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Detect of Hypericin (HyH) gene in *Hypericum erectum* in Korea and Comparison of *H. perforatum* in Europe

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Hypericin (HyH) is a substance which is isolated a medicinal herb, *Hypericum perforatum* L., commonly known as St. John's Wort. *Hypericum erectum* is a long-lived herb that is distributed in Korea. Cloned HyH genes *H. erectum* of were conformed by sequencing. The cDNA Hyp-1 sequence has 732 bp with an open reading frame of 567. Thus coding for a protein of 152 amino acid residues. A BLAST research using the deduced nucleotide sequences in HyH gene produced significant alignments with the *H. perforatum*. Sequences in HyH gene showed significant homology with *Rubus idaeus* putative allergen Rub-i-1 mRNA, Protein sequence comparisons revealed significant homology between Hyp-1 and the phenolic oxidative coupling protein hyp-1 of *H. perforatum* (98%). Additionally, Hyp-1 showed significant homology with various other classes of allergens, including Pru-av-1 (62%) from *Prunus avium* and allergen Bet-v1-Sc3 from *Betula pendula* (60%). Thus, the result of this study may offer an important information to establish an assay system for chemicals of the herbal medicines for *H. erectum* as well as *H. perforatum*.

Key words – Hypericin (HyH), *Hypericum erectum*, *Hypericum perforatum*

Introduction

Herbology is one of the oldest sciences. Human beings have been using herbs for medicines for thousands of years, and almost every culture has had some herbal medicine tradition [3]. Many modern medicines were derived, originally, from herbal remedies. In fact, it is a mystery as to how 'pre-scientific' cultures discovered some of these herbal concoctions. Some of the herbal effects are quite subtle, others require complex preparation, some herbs are only effective when mixed with other agents. How did our ancestors discover these remedies? This ancient knowledge is a true marvel [5,9].

Scientific investigation has shown that herbal efficacy is due to natural chemicals in the herbs. In other words, herbs are drugs. Often some of the chemicals are pharmaceutically active, others are bio-irritants, others are toxins, and still others have little effect on the human body [1]. All of these chemicals can be mixed up in a wild melange in a single herb [8,17]. The mixtures depend not only on the species of plant, but also on individual genetic traits, and on growing conditions. Concentrations of active ingredients therefore vary widely between individual plants [5,6,17].

Hypericin (HyH) is a substance isolated a medicinal herb *Hypericum perforatum* L., commonly known as St. John's Wort [12]. HyH belongs to group of compound known as napthodianthrones [2]. HyH is a secondary plant metabolite of St. John's Wort and the amount of HyH strongly depend upon the source of plant material [1]. Initially HyH was considered to be the antidepressant principle of *H. perforatum*, but according to recent research hyperforin has emerged as antidepressant principle of the herb [2]. HyH has been reported as the active compound found in St. John's Wort and has been shown to act as a monoamine oxidase inhibitor [2,11]. As is true for most anthraqunones, there are no published descriptions of the biosynethetic pathway that leads to HyH production.

HyH is used as strand for identification of genuine plant material and thus has importance from quality control point of view. The standardization of *H. perforatum* is now based on both HyH and Hyperforin content. For example, the herb must contain 0.3% of HyH [4].

Clinical studies with St. John's Wort have shown it to be as effective as the standard anti-depressant drugs but with fewer side effects [19]. Although the exact mechanism of action is not fully understood, most scientists believe it exerts its anti-depressant effect by inhibiting serotonin reuptake. The use of St. John's Wort has increased dramati-

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cally in both Europe and the U.S. More than 60 million daily doses of the herb are prescribed by German physicians

The genus *Hypericum* includes about 370 species of medium sized herbs to shrub distributed globally except for arctic and desert areas and lowland trophics [13]. *Hypericum erectum* Thunberg (Cuttiferae) is a long-lived herb (< 0.6 m in height) that is distributed in natural habitats of fields and mountains. *H. erectum* is diploid (2n=18) and predominantly insect-pollinated that blooms from July to August.

The purpose of this study is to compare a close homology with the cDNA sequences of HyH in *H. perforatum* or those of other plants.

Materials and Methods

Plant materials and cell culture

Leaves were collected from *H. erectum* in Korea. Callus cultures were initiated using leaves from a week old *H. erectum* plant grown *in vitro* as previously described by Bais et al. [2]. Cell suspension cultures were established from the callus cultures and were maintained in 200 ml flasks with 50 ml of nutrient MS [15] medium containing 2,4-dichlorophenoxyacetic acid (0.9 uM), kinetin (0.11 uM), and sucrose (30 g/L) by biweekly sub-culturing on a rotary shaker at 90 rpm maintained at 25±1℃.

RNA extraction and cDNA library construction

Dark-grown cells of *H. erectum* were used for cDNA library construction from cell cultures of *H. erectum*. Using TRI REAGEN (Sigma, St. Louis, MO), total RNA were extracted from leaves, roots, and shoots grown in vitro, and dark-grown cell suspension cultures of *H. erectum*. 1 ug of total RNA was used for cDNA synthesis using reverse transcriptase (RT) (Clontech, Powerscript™ RT). The cDNA was amplified by 20 cycles of long distance PCR. The amplified DNA was phenol-extracted, digested with *Sfi*I, and ligated into a λ-TriplEX2 vector using the SMART™ cDNA construction kit (Clontech, PTR3001). The λ-TriplEX2 vector was packed into an SBET phage using the Packgene® Lamda DNA packaging system (Promega).

Screening for enzymes involved in the synthesis of HyH

A λ-TriplEX2 vector containing *H. erectum* cDNA library was plated with *Escherichia coli* for screening. The percentage of recombinant clones determined by blue/white screening in the presence of isopropyl-1-thio-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Primary screening of the λ- TriplEX2 library for genes involved in the biosynthesis of HyH was performed after 12-h incubation 37℃ under dark conditions by spreading emodin (20 ug/ml) on the bacterial lawns and selecting the phage plaques that turned pale yellow emodin into red-colored HyH. Ten positive plaques were selected and re-screened after amplification. Secondary screening of amplified plaques were performed identified to the primary screening by addition of 20 ug/ml emodin to the overnight grown plaque lawn. Colonies, which turned red instantly, were picked with a micro-tip and then converted to pTriplEx2 in *E. coli* (BM 25.8) by in vivo excision and circularization into a complete plasmid from the recombinant phage. For the large scale conversion of emodin to HyH, selected *E. coli* clones were cultured overnight under dark condition in LB broth media (50 ml). Subsequently, emodin was added (20 ug/ml) to visualize and quantify the bioconverted HyH using LC-MS. Interconverted bacteria cells and selected plaques from secondary and tertiary screening were picked using a micropipette tip. The pTriplEX2 sequencing primers provided with the SMART™ cDNA construction kit (Clontech, PTR30001) were used to perform PCR for the determination of insert sizes. The bacterial cells, cell-free media filtrate, and plaques, obtained from secondary and tertiary screening, were extracted as per the methology described earlier and analyzed by HPLC-MS for HyH identification.

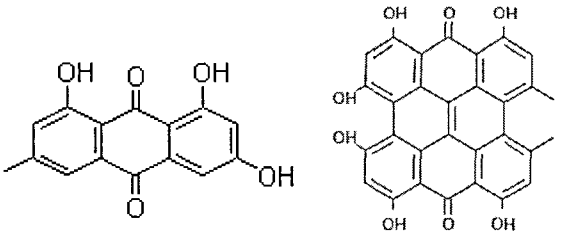
Sequencing

Cloned genes were conformed by sequencing. The DNA was sequenced on a ABI Prism 377 DNA Chemistry protocol. Sequences were aligned using MacVector and Assembly LIGN software (Kodak, International Biotechnologies, Inc., New Haven, CT). The sequence was analyzed, follow by amino acid alignment to compare homologous proteins using VectorNTI Suite software (Informax, Bethesda, MD).

Phyogenetic analysis

A phenetic relationship was performed using the package PAUP 4* (ver. 4.0b8 for 32-bit Microsoft Windows) [16]. The aligned *H. erectum* sequences were analysed by the maximum parsimony (MP, with the branch and bound procedure) and maximum likelihood (ML) methods [7].

Node support was estimated with searches on 1000 bootstrap replicates. Indels were alternatively included and excluded in the analyses, to score the influence of gaps on the results. Equal weight was initially given to transitions and transversions; all analyses were then replicated by imposing a weight to transversions 2, 3, 5, and 10 times that of transitions. The log likelihood scores were finally calculated [7].



(A) (B)

Fig. 1. Structures of emodin (A) and hypericin (B) in *Hypericum perforatum*.

Results

cDNA cloning and precursor-based screening of λ-phages

A cDNA library was constructed by isolating RNA from dark-grown cell suspension cultures of *H. erectum* using the λ-TriplEX2 vector. Primary screening of a cDNA library was performed from the unamplified library using *E. coli* (5α). After a 12-h incubation at 37℃, positive plaques were selected by white/blue color screening to obtain stable transfectants and selected white clones turned red post-emodin administration. The plaques were amplified and used for inter-conversion in *E. coli* cell lines.

H. erectum conversion of emodin to HyH may be due to an enzymatic dimerization reaction. In support of this hypothesis, it was found traces of emodin in *H. erectum* cell cultures (Fig. 1).

Sequence analyses

The cDNA Hyp-1 sequence has 732 bp with an open reading frame of 567 (including stop codon). Thus coding for a protein of 152 amino acid residues (Fig. 2). All positive clones analyzed contained a 3'non-coding region of nucleotides between the stop codon and a poly (A) tail. The alignment resulted in 7 gaps, which were treated as missing data. A BLAST research using the deduced nucleotide sequences in HyH gene produced significant alignments with the *H. perforatum* (Fig. 3). Sequences in HyH gene showed significant homology with *Rubus idaeus* putative allergen Rub-i-1 mRNA, partial codes found a wide variety of species. Nucleotide sequence comparisons revealed homology between Hyp-1 and a *Cichorium intybus* mRNA for vegetative storage protein (VSP) with a 30% homology.

Query	1	ACAACACAAAATACAGAT--A-CA-TTAGTGATCAGTGTTTATATACTTTCATCTTTTAG	56
Sbjct	1	ACAACACAAAATACAGATACATCAGTTAGTGATCAGTGTTTATATACTTTCATCTTTTAG	60
Query	57	CTATTTTAACATTTCTGAATATGGCGGCGTACACCATTGTTAAGGAGGAGGAATCTCCAA	116
Sbjct	61	CTATTTTAACATTTCTGAATATGGCGGCGTACACTATTGTTAAGGAGGAGGAATCTCCAA	120
Query	117	CTGCACCCCACAGGCTGTTTAAGGCATTGGTCCTTG-ACGCCATCAAGTCATTGTTAAGG	175
Sbjct	121	CTGCACCCCACAGGCTGTTTAAGGCATTGGTCCTTGAACGCCATCAAGTCCTTGTTAAGG	180
Query	176	CTGAGCCTCATGTCTTCAAGAGCGGCG-AATTATCGAAGACGATGGAGGTGTCGGCACGG	234
Sbjct	181	CTCAGCCTCATGTCTTCAAGAGCGGCGAAATTATCGAAGGCGATGGAGGTGTCGGCACGG	240
Query	235	TCACCAAAATCACTTTCGTTGATGGACATCCCCTCACGTACATGTTGCACAAGTTTGATG	294
Sbjct	241	TCACCAAAATCACTTTCGTTGATGGACATCCCCTCACGTACATGTTGCACAAGTTTGATG	300
Query	295	AAATTGATGCCGCAAATTTTTATTGCAAGTACACTATTTTTGAAGGTGATGTGTTGCGTG	354
Sbjct	301	AAATTGATGCCGCAAATTTTTATTGCAAGTACACTATTTTTGAAGGTGATGTGTTGCGTG	360
Query	355	ACAATATTGAGAAGGTTGTCTATGAGGTGAAATTGGAAGCCGTAGGCGGAGGAAGCAAGG	414
Sbjct	361	ACAATATTGAGAAGGTTGTCTATGAGGTGAAATTGGAAGCCGTAGGCGGAGGAAGCAAGG	420
Query	415	GTAAGATTACAGTCTCCTATCATCCCAAGCCTGGTTGCACCGTTAATGAAGAAGAAGTCA	474
Sbjct	421	GTAAGATTACAGTCTCCTATCATCCCAAGCCTGGTTGCACCGTTAATGAAGAAGAAGTCA	421
Query	475	ACATCGGGGAAAAGAA-GCCTATGAATTTAACAAGCAAGTGGAAGAATACCTTGCTGCTA	533
Sbjct	481	AGATCGGGGAAAAGAAAGCCTATGAATTTTACAAGCAAGTGGAAGAATACCTTGCTGCTA	540
Query	534	ATCCTGAAGTTTTCGCTTAAAAATTTCTTTGCTC	567
Sbjct	541	ATCCTGAAGTTTTCGCTTAAAAATTTCTTTGCTC	574

Fig. 2. Nucleotide sequence and deduced amino acid sequence of Hyp-1.

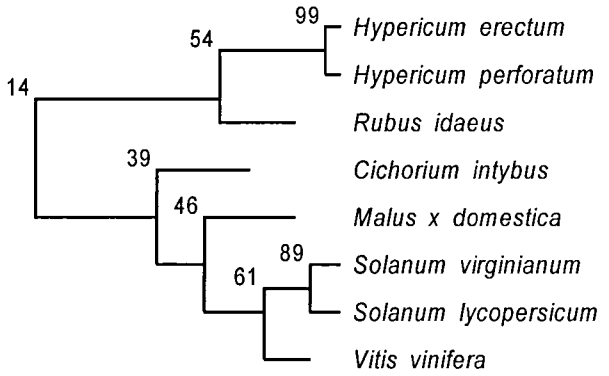


Fig. 3. ML phylogenetic tree of the deduced nucleotide sequences in HyH gene. Query coverage are shown for nodes on the phylogeny.

A BLAST research using the deduced amono acid sequence of Hyp-1 produced significant alignments with the phenolic oxidative coupling protein hyp-1 of *H. perforatum* (98%) (Fig. 3). Hyp-1 showed significant homology with various other classes of allergens, including Pru-av-1.0201, a major cherry allergen (62%), Pru-av-1.0202 (61%) and Pru-av-1.0203 (60%) from *Prunus avium,* and allergen Bet-v1-Sc3 from *Betula pendula* (60%). Additionally, It produced significant alignments with the Mal d1 family of proteins found a wide variety of apple, genus *Malus.* Protein sequence comparisons revealed significant homology between Hyp-1 and a major apple allergen (Mal d1) with a 58% homology, followed by Cor-a-1.04, a major hazelnut (Corylus avellanal) pollen allergen with 60% identity in amino acid sequence to Hyp-1.

The amino acid sequence analysis indicates Hyp-1 has a molecular mass of 17.8 kDa with a PI of 5.54. The motif scan for the sequence of Hyp-1 shows similarity with the Bet.v.1 family (pathogensis-related proteins) signature motif. The sinature G-X_2-[LIVMF]-X_4-E-X_2-[CSTAEN]-$X_{8,9}$-[END]-G-[GS]-X_2-K-X_4-[FY], which is a characteristic of Bet.v.1, is positioned at 89-120 amino acid residues in Hyp-1.

Discussion

The initial steps in the biosynthesis of HyH are derived from acetyl-CoA and malonyl-CoA conversion. These steps are followed by cyclization steps leading to the formation of an anthrone derivative, which may further bifurcate to form emodin and emodine anthrone (Fig. 1). Early laboratory syntheses involved many steps and low yields, but HyH could be prepared with a 66% yield from emodin dianthrone, which is available synthetically from commercially available emodin. I hypothesized that in *H. erectum* conversion of emodin to HyH may be due to an enzymatic dimerization reaction. In support of this hypothesis, I found traces of emodin in *H. erectum* cell cultures.

The amino acid sequence of Hyp-1 has a close homology with the Bet.v.1 family. Bet.v.1 is a major allergen from the white birch tree (*Betula verrulosa*). Proteins belonging to the Bet.v.1 family are the amin cause of pollen-related food allergies [18], and this protein family shows significant (35-60) homology to PR (pathogenesis-related)-10 type proteins [10,20], which are expressed in plant tissues upon microbial infection and chemical treatment [21]. The deduced amino acid sequence for Hyp-1 comprises several of the signature features of Bet.v.1 class allergens [10]. A conserved motif present in Hyp-1 is common throughout all the allergen-coding sequences from the Bet.v.1 family as well as in the PR-10 intracelluar PR proteins [14].

Many plants could live in regions where they were not endemic. Indeed many plant species have been introduced to new locals. This invites the theory that these floral types where once strictly local endemics. Different places may have virtually the same ecosystem and climate. Similar climates and soils tend to host similar vegetation types. However, they do not necessarily share the same species. The deciduous forests of Europe and East Asia including Korea may look similar, but they are not composed of the same species. *H. erectum* is found in Northeast Asian regions such as Japan, central and northeast China and absent in Europe. whereas, *H. perforatum* is found in Europe and America, but the species is not distributed in Northeast Asian regions. Nevertheless, to some degree, *H. erectum* is more or less to similar to *H. perforatum* in habit, petal and sepal size, inflorescence type, style length, stylar hair distribution, leaf base, stem branching pattern, and the result of sequences of HyH gene. However, in many countries there is an economic advantage to herbs like St. John's wort. Herbal remedies are often cheaper than factory made drugs. For example, in many countries including Korean and Chinese naturopathic and homeopathic shops are as common as pharmacies. This reflects the economic reality of Korea just as much as it reflects local beliefs. *H. erectum* has been readily available in all parts of Korean drugs, so is interesting variants of herbal tradition. Scientific investigation of *H. erectum* has shown that HyH is due to natural chemicals in the Korean herbs. Thus, the result of this study may offer an important information to establish an assay system for chemicals of the herbal medi-

cines and provide a quality control for *H. erectum* as well as *H. perforatum*.

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초록 : 한국내 고추나물의 하이퍼리신 유전자(HyH)의 탐색과 유럽의 서양고추나물과 비교

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하이퍼리신(Hypericin, HyH)은 예로부터 성 요한의 풀(St. John's Wort)로 널리 알려져 있는 서양고추나물(*Hypericum perforatum*)에서 추출되는 약리성분이다. 서양고추나물은 국내에서는 자생하지 않으나 같은 속의 고추나물(*H. erectum*) 등이 이속에 속하며 우리나라에 자생한다. 고추나물을 조직배양으로 RNA를 추출하고 cDNA를 합성한 후 Hyp 유전자를 추출하여 서열화한 결과, 전체 크기는 732 bp로 나타났으며 서양고추나물의 HyH-1과 거의 99.8% 서열 일치를 나타내었다. 이 서열의 152개 아미노산 역시 서양고추나물의 항산화효소와 98% 일치하였으며 자작나무와 능금속 식물에서 유발되는 알레르기 유발유전자와 약 60% 일치를 나타내었다. 본 연구 결과 우울증치료제로 사용되는 하이퍼리신 추출에 우리나라 자생종인 고추나물이 이용될 수 있을 것으로 사료된다.