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Full Length Research Paper

Molecular cloning and characterization of the *HDR* gene involved in the methyl-erythritol phosphate (MEP) pathway from sweet potato [*Ipomoea batatas* (L.) Lam]

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1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR, EC 1.17.1.2) catalyses the last committed step of MEP pathway, which provides precursors for biosynthesis of isoprenoids in plant. This research is the first report of cloning full-length cDNA encoding *HDR* from sweet potato [*Ipomoea batatas* (L.) Lam], which is designated as *IbHDR* (Genbank Accession Number: HQ596402). The full length of *IbHDR* was 1725 bp, containing a 1383 bp open reading frame which encoded 461 putative amino acids. The deduced *IbHDR* protein was predicted to possess a chloroplast transit peptide at the N-terminal and showed extensive homology with *HDR*s from other plant species. Quantitative PCR revealed that transcription level of *IbHDR* was highest in tender leaves while lowest in old leaves. Accumulation analysis indicated that carotenoids content was highest in old leaves, followed by tender leaves. Other tissues demonstrated similar carotenoids accumulation which was approximate 7-fold lower than that in old leaves. Cloning and characterization of *IbHDR* gene will facilitate the further understanding of the function of *IbHDR* at the genetic level. This report also will be helpful to elevate carotenoid content in sweet potato cultivators by applying metabolic engineering strategy.

Key words: *Ipomoea batatas,* 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, transcription pattern, carotenoids.

INTRODUCTION

Sweet potato [*Ipomoea batatas (L.)* Lam] is the seventh most important food crop in the world because of it's high level of digestible nutrients including complex carbohydrates, dietary fiber, vitamin C, beta carotene and so on (Noh et al., 2010). Carotenoids are a group of natural pigments in plants, consisting of compounds such as beta carotene, lycopene, lutein and zeaxanthin. Carotenoids are necessary for photosynthesis of plants and also precursors for the synthesis of abscisic acid (ABA) (Rock and Zeevaart, 1991; Chernys and Zeevaart, 2000). Nowadays, Carotenoids are increasingly being paid attention to because of the high-value health benefits for humans. Carotenoids are able to enhance immunity to protect from eye disease and exhibit a kind of anti-cancer activity (Krinsky and Johnson, 2005). Approximately 250000 to 500000 malnourished children in the developing world go blind each year due to the deficiency of vitamin A. As the precursor of vitamin A, it is extremely necessary to investigate the biosynthetic pathway of carotenoids.

Carotenoids are synthesized via the formation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) units (Ramos-Valdivia et al., 1997), both of which are the universal precursors of all

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isoprenoid compounds (Liao et al., 2006). In plants, IPP and DMAPP can be independently synthesized via two pathways, the cytosolic mevalonate (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (Rohmer, 2003; Kuzuyama and Seto, 2003). However, many researches indicated that IPP and DMAPP used for carotenoids biosynthesis were mainly provided by the plastidial MEP pathway (Rohmer, 1999). 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR; EC 1.17.1.2) is the last enzyme of MEP pathway, which catalyses the formation of IPP and DMAPP at a ratio 5:1. It has been demonstrated that HDR is a limiting enzyme for isoprenoid biosynthesis in *E. coli* by overexpressing HDRs from cyanobacteria (*Synechocystis*) and a plant (*Adonis aestivalis*) (Cunningham et al., 2000). *Arabidopsis IspH* null mutant has an albino phenotype. *Arabidopsis* transgenic lines caused by *IspH* gene silencing showed various albino patterns, indicating that the *HDR* was an indispensable gene for plants (Hsieh and Goodman, 2005). Over expression of tomato *IspH* cDNA in *Arabidopsis* plant led to the conclusion that plant *IspH* protein plays a key role in controlling the biosynthesis of plastid isoprenoids (Botella-Pavia et al., 2004). A strong upregulation of *HDR* gene expression was observed in correlation with carotenoid production during both tomato fruit ripening and *A. thaliana* seedling deetiolation. Overexpression of the tomato *HDR* in *Arabidopsis* which upregulated *HDR* gene expression increased carotenoid level in light-grow transgenic plants but did not increase carotenoid level in etioplasts (Botella-Pavia et al., 2004). All these reports suggested that *HDR* may be an ideal target enzyme for the metabolic engineering of carotenoids biosynthesis. Unfortunately, there are no reports on cloning of the *HDR* gene from sweet potato. In the present study, a new *HDR* gene from sweet potato was cloned and characterized. Finally, the tissue expression profile analysis of *IbHDR* will enable us to map and regulate the important enzymatic steps involved in sweet potato carotenoids biosynthetic pathway at the level of molecular genetics in the future.

MATERIALS AND METHODS

Plant materials and growth conditions

Sweet potato (Chongqing) plant was cultured in the fields of Chongqing Sweet Potato Research Center, Chongqing, China. Roots, stems, petioles, flowers and leaves were harvested 100 days after planting (DAP), and the flowers were harvested in the florescent season. The roots were further classified into three sub-categories based on maximum diameter: fibrous roots (diameter < 2 mm), developing storage roots (diameter 2 to 5 mm) and mature storage roots (diameter > 5 mm). The harvest materials were stored in -70°C for RNAs preparation.

Strains and plasmids

Strains of *E. coli* DH5α was stored in our laboratory and pMD18-T vector was purchased from Takara.

Cloning of full-length cDNA of *IbHDR*

Total RNA was isolated from sweet potato according to manufacturer's protocol (TRNzol Reagent, Tiangen, China). After DNase treatment, the first-strand cDNA mixtures were synthesized following the manufacture's protocol (RNA PCR Kit (AMV) Ver. 3.0, Takara). Core fragment of *IbHDR* was amplified using prepared cDNA. Two degenerate primers (FIbHDR and RIbHDR) were designed according to the conserved sequences of *HDR*s from other plants and standard gradient PCR (from 55 to 65°C) was used for amplification core fragment of *IbHDR*. The core fragment was purified and subcloned into pMD18-T vector and transformed into *E. coli* strain DH5α followed by sequencing. Subsequently, the gene specific primers were designed based on the sequence of core fragment to clone the 5'-end and 3'-end of *IbHDR* by the technology of rapid amplification of cDNA ends (RACE).

SMART™ RACE cDNA Amplification Kit (Clontech, USA) was used to prepare 3'-RACE-ready cDNA and 5'-RACE-ready cDNA. Advantage 2 PCR Kit (Clontech) was used for amplification of 3'-end and 5'-end of IbHDR cDNA. For the first PCR amplification of 3'-RACE, IbHDR3 to 1 and M13 were used as the primers and 3'-RACE-ready cDNA was used as template. For the subsequent nested PCR amplification of 3'-RACE, IbHDR 3 to 2 and M13 were used as primers and the product of the first 3'-RACE PCR amplification was used as template. PCR parameters of both amplifications were set as: denaturing at 95°C for 2 min followed by 30 cycles of amplification (94°C for 45 s, 56°C for 45 s, and 72°C for 1 min), and the final procedure was an extension at 72°C for 10 min. For the first PCR amplification of 5'-RACE, IbHDR 5 to 1 and Universal Primer A Mix (UPM) were used as primers and 5'-RACE-ready cDNA was used as template. For the subsequent nested PCR amplification of 5'-RACE, IbHDR 5 to 2 and Nested Universal Primer A (NUP) were used as primers and the product of the first 5'-RACE PCR amplification was used as template. The first and nested PCR amplification of 5'-RACE were carried out at the same conditions: 30 cycles (30 s at 94°C, 30 s at 68°C, 1 min at 72°C) followed by 1 min at 95°C. After nested PCR amplifications of 3'-end and 5'-end, PCR products with expected length were obtained and inserted into the pMD18-T vector (Takara) for sequencing.

Bioinformatic analysis

Sequences of 3'-end, 5'-end and the core fragment were assembled by using Contig Express tool provided by Vector NTI Suite 8.0. The open reading frame (ORF) of *IbHDR* was predicted by ORF Finder on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequence of *IbHDR* was submitted to NCBI for PSI-BLAST searching (http://www.ncbi.nlm.nih.gov/BLAST/). The subcellular location of *IbHDR* was predicted by Target P tool provided by Expasy website (http://www.expasy.org).

IbHDR expression analysis by Quantitative-PCR

Quantitative-PCR was performed to determine the expression levels of *IbHDR* in different tissues, including tubers, fibrous root, old stems, tender stems, tender leaves, old leaves, petiole and flowers. 18S gene was used as internal control. First-strand cDNA was synthesized following the manufacture's protocol PrimeScript™ RT reagent Kit (takara). The gene-specific primers (Table 1) were designed for amplifying a 181 bp-length fragment of *IbHDR*. SYBR® *Premix Ex Taq*™ kit (Takara) was used for Quantitative-PCR amplification. The reactions were run on an Mx3000P® 8 QPCR

Table 1. The oligonucleotide primers used to isolate *HDR* from sweet potato in the study.

Primers	Sequence (5'-3')
FIbHDR	GT(T/C)GAGCG(T/G)GC(T/G/C/A)GT(C/G/T)CAGAT
RIbHDR	GTCAA(T/C)CCA(G/A)TA (T/A)GA(G/T/C)GGAAT
IbHDR 5-1	GCTAGAACCATCAAGCTGACCTCCAAGA
IbHDR5-2	CATGGGCAGGTCGTATCAACTATCTGTA
IbHDR3-1	GAGGACTATGATGCGGAAGTATGG3
IbHDR3-2	CTTATGTTGGTGATTGGTGGATGG
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
NUP	CTAATACGACTCACTATAGGGC
FqIbHDR	AAGCAGTGGTATCAACGCAGAGT
RqIbHDR	GGACTATGATGCGGAAGTATGG
18S-F	ATCTCCTGTAGGTATGAAGTGTTG
18S-R	ATGATAACTCGACGGATCGCCTTGGATGTGGTAGCCGTTT

System (Eppendorf). The procedures were set as: denaturating at 95°C for 2 min followed by 40 cycles amplification (denaturation at 95°C for 20 s, annealing at 56°C for 15 s and extension at 68°C for 20 s). Fluorescence signals were collected at each polymerization step. The expression analysis of the *IbHDR* was calculated according to the method of $2^{-\triangle\triangle Ct}$ (Livaka and Schmittgenb, 2001). The mean Ct values of both *IbHDR* and 18S were calculated after triplicate independent PCRs.

Measurement of carotenoids quantification

Extraction and measurement of Carotenoids were performed following the protocols of Wellburn (Wellburn, 1994). The fresh plant samples (2 g) were ground using 3 ml 80% acetone replication five times and then added to the final volume of 25 ml. Samples were centrifuged (8000 g at 25°C for 5 min), the supernatant that contained the carotenoids was used for optical absorbance measurement at 440 nm (U-1100, HITACHI Co., Japan) to determine the Carotenoids content (Lichtenthaler et al., 1983). And the supernatant that contained the total chlorophyll was used for optical absorbance measurement at 652 nm act as interferences avoid other pigments absorb at 440 nm. All the measurements were repeated in triplicate and their averages were determined. Carotenoids contents based on Lambert-beer law and the calculation accordance with the method in the Pharmacopoeia of China (1995). Each plot indicates the standard errors of the average of carotenoids.

RESULTS AND DISCUSSION

Molecular cloning of the full-length cDNA of *IbHDR*

An 828-bp core fragment of *HDR* was cloned from sweet potato using two degenerate primers designed base on conserved region of other plant *HDR*s. The BLAST analysis on NCBI showed that the core fragment of *IbHDR* had high similarity with other *HDR*s. The 595 bp-length 3'-end and the 667 bp-length 5'-end sequence of *IbHDR* were obtained through RACE technology. By assembling three fragments on Contig Express (Vector NTI Suite 8.0), the 1725 bp deduced full-length cDNA sequence of *IbHDR* was obtained, which consisted of a 1725 bp-length open reading frame encoding 461 amino acids, a 301 bp-length 3' UTR and a 38 bp-length 5' UTR (Figure 1). The gene was designed as *IbHDR* and was submitted to GenBank (Accession Number: HQ596402). To our knowledge, this is the first report of successfully cloning *HDR* from sweet potato.

Comparative and bioinformatic analysis of *IbHDR*

BLAST analysis of *IbHDR* on NCBI showed that *IbHDR* shared high similarity with HDRs from other plants, such as *Lycopersicon esculentum* (85% identities), *Solanum tuberosum* (84% identities), *Nicotiana langsdorffii* × *Nicotiana sanderae* (84% identities), *Nicotiana tabacum* (84% identities), *Vitis vinifera* (80% identities), *Catharanthus roseus* (80% identities), *Arabidopsis thaliana* (74% identities), *Sorghum bicolor* (74% identities) and *Zea mays* (73% identities). The subcellular prediction using TargetP showed that *IbHDR* possessed a 63 aa plastidial transit peptide at the N-terminal. This is consistent with the outcome reported previously: transient expressing 100 N-terminal aa residues of HDRs from *Ginkgo biloba* and *Pinus taeda* fused with GFP protein in *Arabidopsis* was located in chloroplast (Kim et al., 2008). Analysis results indicate that *IbHDR* is a plant HDR protein involved in the mevalonate-independent biosynthetic pathway. The multiple alignment of amino acids of HDRs indicated that all HDRs had the four conserved cysteine residues (Figure 2) that might participate in the coordination of the iron-sulfur bridge (Seemann et al., 2002). No obvious similarity of N terminal of HDRs was found, while, the catalytic regions after the plastidial transit peptides showed high similarity. Using MEGA 4.0 based on CLUSTAL X alignments, a phylogenetic tree of HDRs was constructed from different organisms including plants, algae and bacteria. Stuart et al. (2003) reported multiple-species network imply a

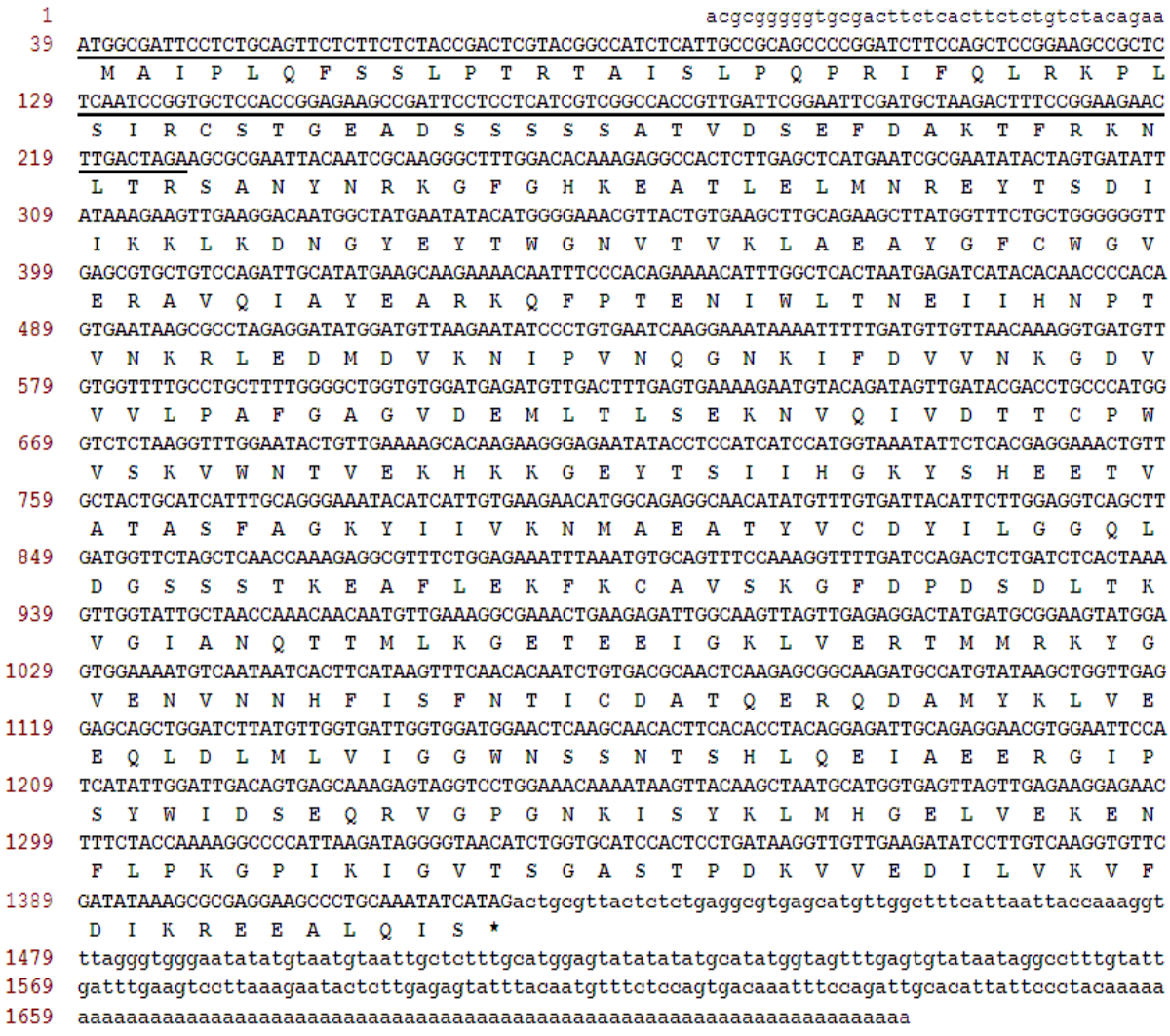

Figure 1. The full-length cDNA sequence and the deduced amino acid sequence of IbHDR. The coding sequence and its deduced amino acid sequence are shown in capital letters. The stop codon (TAG) is marked with an aster, the plastidial transit peptide is underlined.

functional relationship based on evolutionary conservation. The results demonstrated that HDRs were derived from an ancestor gene and evoluted into three groups including plants, algae and bacteria HDR group. IbHDR had higher identity with plant HDRs than bacterium and algae HDRs (Figure 3). All the analysis results strongly suggest that *IbHDR* is a plant HDR protein involved in the mevalonate-independent biosynthesis.

Expression patterns of *IbHDR*

Quantitative PCR was performed to investigate the expression patterns of *IbHDR* in sweet potato. Total RNAs were isolated from different tissues of sweet potato, including tubers, fibrous roots, tender stems, old stems, tender leaves, old leaves, petioles and flowers. FqIbHDR and RqIbHDR were used as specific primers (Table 1). The house-keeping gene (18S rRNA) was used as an internal control. The results showed that expression level of *IbHDR* was highest in tender leaves; tender stems took the second place and then followed by flowers, tubers, petioles and fibrous roots (Figure 4). This result demonstrated that expression patterns of *IbHDR* varied with different organs, which was most abundant in immature tissues and extremely low in old tissues. This research is consistent with the fact that HDR protein showed highest level in young leaves of *A. thaliana* (Guevara-Garcia et al., 2005). Similarly, RT-PCR analysis of *Taxus media HDR* gene expression also demonstrated that the highest expression level of *TmHDR* was found in leaves (Sun et al. 2009). However, expression pattern of HDRs in other species demonstrated different results. RT-PCR analysis of *Camptotheca acuminata HDR* gene expression revealed that *CaHDR* expressed differently in all tested plant

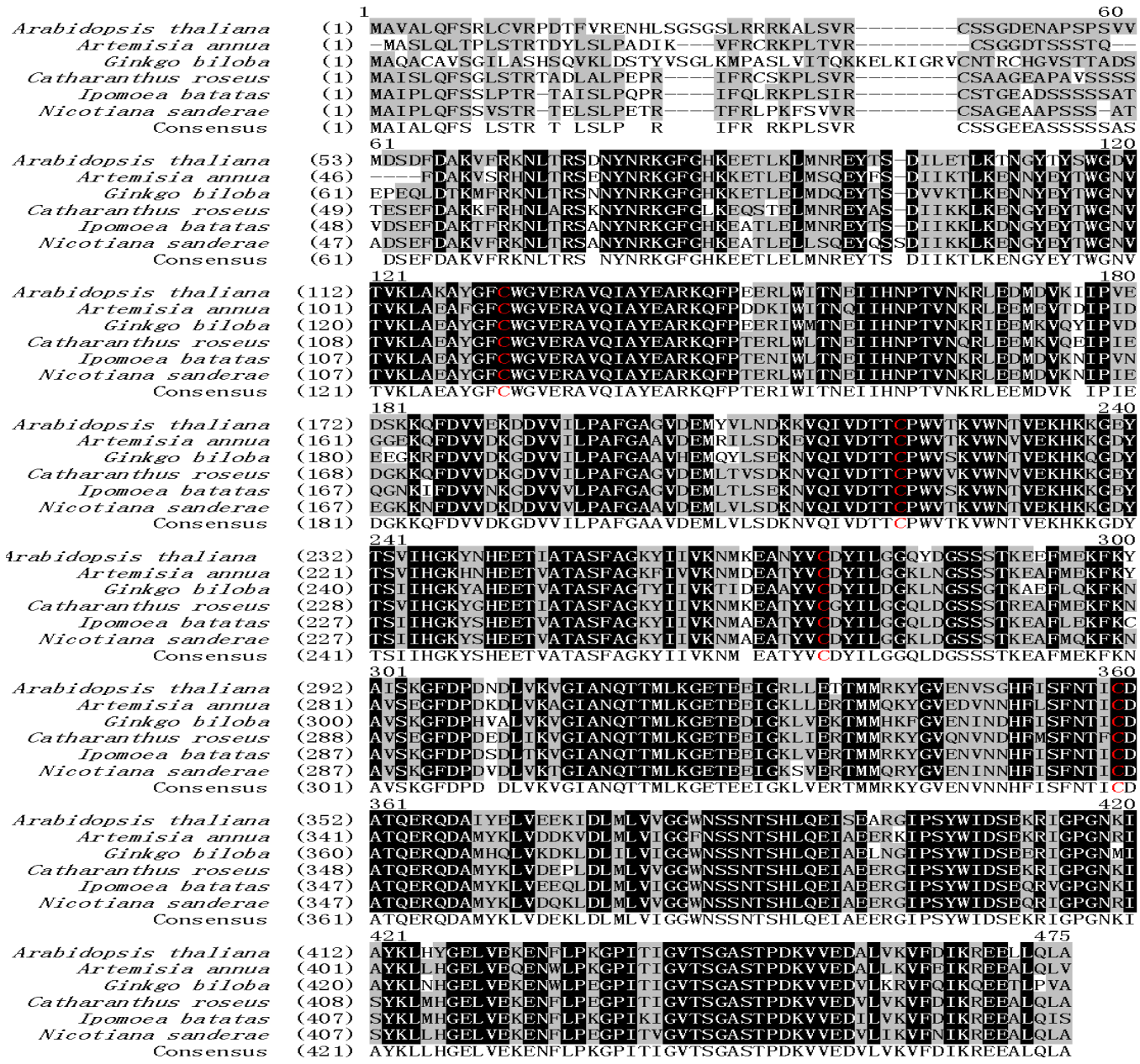

Figure 2. Multiple alignments of amino acid sequences of HDRs. The identical amino acids are shown in white with black background and the conserved amino acids in black with gray background. Numbers indicate the position of amino acid residues. Iron-sulfur bridges are shown in red with black background.

organs with the highest expression level in flowers (Wang et al., 2007). Expression profiling analysis of *Rauvolfia verticillata HDR* revealed that the highest expression level of *RvHDR* was found in flowers, followed by leaves, fruits and roots; the lowest expression level was found in stems (Chen et al. 2010). The above results indicated that expression pattern of HDRs displayed a disparity in different species. The low accuracy of RT-PCR might serve as an alternative possible explanation for the phenomenon.

Carotenoids accumulation analysis

The carotenoid contents of different tissues including flowers, old stems, mature leaves, petioles, tender stems, tender leaves, tuber roots and fibrous roots have been recorded in Figure 4. By detecting optical absorption at 440 nm, carotenoids content in various tissues were calculated. The results demonstrated that carotenoids were mainly accumulated in the leaves, and more specifically, their level was the highest in old leaves,

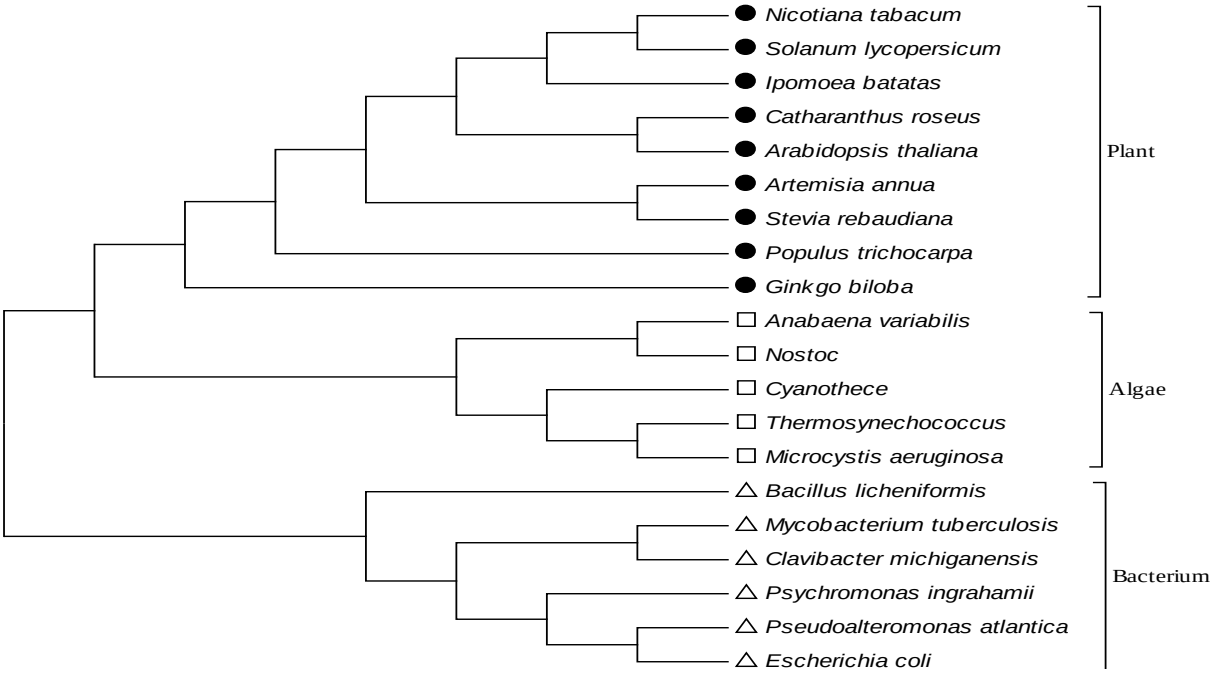

Figure 3. A phylogenetic tree of IspHs from different organisms including plants, algae and bacteria constructed by the neighbor-joining method on MEGA 4.0. HDRs from plants are marked with●, IspHs from algaes marked with □, and the others from bacteria marked with△. The numbers on the branches represented bootsrap support for 1000 replicates. The sequences used are listed bellow with Accession number: *Nicotiana tabacum*(AF159699.2), *Solanum lycopersicum*(GU086226.1), *Catharanthus roseus*(DQ848676.1), *Arabidopsis thaliana* (AY168881.1), *Artemisia annua*(EU332141.1), *Stevia rebaudiana*(DQ269451.4), *Populus trichocarpa*(EU693025.1), *Ginkgo biloba*(DQ364231.1), *Anabaena variabilis*(YP_323455.1), *Nostoc*(NP_485028.1), *Cyanothece*(ZP_01731309.1), *Thermosynechococcus*(NP_681832.1), *Microcystis aeruginosa*(CAO90213.1), *Bacillus licheniformis*(YP_079844.1), *Mycobacterium tuberculosis*(YP_177788.1), *Clavibacter michiganensis*(YP_001222973.1), *Psychromonas ingrahamii*(ABM04955.1), *Pseudoalteromonas atlantica*(ABG41681.1), *Escherichia coli*(AY062212.1).

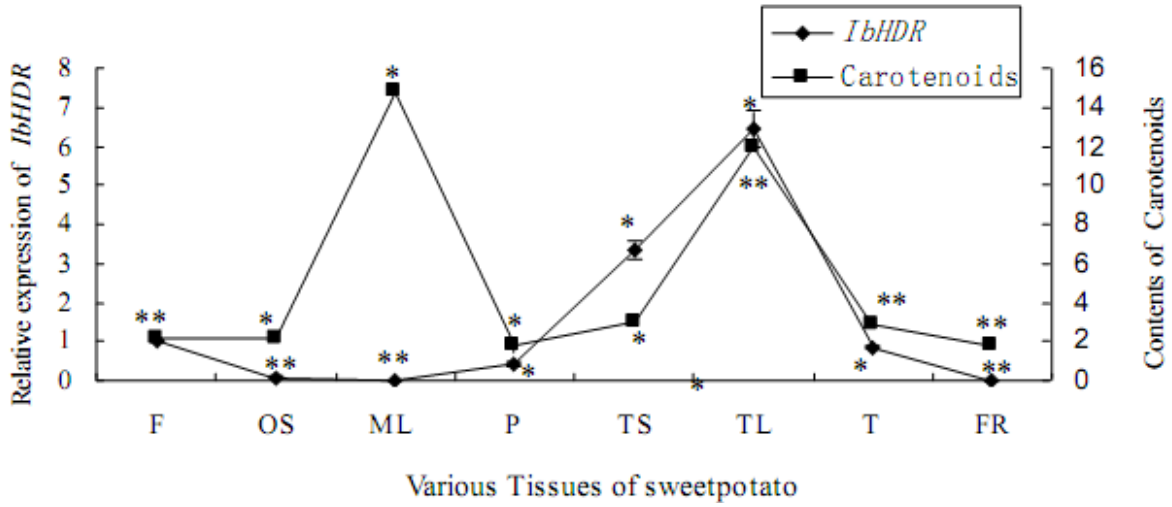

Figure 4. Carotenoids accumulation and expression of *IbHDR* genes in different tissue sampled from sweet potato. Contents of carotenoids (■) was determined by optical absorbance measurement at 440 nm and 652 nm and relative expression of *IbHDR* (◆) was measured by real-time quantitative PCR in flower (F), old stem (OS), mature leaf (ML),petiole (P), tender stem (TS), tender leaf (TL), tuber roots (T), fibrous root (FR). (Average values are given; error are represented as vertical lines. n=3,** P<0.05, * P<0.1).

followed by tender leaves. However, carotenoids content was very similar in other tissues and was significantly lower compared with that in old leaves. Because the expression of HDR was highest in tender leaves, accordingly, it is assumed that carotenoids increasingly accumulate in old leaves along with the process of leaf development and senescence. The similar phenomenon has been previously reported in tomato. Ronen et al. (2000) reported a cumulative process of β-carotene with the fruit development in tomato. The present study is also the first report of analysis of carotenoids content in various tissues of sweet potato. In addition, high accumulation of carotenoids in leaves recorded in present study strongly proves the fact that the carotenoids are indispensable for photosynthetic functions (Bartley et al., 1995). In summary, cloning and characterization of HDR gene from sweet potato will facilitate the further understanding of carotenoids biosynthesis at the molecular level. Meanwhile, the present study also provides an important gene of great interest for metabolic engineering of carotenoids biosynthetic pathway in sweet potato.

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