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ORIGINAL PAPER

Molecular characterization of the *Oncidium* orchid *HDR* gene encoding 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase, the last step of the methylerythritol phosphate pathway

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Abstract Two pathways are used by higher plants for the biosynthesis of isoprenoid precursors: the mevalonate pathway in the cytosol and a 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastids, with 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (HDR) catalyzing the last step in the MEP pathway. In order to understand the contribution of MEP pathway in isoprenoid biosynthesis of *Oncidium* orchid, a full-length cDNA corresponding to HDR from the flower tissues of *Oncidium* Gower Ramsey was cloned. The deduced OncHDR amino acid sequence contains a plastid signal peptide at the N-terminus and four conserved cysteine residues. RT-PCR analysis of *HDR* in *Oncidium* flowering plants revealed ubiquitous expression in organs and tissues, with preferential expression in the floral organs. Phylogenetic analysis revealed evolutionary conservation of the encoding HDR protein sequence. The genomic sequence of the HDR in *Oncidium* is similar to that in *Arabidopsis*, grape, and rice in structure. Successful complementation by OncHDR of an *E. coli hdr*$^{-}$ mutant confirmed its function. Transgenic tobacco carrying the *OncHDR* promoter-*GUS* gene fusion showed expression in most tissues, as well as in reproductive organs, as revealed by histochemical staining. Light induced strong GUS expression driven by the *OncHDR* promoter in transgenic tobacco seedlings. Taken together, our data suggest a role for *OncHDR* as a light-activated gene.

Keywords Isoprenoid biosynthesis · *Oncidium* Gower Ramsey · Promoter reporter fusion · Transgenic tobacco · Phylogenetic analysis · *E. coli hdr* complementation

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The nucleotide sequence reported in this paper has been submitted to (NCBI) under accession numbers EU908201 and EU908200.

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Introduction

Orchids (Orchidaceae) represent the second largest family of plants, and the number of species may exceed 20,000–25,000 (Pridgeon and Chase 1995). Hybrids of the genus *Oncidium* and its hybridization compatible alliance genera *Odontoglossum* and *Miltonia* are grown widely for production of commercial cut flowers and potted plants because of their showy and colorful flowers. The pigments in the *Oncidium* flower consists of anthocyanins and carotenoids, the latter being the main component in the labellum or lips in a flower (Hieber et al. 2006). Currently, only limited information on the biosynthesis of carotenoids in *Oncidium* flowers is available. Hieber et al. (2006) reported on the mixtures of carotenoids and anthocyanin pigments present in *Oncidium* flowers; with the isomers of

violaxanthin representing the predominant yellow pigment in the lip tissue.

Carotenoids are derived from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoid compounds (Cunningham and Gantt 1998; Lange et al. 2000). There are two pathways leading to biosynthesis of both IPP and DMAPP in higher plants, namely, the mevalonic acid (MVA) pathway, which produces cytosolic IPP; and the plastid 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (for review see Eisenreich et al. 2001; Lichtenthaler 1999; Rodríguez-Concepción and Boronat 2002).

The MVA pathway provides precursors for many isoprenoid compounds (Edwards and Ericsson 1999), and some have been shown to be essential for growth and development. As well, MVA biosynthesis appears to be essential for cell cycle progression (Hemmerlin and Bach 1998) and in response to wounding (Maldonado-Mendoza et al. 1997).

The plastidial MEP pathway has been studied by a combination of biochemical and genomic approaches in some plants (Rodríguez-Concepción 2006; Rodríguez-Concepción and Boronat 2002). The precursors for monoterpenes, diterpenes, carotenoids, tocopherols, and the prenyl moiety of chlorophyll are all derived from the MEP pathway (Eisenreich et al. 2001) (Fig. 1).

Currently, there is no information available for MEP pathway to operate in orchids. We are working on isolating and characterizing the genes from this pathway in *Oncidium*. In this work, we aimed to clone the *Oncidium HDR* gene of the MEP pathway and characterize its expression and regulation, due in part to more complete data available for this gene. A second reason for choosing the HDR gene is its importance being considered as the controlling point for MEP pathway and limiting step for isoprenoid biosynthesis in *E. coli* (Cunningham et al. 2000; Rodríguez-Concepción 2006). We analyzed *HDR* expression pattern

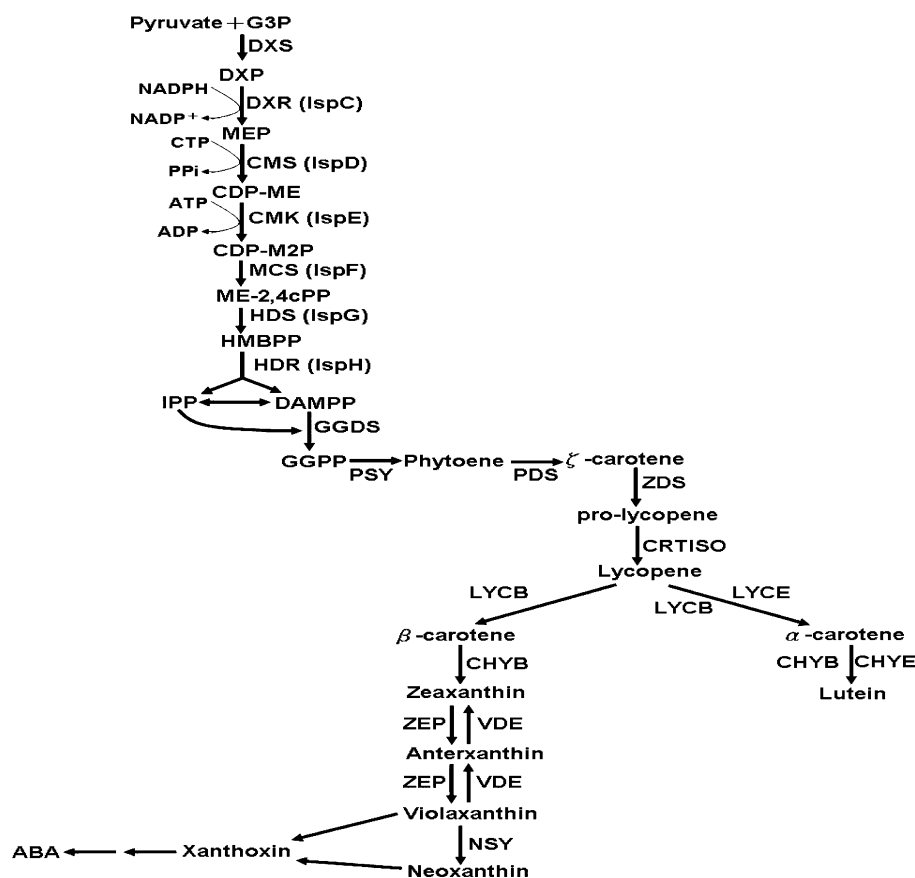

Fig. 1 The pathway for carotenoid biosynthesis in plants. *GAP* glyceraldehyde 3-phosphate, *DXP* deoxyxylulose 5-phosphate, *MEP* methylerythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-methylerythritol, *CDP-MEP* 4-diphosphocytidyl-methylerythritol 2-phosphate, *ME-cPP* methylerythritol 2,4-cyclodiphosphate, *HMBPP* hydroxymethylbutenyl 4-diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *GGPP* geranylgeranyl diphosphate, *ABA* abscisic acid. Enzymes are indicated in *bold*: *DXS* DXP synthase, *DXR* DXP reductoisomerase, *CMS* CDP-ME synthase, *CMK* CDP-ME kinase, *MCS* ME-cPP synthase, *HDS* HMBPP synthase, *HDR* HMBPP reductase, *GGDS* GGPP synthase, *PSY* phytoene synthase, *PDS* phytoene desaturase, *ZDS* ζ-carotene desaturase, *CRTISO* carotenoid isomerase, *LCYB* lycopene β-cyclase, *LCYE* lycopene ε-cyclase, *CHYB* carotenoid β-ring hydroxylase, *CHYE* carotenoidε-ring hydroxylase, *ZEP* zeaxanthin epoxidase, *VDE* violaxanthin de-epoxidase, *NSY* neoxanthin synthase

by RT-PCR and promoter reporter fusion, and subsequent transformation into the tobacco genome. *HDR* was expressed in most tissues of *Oncidium* plants and *OncHDRp::GUS* in transformed tobacco seedlings. We further confirmed the function of the *Oncidium HDR* by complementation study in a bacteria mutant defective in the *HDR* gene.

Materials and methods

Plant materials and RNA isolation

Plants of *Oncidium* Gower Ramsey orchid were grown in the fan-and-pad greenhouse of National Pingtung University of Science and Technology (Pingtung, Taiwan) under shade light and controlled temperature ranging from 23 to 27°C. For all experiments, young flower buds and other tissues were harvested and frozen in liquid nitrogen, then stored at −80°C until use. Total RNAs were isolated and purified by use of TRIZol reagent according to the manufacturer's instructions (Invitrogen). RNA precipitates were resuspended in DEPC-treated sterile water and precipitated with LiCl (at a final concentration of 2 M). The supernatant was treated with RNase-free DNase (Promega) at 37°C for 30 min to remove residual genomic DNA. Purified RNAs were quantified by spectrophotometry (U2000, Hitachi) and quality was checked by agarose gel electrophoresis (Chen et al. 2005).

Cloning of *Oncidium* Gower Ramsey *HDR* cDNA

Total RNA was isolated from unopened flower buds of *Onicidium*. An amount of 1 μg RNA was used to synthesize double-stranded (ds) cDNA by use of the SMART cDNA PCR Synthesis Kit (Clontech). Synthesized dscDNA fragments were used as templates for PCR. *HDR* degenerate primers (Supplementary Table 1), designed from conserved amino acid sequences found in GenBank, were used to amplify regions of interest. The amplified fragment was cloned into pGEM-T Easy vector (Promega) and sequenced. Gene-specific primers (Supplementary Table 1) were designed from the known sequence of the *OncHDR* fragment for 5′ and 3′ rapid amplification of cDNA ends (RACE) by use of the SMART RACE cDNA kit (Clontech) according to the manufacturer's instructions. The 5′-RACE and 3′-RACE products were cloned into the pGEM-T Easy vector and sequenced. On the basis of the RACE sequence data, the Onc.HDR-F and Onc.HDR-R primers (Supplementary Table 1) were designed for amplification of full-length cDNAs by use of Ex Taq (Takara, Tokyo).

DNA blot analysis

Genomic DNA (30 μg/sample) was extracted from young *Oncidium* leaves by the CTAB DNA extraction method (Doyle and Doyle 1990) and digested overnight with *Eco*RI and *Hin*dIII (BioLabs). The product was fractionated by 0.8% agarose gel electrophoresis, transferred to Hybond N^+ membranes (Amersham International, Buckinghamshire, UK), and hybridized with a ^{32}P-labeled 500-bp fragment containing the partial *OncHDR* coding region excised from plasmid DNA, purified by gel electrophoresis and labeled by random priming of hexamers. Pre-hybridization and hybridization followed standard protocols (Sambrook and Russell 2001).

Transcript analysis by semi-quantitative RT-PCR

Total RNA was isolated from various tissues of *Oncidium* as described above. For cDNA synthesis, total RNA (1 μg) was reverse-transcribed in a 20 μL reaction mixture by use of the ImProm-II™ Reverse Transcription System (Promega). The primers used were vHDR-F and vHDR-R (Supplementary Table 1). As an internal control, *actin 1* gene was amplified with the primers Actin-2 and Actin-3 (Supplementary Table 1). Five microliters of cDNA sample from the RT reaction was used for 26 cycles of PCR: denaturation at 94°C (30 s), annealing at 60°C (30 s), and extension at 72°C (40 s). The PCR product (25 μL) from each reaction was analyzed by electrophoresis in 1.2% agarose gel.

Quantification of transcript levels by real-time PCR assay

Quantitative real-time PCR and data analysis were performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Total RNA was isolated from *Oncidium* flowers. Total RNA (5 μg) was treated with DNase I and used for first-strand cDNA synthesis by priming with oligo d (T_{15}) and catalyzed with Superscript III Reverse Transcriptase (Invitrogen) at 42°C for 1.5 h. The gene-specific forward and reverse primers (HDRr-F and HDRr-R) (Supplementary Table 1) were predicted using the Primer Express 2.0 (Applied Biosystems). The real-time PCR conditions were: 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Before running real-time PCR, primer efficiency was evaluated by using both *HDR* and 26S *rRNA* at 50, 150 and 300 nM combinations. The 150 nM for both gene was chosen as most suitable combination. Each sample was amplified in triplicate and all PCR reactions were performed on ABI PRISM 7000 Sequence Detection System.

With a housekeeping gene *26S rRNA*, the relative amount of the *OncHDR* transcript is presented as $2^{-\Delta CT}$ according to the ΔC_T method described in the Real-Time PCR Applications Guide (Applied Biosystems).

Pigment isolation and quantification

Fifteen milliliters of hexane/acetone/ethanol (2:1:1) was added to 0.3–0.6 g of fresh *Oncidium* tissues (flower buds, leaves and roots) and mixed until the tissue was bleached. The organic solvent phase was separated by the addition of 2 mL water and hexane fraction dried in a stream of nitrogen gas at room temperature and resuspended in acetone. Total carotenoids and chlorophylls were determined spectrophotometrically according to Arnon et al. (1954).

Complementation of the *E. coli HDR* mutant with *OncHDR*

The *E. coli HDR* mutant strain MG1655 *ara*< >*ispH* was maintained on Luria–Bertani (LB) medium containing 50 mg L^{-1} kanamycin and 0.2% (w/v) arabinose (Ara) (McAteer et al. 2001). The pQE-30-*OncHDR* plasmid was transformed into the *E. coli HDR* mutant and selected on LB plates containing 50 mg L^{-1} kanamycin, 50 mg L^{-1} ampicillin, 0.2% glucose (Glc), and 0.5 mM IPTG. The surviving colonies containing the pQE-30-*OncHDR* plasmid were identified. The empty pQE-30 vector was transformed into the *E. coli HDR* mutant as a control and selected on LB plates containing 50 mg L^{-1} kanamycin, 50 mg L^{-1} ampicillin, and 0.2% Ara. The transformants containing the control vector could not grow on 0.2% Glc-containing medium.

Sequence analysis

Protein sequences of HDR homologues from other species were retrieved from GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by the TBLASTN algorithm. Multiple alignments of amino acid sequences involved use of the ClustalW program (http://www2.ebi.ac.uk/clustalw). Plastid-targeting peptides were predicted by the ChloroP program (http://www.cbs.dtu.dk/services/ChloroP).

Isolation of *OncHDR* promoter by genomic walking

The *OncHDR* promoter was isolated according to the instructions of the GenomeWalker Universal Kit (Clontech). Genomic DNA was extracted from young *Oncidium* leaves by the CTAB DNA extraction method (Doyle and Doyle 1990). Orchid genomic DNA was digested with four restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II, and *Stu*I), and the products were ligated to a Genome Walker adaptor before being used as templates for PCR reactions. The first PCR involved use of an adaptor primer AP1 and one gene-specific primer, HDRpGSP1 (Supplementary Table 1), designed on the basis of the *OncHDR* full-length cDNA sequence. In addition, the other adaptor primer AP2 and the second gene-specific primer, HDRpGSP2 (Supplementary Table 1), were used in secondary PCR. The amplification conditions for the first PCR were seven cycles at 94°C (for 10 s) and 72°C (for 3 min), 32 cycles at 94°C (for 10 s) and 67°C (for 3 min), then a final extension at 67°C (for 7 min). The conditions for the second PCR were the same as for the first, except that the number of cycles was reduced to 25. The secondary PCR products were analyzed by electrophoresis on 1% agarose gels. The major product band (s) from independent second PCR reactions was gel-purified with use of QIAEXII Gel Extraction Kit (Qiagen), cloned into pGEM-T Easy vector and sequenced.

Construction of the *OncHDR* promoter reporter gene fusion

The 1.7 kb promoter fragment of *OncHDR* spanning the coding region was amplified by PCR with the primers HDRp-F and HDRp-R (Supplementary Table 1), which contained *Hin*dIII and *Bam*HI restriction enzyme sites at their 5′ and 3′ ends, respectively. The PCR products were first digested by *Hin*dIII and *Bam*HI and then cloned into the *Hin*dIII and *BamH*I sites in a promoter-less binary vector pCAMBIA1391Z (Cambia GPO, Canberra, Australia) that contained the intron-GUS gene. The construct was designated pCAMBIA1391Z-*OncHDRp*.

Tobacco transformation

The constructed plasmid pCAMBIA1391Z-*OncHDRp* was transformed into *A. tumefaciens* LBA4404 by electroporation (Nagel et al. 1990). Leaf discs of tobacco (*Nicotiana tabacum* cv. Petit-Havana SR1) were transformed as described previously (Horsch et al. 1985). Transformed plants were selected and regenerated on MS medium containing cefotaxime (250 mg L^{-1}) and hygromycin (40 mg L^{-1}).

Analysis of GUS gene expression

Histochemical localization of GUS gene expression was analyzed according to Jefferson et al. (1987). Briefly, tissues of in vitro-grown seedlings of transgenic tobacco were immersed in GUS assay buffer containing X-gluc. The GUS-stained tissues and seedlings were photographed by use of a digital camera.

Results

Characterization of *OncHDR* cDNA and deduced OncHDR protein

We cloned the full-length *OncHDR* cDNA from *Oncidium* Gower Ramsey flower tissues using degenerate primers designed on the basis of seven plant HDR proteins conserved in a core region. A cDNA fragment of about 500 bp was amplified by RT-PCR, with total RNA isolated from young floral buds being used as templates. On the basis of this sequence, we designed two gene-specific primers for RACE, which produced two PCR products of about 956 bp and 588 bp. A full-length cDNA (Accession no. EU908200) consisting of a 1,392 bp open reading frame (ORF) was obtained by combining the sequence information from 3′ and 5′ RACE. The deduced protein sequence of OncHDR consisted of 463 amino acid residues, of 52.1 kDa. The amino acid sequence of the HDR of *Oncidium* Gower Ramsey showed high homology

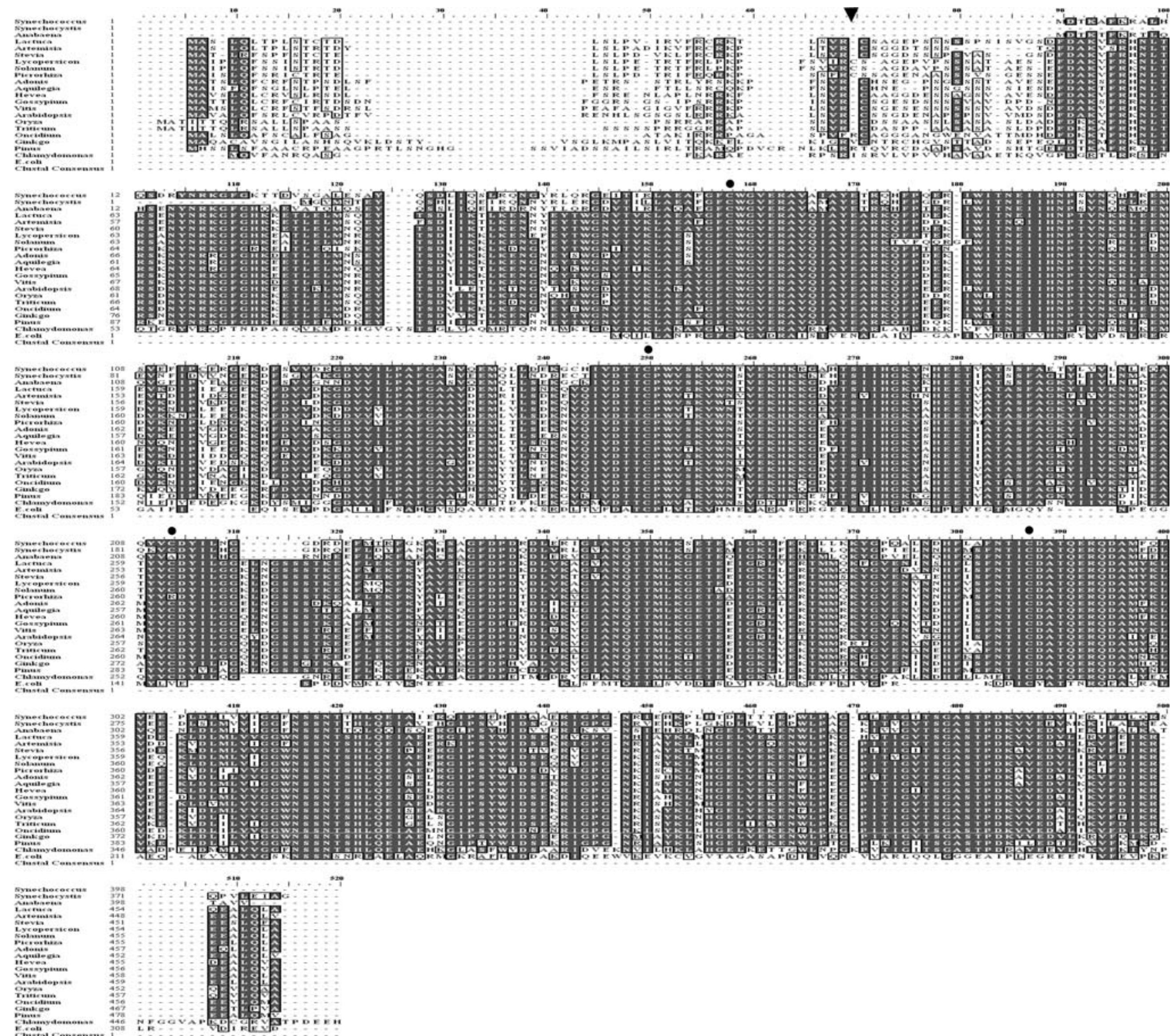

Fig. 2 Multi-alignment of amino acid sequences of Onc.HDR and other plant HDRs. *Synechococcus* (YP_172141), *Synechocystis* (Q55643), *Anabaena* (YP_323455), *Adonis* (AAG21984), *Aquilegia* (TC9243), *Lactuca* (TC12240), *Stevia* (ABB88836), *Lycopersicon* (TC124188), *Solanum* (ABB55395), *Arabidopsis* (AAN87171), *Gossypium* (TC27556), *Oryza* (ABF98702), *Triticum* (TC232642), *Oncidium* (EU908200), *Ginkgo* (ABC84344), *Chlamydomonas* (TC48149), *Picrorhiza* (EF199770), *Vitis* (GSVIVT00036436001) and *E. coli* (P22565) sequences deposited in the TIGR, NCBI, Grape Genome Browser and Swiss-Prot databases. Conserved residues are highlighted (with black when present in all sequences). The plastid-targeting peptide cleavage site predicted by the ChloroP algorithm is marked with a *black arrow*. *Black circles* mark the position of cysteine residues

throughout the entire coding region to that of *Hevea brasiliensis* (77.5%), *Oryza sativa* (77.3%), *Vitis vinifera* (77.3%) *Lycopersicon esculentum* (76.6%), *Aquilegia* (76.5%), *Arabidopsis thaliana* (73.8%) and *Ginkgo biloba* (71.1%) (Fig. 2). The deduced OncHDR sequence contains an N-terminal chloroplast signal peptide consisting of 33 residues predicted by the ChloroP program (Emanuelsson et al. 1999), as do those of putative HDR homologues of other plants (Fig. 2). All plant HDRs, including that of *Oncidium* orchid, showed four conserved cysteine residues, which supposedly participate in the coordination of the iron–sulfur bridge proposed to be involved in enzymatic catalysis (Grawert et al. 2004; Rohdich et al. 2002; Wolff et al. 2003). The position of one of these cysteine residues is not conserved in bacterial proteins and the protein of one plant species, *Picrorhiza kurrooa* (Fig. 2).

Phylogenetic relationship of OncHDR

We constructed a phylogenetic tree using ClustalW (http://www.ebi.ac.uk/clustalw/). The tree (Fig. 3) shows the eukaryotic HDR homologues clustered into two clades, with dicots and monocots; cyanobacteria into two subgroups; and green algae into another group. The result suggests that the OncHDR is conserved evolutionarily with other HDRs.

Expression of *OncHDR* correlates with carotenoid accumulation in *Oncidium* flowers

To investigate the expression profile of *OncHDR* in different tissues of *Oncidium* Gower Ramsey plants, total RNA isolated from roots, leaves, peduncle, labellum (lip), whole flowers was analyzed by two-step RT-PCR, and flower buds at different stages (Fig. 4a) by real-time RT-PCR. *OncHDR* expression could be detected in all tissues examined, with weaker expression in roots (Fig. 4b). The transcript of *OncHDR* reached their highest level at the half-open state, and then decreased in fully open flowers (Fig. 5a). The trend of transcript expression level is well correlated with the accumulation of total carotenoid at different developmental stages of flower buds (Fig. 5a, b).

Genomic structure of *OncHDR* in *Oncidium* similar to other plants

A genomic sequence of the *Oncidium HDR* gene was isolated after PCR amplification of genomic DNA with specific primers (Onc.HDR-F and Onc.HDR-R, Supplementary Table 1) based on the *OncHDR* cDNA sequence. The *OncHDR* genomic sequence spans approximately 5.2 kb (Accession no. EU908201), whereas the length of *Arabidopsis* (Guevara-García et al. 2005; Hsieh and

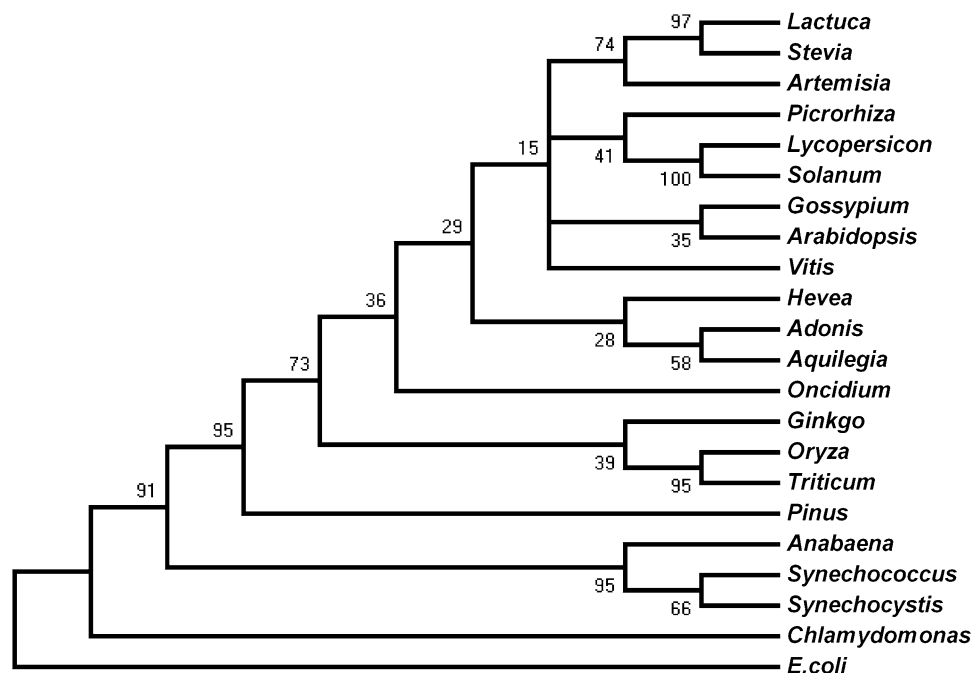

Fig. 3 Phylogenic relationship between the HDR of *Oncidium* Gower Ramsey and those of other organisms. The numbers on the branches represent bootstrap support for 1,000 replicates. The species and corresponding accession number are as follows: *Synechococcus* (YP_172141), *Synechocystis* (Q55643), *Anabaena* (YP_323455), *Adonis* (AAG21984), *Aquilegia* (TC9243), *Lactuca* (TC12240), *Stevia* (ABB88836), *Lycopersicon* (TC124188), *Solanum* (ABB55395), *Arabidopsis* (AAN87171), *Gossypium* (TC27556), *Oryza* (ABF98702), *Triticum* (TC232642), *Oncidium* (EU908200), *Ginkgo* (ABC84344), *Chlamydomonas* (TC48149), *Picrorhiza* (EF199770), *Vitis* (GSVIVT00036436001) and *E. coli* (P22565)

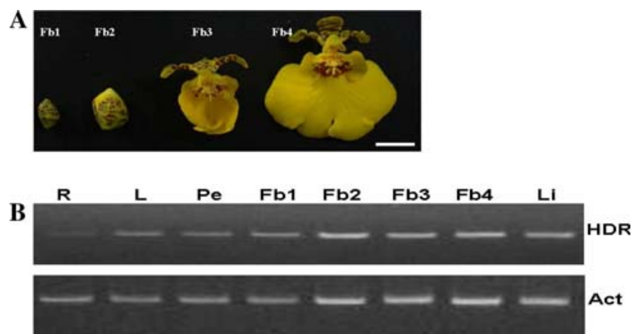

Fig. 4 **a** Developmental stages of flower buds (Fb1–Fb4) of *Oncidium* Gower Ramsey. **b**. RT-PCR analysis of expression of *HDR* gene in various tissues and flower development stages of *Oncidium* plants. *R* roots, *L* leaves, *Pe* peduncle, *Fb1–Fb4* flower-bud stages, *Li* labellum

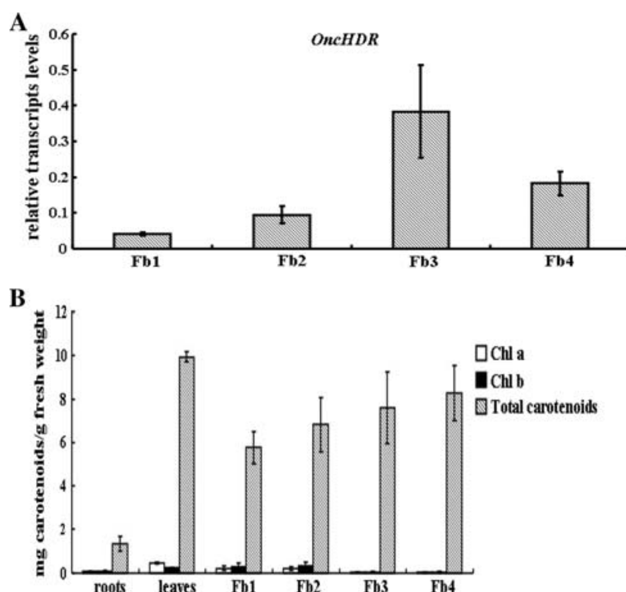

Fig. 5 **a** Transcript levels of *OncHDR* in *Oncidium* floral stages analysed by real-time PCR. Flower bud stages: Fb1: <0.2 cm buds, Fb2: >0.2 cm bubs; Fb3: half-open flower, Fb4: fully open flower. **b** Chlorophyll *a*, *b* and carotenoid contents in roots, leaves and flower buds of *Oncidium* Gower Ramsey. *Fb1* <0.2 cm Buds, *Fb2* >0.2 cm bubs, *Fb3* half-open flower, *Fb4* fully open flower

Goodman 2005) and rice homologues is 2.4 and 2.8 kb, respectively. These gene homologues all possess nine introns, and their relative distribution is similar among the four species compared. The only difference in these homologues is the variation in their intron size (Fig. 6a). Introns 2, 7, and 9 of *OncHDR* are longer than those of *Arabidopsis* and rice homologues (Fig. 6a, b). All introns in *OncHDR* followed the GT/AG splicing rule (Brendel et al. 1998).

To examine the copy number of *OncHDR* in *Oncidium*, we used DNA blot analysis of the *Oncidium* genomic DNA digested with two different restriction enzymes. Use of a partial cDNA fragment, spanning from 400 to 920 bp, of *OncHDR* as a probe revealed more than three hybridized bands in the blot (Supplementary Fig. 1), which suggests that more than one copy of *OncHDR* exists in the *Oncidium* Gower Ramsey genome.

Oncidium HDR complements the *E. coli hdr* (*IspH*) mutant

To further characterize the function of *OncHDR*, we use a complementation assay with an *E. coli hdr*$^-$ mutant lacking

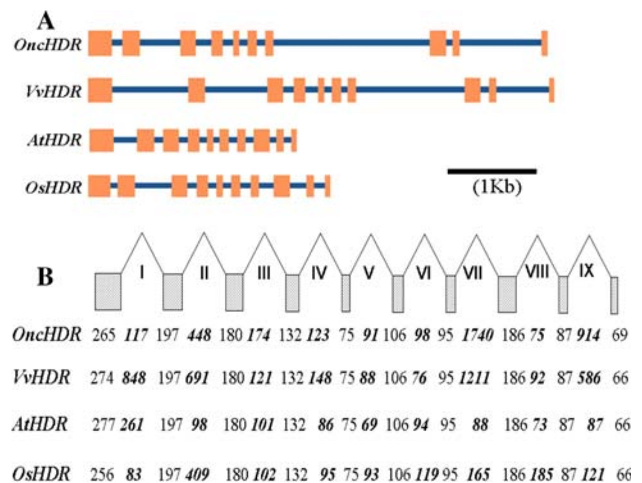

Fig. 6 **a** Comparison of genomic structure of *Oncidium* (*OncHDR;* EU908201), *Vitis* (*VvHDR*; GSVIVT00036436001), Arabidopsis (*AtHDR*; AY883838) and rice (*OsHDR*; AP008209) homologs. **b** Schematic diagrams of exon and intron structures in different plant *HDR* genes

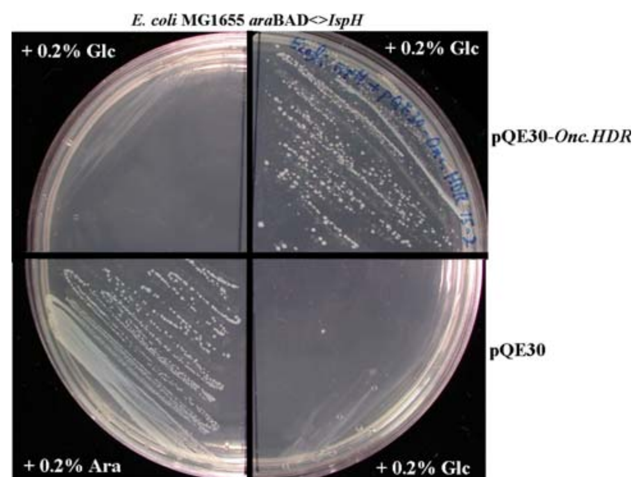

Fig. 7 Complementation assay of *Oncidium HDR* with the *E. coli HDR* mutant. The *E. coli HDR* mutant strain MG1655 *ara*< >*IspH* transformed with *Oncidium HDR* but not the control mutant carrying pQE30 was able to grow in LB medium containing 0.2% glucose (right). The *E. coli* mutant was able to grow on medium with 0.2% arabinose but not glucose (*left*)

HDR activity (Hsieh and Goodman 2005) to determine whether the deduced OncHDR is functional in the bacteria. In *E. coli hdr* mutant strain MG1655 *ara* <> *ispH*, a kanamycin^R cassette was used to replace the endogenous *HDR* gene, and a single copy of *ispH* was present on the bacterial chromosome under control of the *araBAD* promoter (McAteer et al. 2001). Since the *HDR* gene is essential for bacterial survival, the *E. coli hdr* mutant strain could grow only in medium containing arabinose but not in glucose-containing medium (Fig. 7). Transforming the bacterial mutant with the pQE30-*OncHDR* plasmid, but not the empty pQE-30 vector, enabled the transformants to grow on LB medium containing glucose, which demonstrates the catalytic competence of the expressed OncHDR protein in the bacterial cells (Fig. 7).

Sequence characterization of the *OncHDR* promoter

The promoter region, about 1.7 kb long, of the *Oncidium HDR* gene was isolated from *Oncidium* genomic DNA by genome walking. We searched for a promoter motif to define putative *cis*-elements in the *OncHDR* promoter

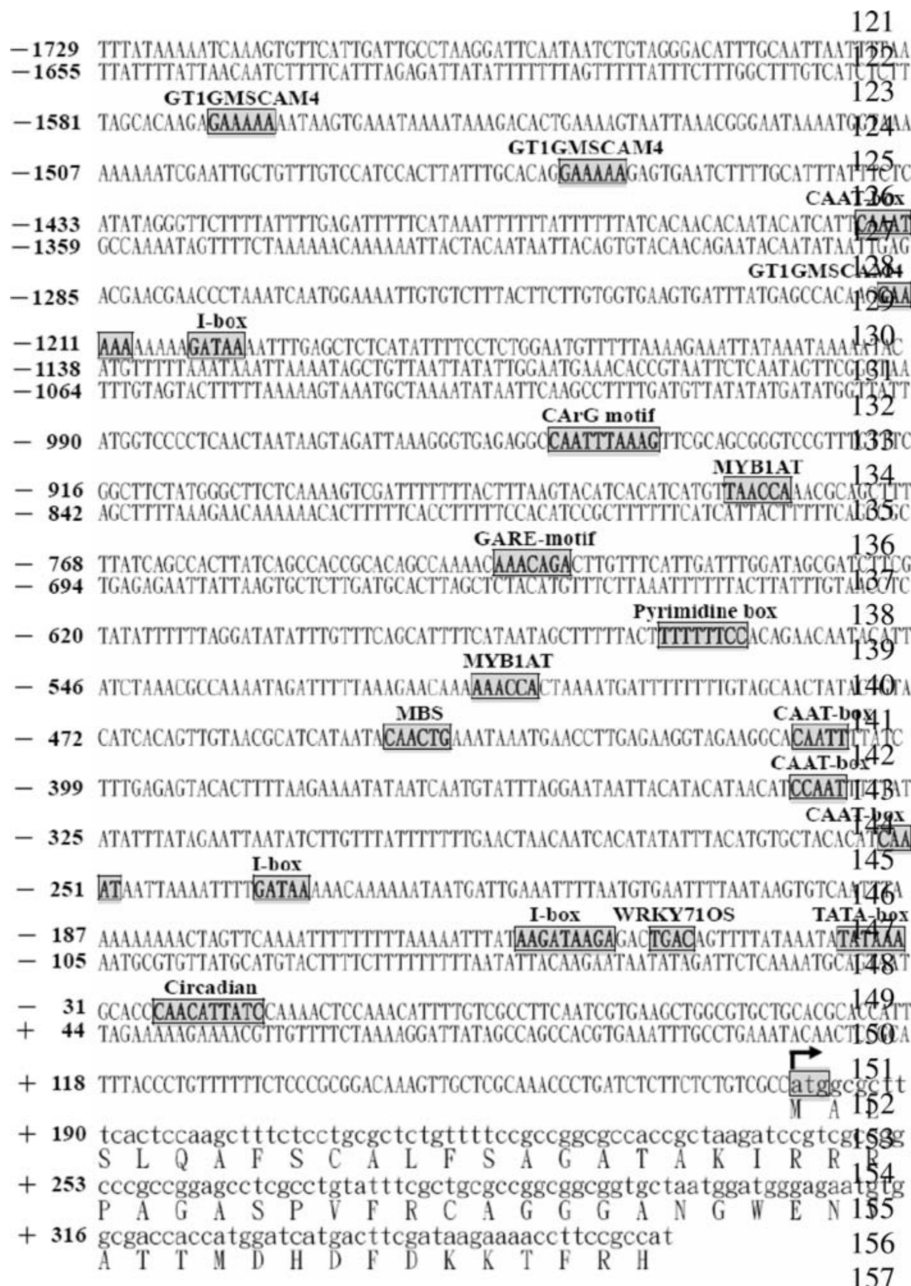

Fig. 8 *OncHDR* promoter sequence and its putative *cis*-elements. *Numbers* indicate the positions relative to the translation start site. The translation initiation codon is *shaded* and indicated by an *arrow*. The putative TATA box, CAAT box, and other important putative *cis*-elements are shown in *bold* and labeled

sequence using the software programs PLACE (Higo et al. 1999) and PlantCARE (Lescot et al. 2002; Rombauts et al. 1999) and found a number of potential regulatory motifs corresponding to known *cis*-elements of eukaryotic genes (Fig. 8). A potential TATA-box and CAAT-box were detected at positions −111 to −106 and −254 to −250 upstream, respectively, of the translation start codon ATG (Fig. 8, Table 1); these boxes may function as basal promoter elements for transcription. Furthermore, several other potential regulatory elements with roles in regulating gene expression are present in the *OncHDR* promoter (Table 1). These include a circadian element (CAACATTATC) at positions −26 to −17 (Piechulla et al. 1998), a WRKY-type transcription factor recognition sequence (TGAC) at positions −128 to −125 (Xie et al. 2005), a pyrimidine box (TTTTTTCC) at positions −569 to −562 (Cercós et al. 1999), a GARE-motif (AAACAGA) at positions −732 to −726 (Ogawa et al. 2003), and a MADS factor recognition site (CAATTTAAAG) at positions −949 to −940 (Tang and Perry 2003). In addition, the *OncHDR* promoter contains two MYB binding sites (Abe et al. 2003), three light-responsive elements and three GT-1-like transcription factor recognition sites (Terzaghi and Cashmore 1995; Park et al. 2004).

Light up-regulates *OncHDRp*::GUS expression in transgenic tobacco seedlings

To investigate the temporal and spatial regulation of the *OncHDR* promoter, transgenic tobacco plants carrying the *GUS* reporter gene fused to the *OncHDR* promoter fragment were grown in the greenhouse, and flowering plants and in vitro T_1 seedlings were histochemically assayed for GUS activity. Positive GUS staining of whole seedlings revealed ubiquitous expression of the *OncHDR* promoter driving GUS in most tissues, including cotyledons, true leaves, petioles, hypocotyls, and roots (Fig. 9). Root tips did not show any GUS activity, but weak GUS expression was detected in the root hairs (Fig. 9d). In 6- and 15-day-old seedlings, strong GUS staining appeared in the vascular tissues of hypocotyls, cotyledons, true leaves and petioles (Fig. 9a–c, f). In the reproductive organs, strong GUS activity was observed in stigma (Fig. 9g). GUS staining was also visible in petals and sepals (Fig. 9g, h).

The *OncHDR* promoter harbors three I-boxes (Table 1) and an ATCTA motif at positions −541 to −546 relative to the translational start site that were predicted to be light-responsive elements. To determine the effect of light on GUS activity driven by the *OncHDR* promoter in

Table 1 Regulatory motifs found in the *Onc.HDR* promoter region

Motif	Sequence[a]	Location	Function	Reference
CAAT-box	CAAAT	−254 to −250	Common *cis*-acting element in promoter and enhancer regions	Shirsat et al. (1989)
		−1364 to −1360		
	CCAAT	−336 to −332		
	CAATT	−409 to −405		
CArG motif	CWWWWWWWWG	−949 to −940	MADS factor recognition site	Tang and Perry (2003)
Circadian	CAANNNNATC	−26 to −17	*cis*-acting regulatory element involved in circadian control	Piechulla et al. (1998)
GARE-motif	AAACAGA	−732 to −726	Gibberellin-responsive element	Ogawa et al. (2003)
GT1GMSCAM4	GAAAAA	−1214 to −1209	GT-1-like transcription factor recognition site	Park et al. (2004)
		−1465 to −1,460		
		−1571 to −1566		
I-box	aAGATAAGA	−140 to −132	Part of a light responsive element	Terzaghi and Cashmore (1995)
	GATAA	−237 to −233		
		−1203 to −1199		
MBS	CAACTG	−446 to −441	MYB binding site involved in drought-inducibility	Abe et al. (2003)
MYB1AT	WAACCA	−512 to −507	MYB binding site involved in drought-inducibility	Abe et al. (2003)
		−859 to −854		
Pyrimidine box	TTTTTTCC	−569 to −562	GA induction	Cercós et al. (1999)
WRKY71OS	TGAC	−128 to −125	A core of TGAC-containing W-box	Xie et al. (2005)

[a] N indicates A, C, G or T; W indicates A or T

transgenic tobacco, we compared GUS expression in 15-day-old seedlings grown in constant dark and those grown under 16-h/8-h light/dark conditions. The activity of *OncHDRp::GUS* was higher under light–dark conditions than under dark conditions (Fig. 9i), which indicated a strong activation of the *OncHDR* promoter by light. GUS staining was associated only with stems and petioles of seedlings grown in constant dark (Fig. 9i left). To compare the light-mediated induction kinetics of the *OncHDR* promoter, transgenic 15-day-old tobacco seedlings were grown in the dark for 3 days, then exposed to white light for 0, 1, 6, and 24 h, and light–dark-grown seedlings were exposed to light for 24 h. GUS activity was observed in stems and petioles but not roots of seedlings grown in the dark (Fig. 9j–l). After 1, 6, and 24 h of light treatment, *OncHDRp::GUS* expression was evident in leaves and roots of transgenic tobacco plants (Fig. 9J-2–4). These results indicate that the inducibility of *OncHDR* promoter was significantly promoted in light-grown seedlings.

Discussion

HDR genes have been cloned and characterized from organisms such as bacteria, cyanobacteria, and a few plants, but no report did exist on the cloning of *HDR* genes from orchid species such as *Oncidium*. Our report probably is the first to describe the cloning and characterization of the *Oncidium* gene encoding HDR.

Among all the MEP pathway enzymes found in the *Arabidopsis* and rice genomes, only *DXS* might be encoded by more than one gene (Rodríguez-Concepción and Boronat 2002). To determine the genomic organization of *OncHDR*, our DNA blot analysis suggested at least two *OncHDR* genes in *Oncidium* (Supplementary Fig. 1), which indicates that *OncHDR* belongs to a small gene family.

The deduced amino acid sequence of OncHDR is similar to that in other plant species. The functional activity of OncHDR was confirmed via complementation assay with the *E. coli HDR* mutant, MG1655 *ara< >ispH*, whereby expression of the *OncHDR* could rescue the lethal phenotype of the *E. coli ispH* mutant (Fig. 7). Thus, OncHDR and *E. coli* IspH (HDR) proteins may have similar enzymatic activity in catalyzing the formation of IPP and DMAPP.

We were able to detect *OncHDR* expression in different tissue types of *Oncidium* Gower Ramsey, with moderate higher transcription level in most tissues and lower level in root tissues (Fig. 4b). Low *HDR* transcript level was also

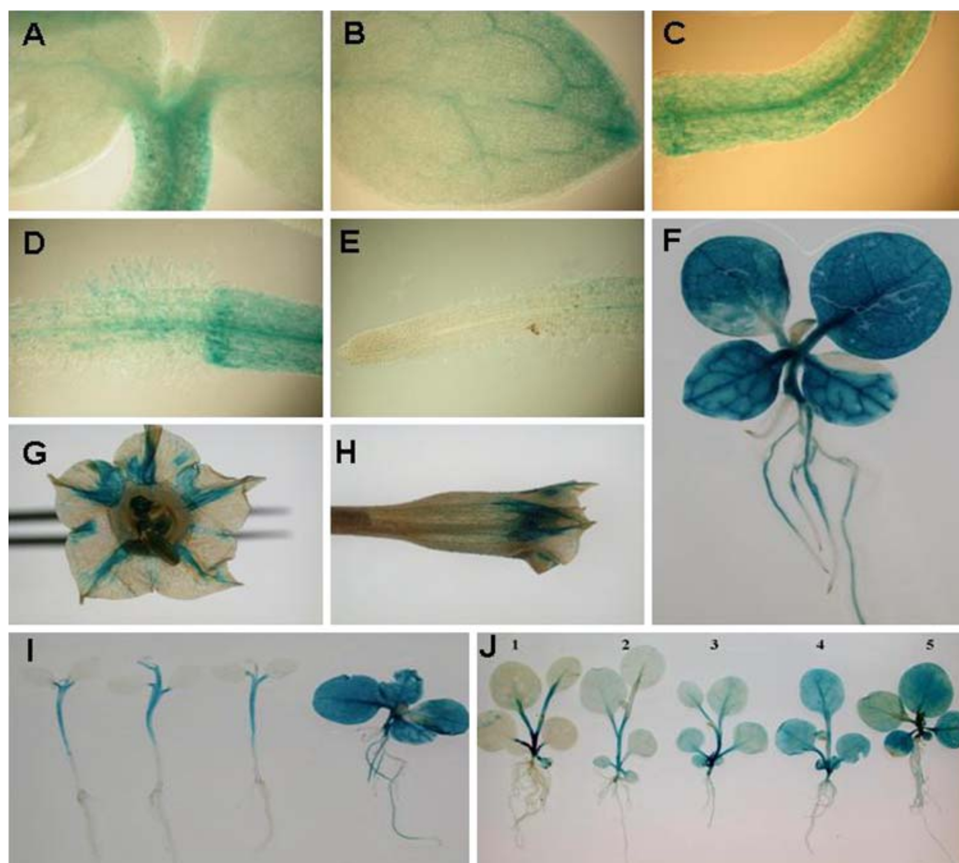

Fig. 9 Histochemical localization of *GUS* gene expression driven by the *OncHDR* promoter in transgenic tobacco. **a** hypocotyl. **b** cotyledon. **c** lower hypocotyl. **d** root. **e**, root tip. **f**, *OncHDRp::GUS* expression in 15-day-old seedling. **g**–**h**, *OncHDRp::GUS* expression in flower tissue. **i** Left are 15-day-old seedlings grown in constant dark; right are 15-day-old seedlings grown in 16-h/8-h light/dark. **j** 1 is 15-day-old seedling and dark adaptive for three days; 2 is a 3-day dark-adaptive seedling, then exposed to 1 h of light treatment; 3 is dark-adaptive seedling, then exposed to 6 h of light treatment; 4 is a dark-adaptive seedling, then exposed to 24 h of light treatment; 5 is a 15-day-old seedling treated with 24 h of continuous light

observed in roots of *Arabidopsis* (Hsieh and Goodman 2005). The expression of *OncHDR* in leaf, peduncle, and flower bud, and to some extent in roots, might explain the gene's basic physiological functions in chloroplast biogenesis and metabolism for the building blocks of carotenoid in chromoplasts (Guevara-García et al. 2005; Hsieh and Goodman 2005; Tambasco-Studart et al. 2005). Because *Oncidium* roots possess some photosynthetic capacity (Hew and Yong 2004), OncHDR may help protect the plant under natural light conditions. The *Arabidopsis ispH* (*HDR*) null mutant showed albino phenotype (Guevara-García et al. 2005; Hsieh and Goodman 2005) due to defect in carotenoids biosynthesis. Complementation of the null mutant with *HDR* gene suggests its role in synthesizing isoprenoid units to produce carotenoid (Guevara-García et al. 2005; Hsieh and Goodman 2005). In tomato, the strong expression of *LeHDR* was related to carotenoid production during tomato fruit ripening and Arabidopsis seedling deetiolation process (Botella-Pavía et al. 2004). Similarly, our results suggest that OncHDR is also involved in carotenoid biosynthesis of *Oncidium* plants, as we detected an increase of total carotenoids during flower development (Fig. 5a), and this pigment accumulation was correlated with the *OncHDR* expression pattern (Fig. 5b).

In conclusion, we have demonstrated the potential function of the *OncHDR* gene by complementing to the bacterial *HDR* mutant, by genomic structure comparison and conserved amino acid sequence. The expression pattern of the *OncHDR* promoter driving the *GUS* reporter gene in transgenic tobacco plants indicated that the *OncHDR* promoter is differentially regulated in various plant organs. Strong GUS activity driven by the *OncHDR* promoter localized in young seedlings and petals as well as vascular bundles in transgenic tobacco (Fig. 9). Furthermore, we have demonstrated the light-induced up-regulation of the *OncHDR* promoter by a transgenic approach.

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