and scopolamine are widely used as anticholinergic agents(1). Because hyoscyamine has undesirable effcts on the central nervous system, the use of scopolamine is preferred (3). Thus, there has been a long–standing interest in increasing the content of scopolamine in cultivated medicinal plants (1).

Roots of several solanaceous plants produce the above-mentioned alkaloids, which are then translocated to the aerial parts of plant (2). Rapid growing root cultures can be established either by manipulation of auxin levels in culture medium, or by genetic transformation of plants with *Agrobacterium rhizogenes* (1,4). Hyoscyamine 6β-hydroxylase (H6H), a 2-oxoglutarate–dependent dioxygenase, catalyzes the hydroxylation of hyoscyamine to scopolamine (5). H6H activity is found at high levels in cultured roots of the *Duboisia* and *Hyoscyamus* species and at very low levels in their cultured shoots and calli (2).

A rough correlation has been found between the H6H activity and the ratio of scopolamine to hyoscyamine contents in scopolamine–producing roots. Thus, H6H is a promising target enzyme which, when expressed strongly in hyoscyamine–accumulating plant tissues, increases contents of scopolamine in the transformants (1). This paper describes the isolation of H6H gene from genomic DNA of cultured root of *Hyoscyamus niger*.

MATERIALS AND METHODS

Chemicals: The indolebutyric acid (IBA), cethyltrimethylammonium bromide (CTAB) and ethidium bromide were purchased from Merck Chemical Co., Ltd (Germany). The agarose, DNA molecular weight marker XVII and PCR kit were from Boehringer and Mannhaim Chemical Co., Ltd (Germany). The two primers were obtained from National Research Center for Genetic Engineering and Biotechnology (Iran).

Correspondence: H. Ebrahimzadeh, Department of Biology, Faculty of Science, Tehran University, Tehran, Iran.
Email: Ebizadeh@khayam.ut.ac.ir.

Plant materials: Seeds of *Hyoscyamus niger* were harvested from Kandowan region (2400 meters high) and after sterilization (6), were treated for 48 hours at -20°C to make rapid germination. Roots were transferred to modified liquid B_5 medium (macro- and micro-elements of B_5 medium, ferrous–EDTA and vitamins of MS medium) containing 1μM indolebutyric acid and 3% sucrose and maintained on a rotary shaker at 100 rpm in the dark at 25°C (1,7). Following appearance of the lateral roots, samples were transferred to a liquid modified B_5 medium free hormone, and after a week used for extraction of genomic DNA.

Polymerase chain reaction: The presence of the H6H gene in root tissued of *H.niger* was analyzed by PCR. Genomic DNA was extracted from root samples (1). PCR was achieved by using taq polymerase, 0.2 μg of genomic DNA and two primers that would anneal at the start of the gene.

Primer 1:
GAGGATCCATTTGATGGCTACTTTTGTG (28mer)

Primer 2:
TGCCTTAAGTTAGACATTGATTTTATATGG (30 mer)

The reaction conditions were: 95°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes (25 cycles).

Electrophoresis of PCR product: The genomic DNA and its PCR product were detected on a 1.5% agarose gel. Also by using a DNA molecular weight marker XVII (as a size marker 4.3 Kb) (8), the correct amplification of the favored gene, was determined. Five μg of total genomic DNA, 1 μg of DNA size marker and 5-10μl of PCR product were loaded in the wells. Visualization of the DNA bands was accomplished by using ethidium bromide (0.5μg/ml) (9).

RESULTS AND DISCUSSION

In vitro cultures of *Hyoscyamus* species have been examined for their tropane alkaloid production by various investigators. The influence of phyto-hormones and light on the production of tropane alkaloids in transformed roots of *Hyscyamus* have been reported in details (10,11).

It was reported that low pH (3.5), MS medium vitamin, and a 1% sucrose have the best effect on growth, morphogenesis and alkaloid production in suspension cultures of *H. muticus* (12). The effects of various nutrient conditions to optimize root growth and scopolamine production in normal root of *H. niger* has also been examined. It hs been reported that nitrate or phosphate concentration (in B_5 medium) has little effect on the root growth and only the low levels of these compounds enhanced specific scopolamine content by 44% and 39% respectively (3). Also it has been reported that auxins, especially IBA, promoted the growth of cultured *Hyoscyamus* roots mainly by stimulating lateral root induction and biosynthesis of scopolamine was inhibited when the concentration of IBA in the culture medium was increased (7).

Considering all above mentioned reports, in this project, 1μM IBA was used to induce lateral roots. After appearance of the lateral roots, samples were transferred to a liquid modified B_5 medium free hormone, and after a week, used for extraction of genomic DNA. This modified B_5 medium had a very desirable effect on the growth and induction of lateral roots of *H.niger* (Fig.1). The absorption spectrum of DNA preparation was determined by spectrophotometric method at a range of 200-300 nm (Fig 2). The H6H gene (with 3 exons and 2 introns) codes a 38KD protein composed of 344 amino acids (2, 13). The gene specific primers were designed based on the DNA sequence of H6H (to anneal the start site of the gene).

Expression of H6H gene in pericycle and the complete size of this gene have been reported as 4.3 Kb (8). The results of the present study are in accords with above respected results (Fig. 3, 4, 5). Our future program is further recognition of this gene, its separation from plant, and study of its effect on alkaloid biosynthesis.

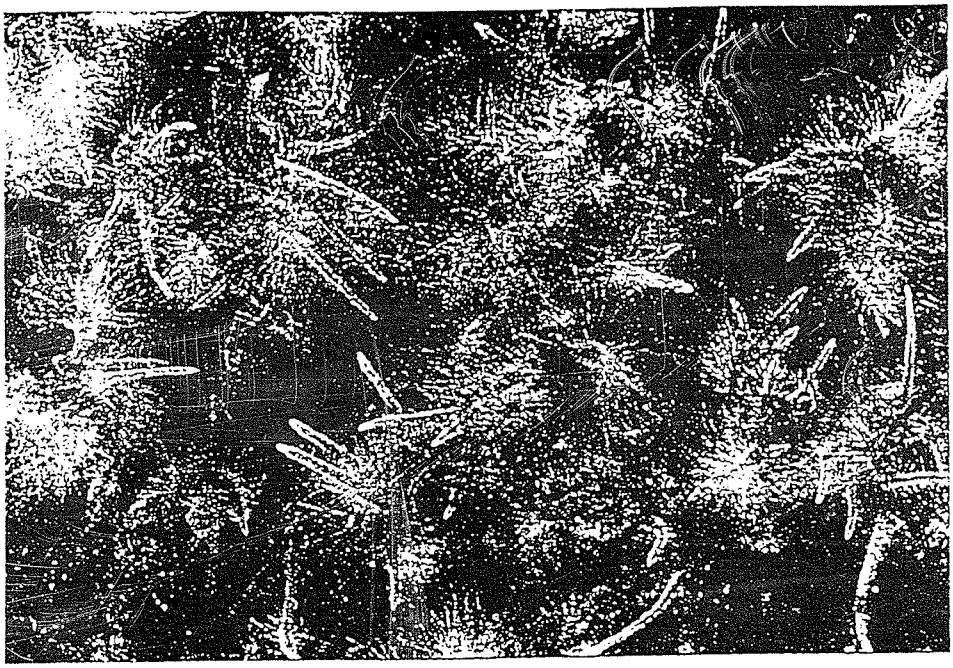

Fig 1. Cultured roots of sterile seedlings of *H. niger* that were used for genomic DNA extraction. (a week after transfering to a medium free hormone).

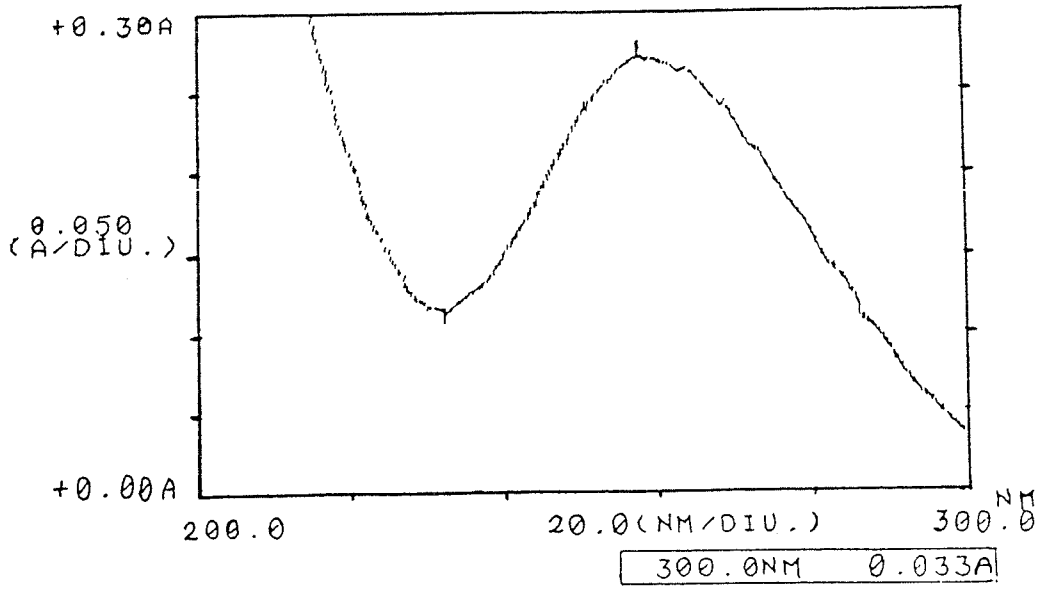

Fig 2. The absorption spectrum of DNA preparation. The maximum absorption: 257.5 nm. The minimum absorption: 238 nm.

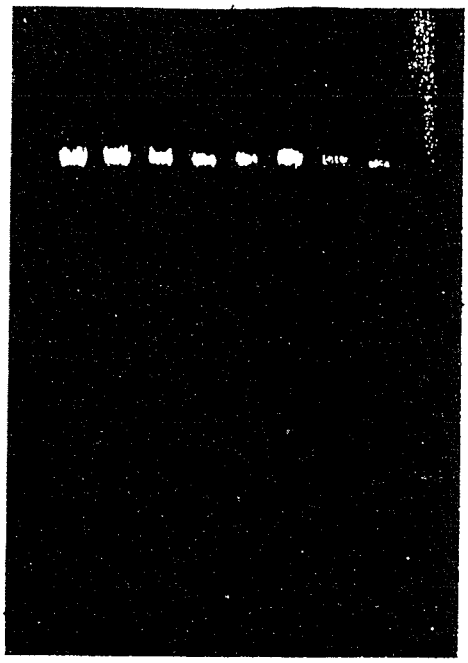

Fig 3. Genomic DNA samples of cultured roots of *H. niger* on a 0.5% agarose gel (to show the accuracy of DNA). Lane 1-8: genomic DNA samples of *H. niger*.

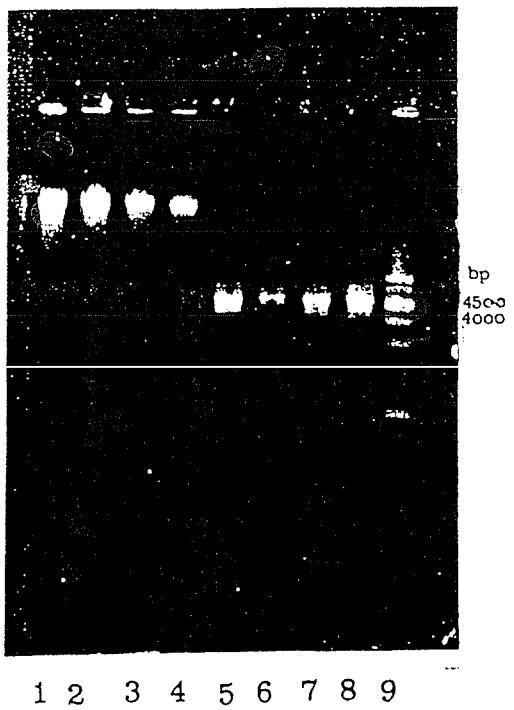

Fig 4. The detection of amplified H6H gene by PCR on a 1.5% agarose gel.
Lanes 1-4: genomic DNA samples of *H. niger*. Lanes 5-8: amplified H6H gene (4.3 Kb). **Lane 9: a** DNA size marker (contained 10 bands, at a range of 500-5000bp bands).

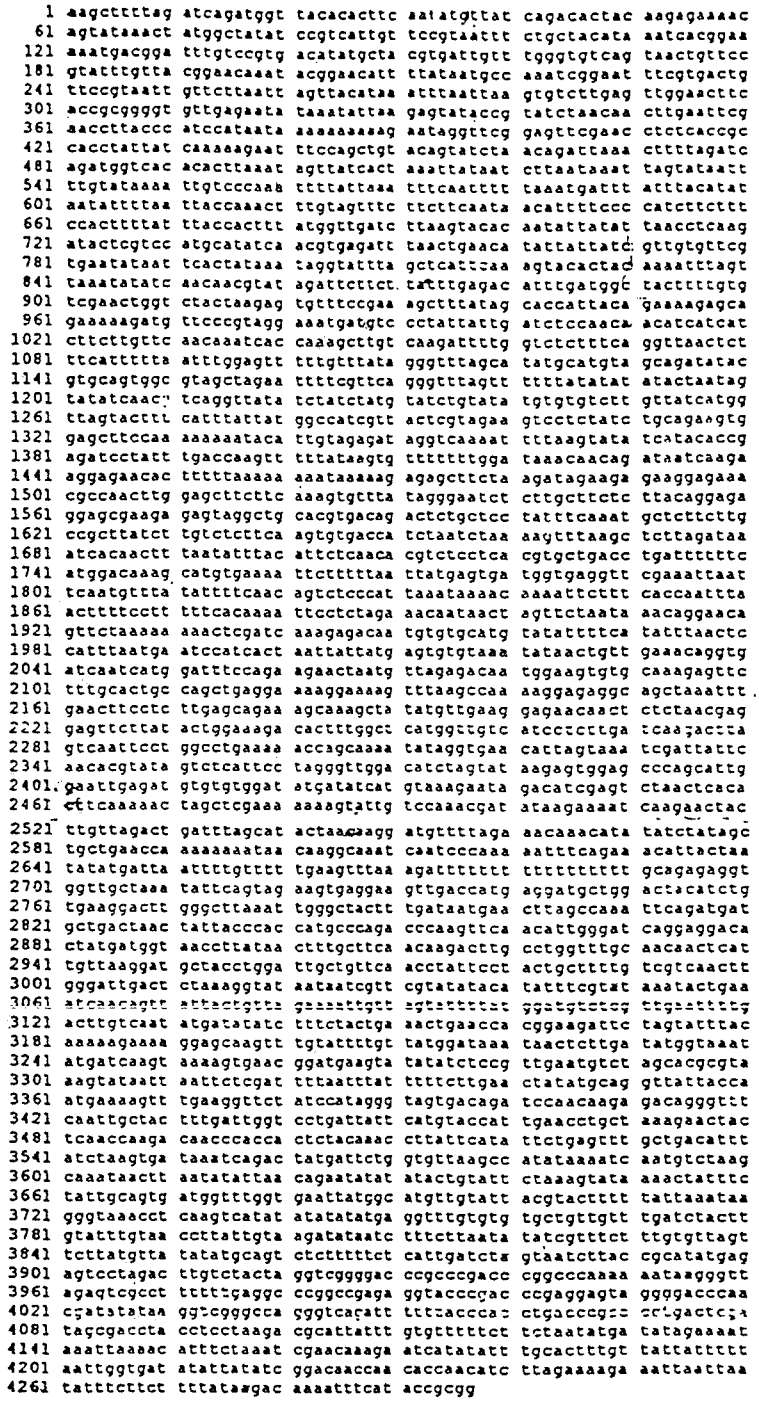

Fig 5. The nucleotide sequences of H6H gene of *H. niger* (with introns)(4).

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