Plant Biotechnology 27, 199–204 (2010)

Gene Note

An aromatic prenyltransferase-like gene *HlPT-1* preferentially expressed in lupulin glands of hop

Yusuke Tsurumaru[1], Kanako Sasaki[1], Tatsuya Miyawaki[1], Takayuki Momma[2], Naoyuki Umemoto[2], Kazufumi Yazaki[1,*]

[1] Laboratory of Plant Gene Expression, Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan; [2] Central Laboratories for Frontier Technology, Kirin Holdings Co., Ltd., Sakura,Tochigi 329-1414, Japan

* E-mail: yazaki@rish.kyoto-u.ac.jp Tel: +81-774-38-3617 Fax: +81-774-38-3623

Received January 20, 2010; accepted February 16, 2010 (Edited by J. Yamaguchi)

Abstract Prenylated aromatic compounds represent the chemical components in the glandular trichomes (lupulin glands) of hops (*Humulus lupulus*, Cannabinaceae), which give the characteristic flavor and taste of beer. To isolate cDNAs for prenyltransferase recognizing aromatic substrates in hops, we constructed a cDNA library from the lupulin glands and randomly sequenced 11,233 EST clones, to obtain 6,613 non-redundant EST sequence information. Among them, we found an aromatic prenyltransferase-like gene (*HlPT-1*), which possessed three features of the plant aromatic prenyltransferase family, i.e., a D-rich motif, membrane-spanning domains, and a transit peptide. The tissue-specific expression study of *HlPT-1* in the intact plant revealed this gene to be highly expressed in hop corns (female flowers), especially in the lupulin glands. Subcellular localization analysis using GFP fusion proteins suggested that HlPT-1 was localized to plastids. Phylogenetic analysis predicted that the *HlPT-1* gene evolved from homogentisate prenyltransferases involved with vitamin E and plastoquinone biosynthesis.

Key word: Aromatic prenyltransferase, bitter acids, *Humulus lupulus*, trichome, xanthohumol.

Hops are an essential ingredient in beer. They give the characteristic flavor and bitter taste to beer, and therefore are consumed worldwide in large amounts. Hop (*Humulus lupulus*, Cannabinaceae) is a perennial and dioecious plant, and the female plants are cultivated to harvest flowers (hop cones) to use as raw material in beer brewing. The basal part of the hop cone bract is covered with many yellow glandular trichomes, which are also called lupulin glands where a variety of essential oils and aromatic compounds are biosynthesized and accumulated. Biosynthesis of essential oil (mono- and sesquiterpenes) in lupulin glands has been recently uncovered at the molecular level by trichome-specific ESTs analysis (Wang et al. 2008); however, the prenyltransferase involved in the synthesis of prenylated aromatic compounds is still largely unknown. In fact, lupulin is a rich source of prenylated aromatic compounds, as exemplified by a wide variety of prenylated flavonoids (xanthohumol, XN; isoxanthohumol, IX; desmethylxanthohumol, DMX; 8-dimethylallylnaringenin, 8DN; 6-dimethylallylnaringenin, and prenylated phloroglucinols also known as bitter acids; humulone, lupulone) that have been detected in lupulin glands (Stevens et al. 2000; Keukeleire et al. 2003).

In the field of health and food sciences, prenylated aromatic compounds have recently received a large amount of attention since they were shown to have divergent biological activities beneficial for humans. For example, these compounds were shown to have phytoestrogen and radical scavenging activities, as well as Cyp1A inhibition, inhibition of iNOS induction, Cox-1 inhibition and anti-tumor activities (Cleemput 2009; Gerhäuser 2005; Milligan 2000; Stevens and Page 2004; Zanoli and Zavatti 2008). It is also suggested that prenyl residues attached to aromatic rings play an important role in these activities. For instance, prenylated aromatic compounds of hops show quinone reductance activities in mouse Hepa 1c1c7 cells; however, the non-prenylated compounds do not have this activity, indicating that the prenyl substitutes raise the activity of aromatic compounds by increasing their hydrophobicity and thereby increasing the cellular uptake of these compounds (Miranda et al. 2000).

Recent studies have revealed the molecular basis in

Abbreviation: DMAPP, dimethylallyl diphosphate; EST, expressed sequence tag; GPP, geranyl diphosphate; PIVP, phlorisovalerophenone; XN, xanthohumol.

This article can be found at http://www.jspcmb.jp/

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

plant prenyltransferases of aromatic compounds. Homogentisate phytyltransferases (e.g., AtVTE2-1), which are responsible for the tocopherol biosynthesis, and the *p*-hyroxybenzoate geranyltransferase (LePGT1), which is responsible for the biosynthesis of a naphthoquinone shikonin, were discovered in *Arabidopsis thaliana* and *Lithospermum erythrorhizon*, respectively (Collakova and DellaPenna 2003; Yazaki et al. 2002). More recently, the naringenin 8-dimethyallyltransferase gene has been cloned as the first flavonoid-specific prenyltransferase from a medicinal plant *Sophora flavescens* (Sasaki et al. 2008). Subsequently, glycinol 4-dimethylallyltransferase cDNA was also isolated from the soybean (*Glycine max*) (Akashi et al. 2009); however, no prenyltransferase gene for prenylated flavonoids has been isolated from non-legume plants, including hops.

In this study, we prepared a cDNA library from lupulin-enriched material and EST analysis was carried out to find a gene coding for prenyltransferases accepting aromatic compounds in hops. Total RNA was isolated from lupulin gland-rich fraction of flower bracts of a hop cultivar (cv. Kirin II) by the CTAB method and was purified using the RNeasy Mini Kit (Qiagen). We constructed a cDNA library using the RNA sample and the *E. coli*-yeast shuttle vector pDR196 (Wipf et al. 2003), which was advantageous for the functional analysis of cDNAs encoding membrane proteins, and the plasmid library was then introduced into *Escherichia coli* DH10B. Subsequently, 11,233 ESTs were randomly sequenced from the primary library. The ESTs were finally assembled into 6,613 non-redundant genes (1,637 contigs and 4,978 singletons).

Plant flavonoid prenyltransferases are, to our knowledge, mostly membrane-bound enzymes and share moderate homology with homogentisate prenyltransferases involved in the biosynthesis of vitamin E and plastoquinone. Homology search against the EST data with AtVTE2-1 (AY089963, a vitamin E biosynthetic enzyme) as the query, revealed a putative aromatic prenyltransferase gene (*HlPT-1*) encoding a polypeptide of 411 amino acids (accession number AB543053). Aromatic substrate prenyltransferases have three critical features i.e. a D-rich motif, membrane-spanning domains, and a transit peptide. The HlPT-1 polypeptide possessed all those features (Figure 1), i.e., a conserved D-rich motif NQxxDxxxD (motif 1), and another characteristic conserved sequence KD(I/L)xDx(E/D)GD (motif 2), nine putative transmembrane α-helices predicted by the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/), and a putative transit peptide sequence (91 amino acids at the N terminus) predicted by the ChloroP program (http://www.cds.dtu.dk/services/ChloroP/). The same sequence has recently appeared in a patent, in which it has been shown that HlPT-1 catalyzes the transfer of the dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) to phlorisovalerophenone (PIVP), phlorisobutrylphenone (PIMP) and naringenin chalcone (Patent number WO/2009/114939).

Prenylated aromatic compounds were specifically accumulated in lupulin glands, while a small quantity was detectable also in leaves (Nagel et al. 2008). In the biosynthesis of bitter acids, for example, valerophenone synthase (VPS) was shown to catalyze the condensation reaction of malonyl-CoA with isovalenyl-CoA or isobutyl-CoA to form aromatic intermediates of bitter acids; *VPS* was also shown to be specifically expressed in lupulin glands (Okada and Ito 2001; Zuurbier et al. 1995). As for the prenyl substrate, the heterodimeric geranyl (or geranylgeranyl) diphosphate synthase gene is also preferentially expressed in lupulin glands (Wang and Dixon 2009). Thus, the prenylation of aromatic substrates is presumed to occur in the lupulin glands of hops.

To study the *HlPT-1* expression level in different tissues of hop plants, semi-quantitative RT-PCR was carried out. Total RNA was isolated and was purified from various organs of hop plants (cv. Kirin II), and cDNAs were synthesized using a Superscript III RT (Invitrogen). PCR was carried out using Go *Taq* DNA polymerase (Promega) and specific primers (HlPT-Fw: 5'-GGCCATTTTGAGTGGGACTA-3', HlPT-Rv: 5'-C-GTATTCCGCAGAGAAGAGG-3', Actin-Fw: 5'TTGT-CCGTGACATGAAGGAG, Actin-Rv: 5'AATCCACA-TCTGCTGGAAGG-3'). The reaction was initiated with denaturation at 95°C for 1 min, followed by 27 cycles of a 3-step incubation (94°C, 1 min; 55°C, 1 sec; 72°C, 30 sec), in which the cycle number was adopted after a preliminary experiment to obtain linear amplification. The PCR products were visualized after electrophoresis on a 1.0% (w/v) agarose gel stained with ethidium bromide, and the intensity of the bands was quantified by using MutiGauge software (Fujifilm). A high expression of *HlPT-1* was detected in early stage cones (EC) and in the lupulin fractions of mature cones (MLp), whereas the highest expression was obtained in lupulins of early stage cones (ELp), as shown in Figure 2. In leaves, only low levels of *HlPT-1* expression were detected, but *HlPT-1* expression was undetectable in the root, stem and 1st stage of flowers (Figure 2). The male flowers were also tested but no expression was observed (data not shown). These results strongly support our hypothesis that the prenylation step of aromatic compounds takes place in the lupulin glands. In xanthohumol (XN) biosynthesis, it has been shown that the final step is the methylation of prenylated chalcone (DMX), and the methyltransferase is also preferentially expressed in lupulin glands (Nagel et al. 2008).

HlPT-1 was predicted to have a putative transit peptide sequence at the N-terminus. In order to prove its subcellular localization experimentally, the putative

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

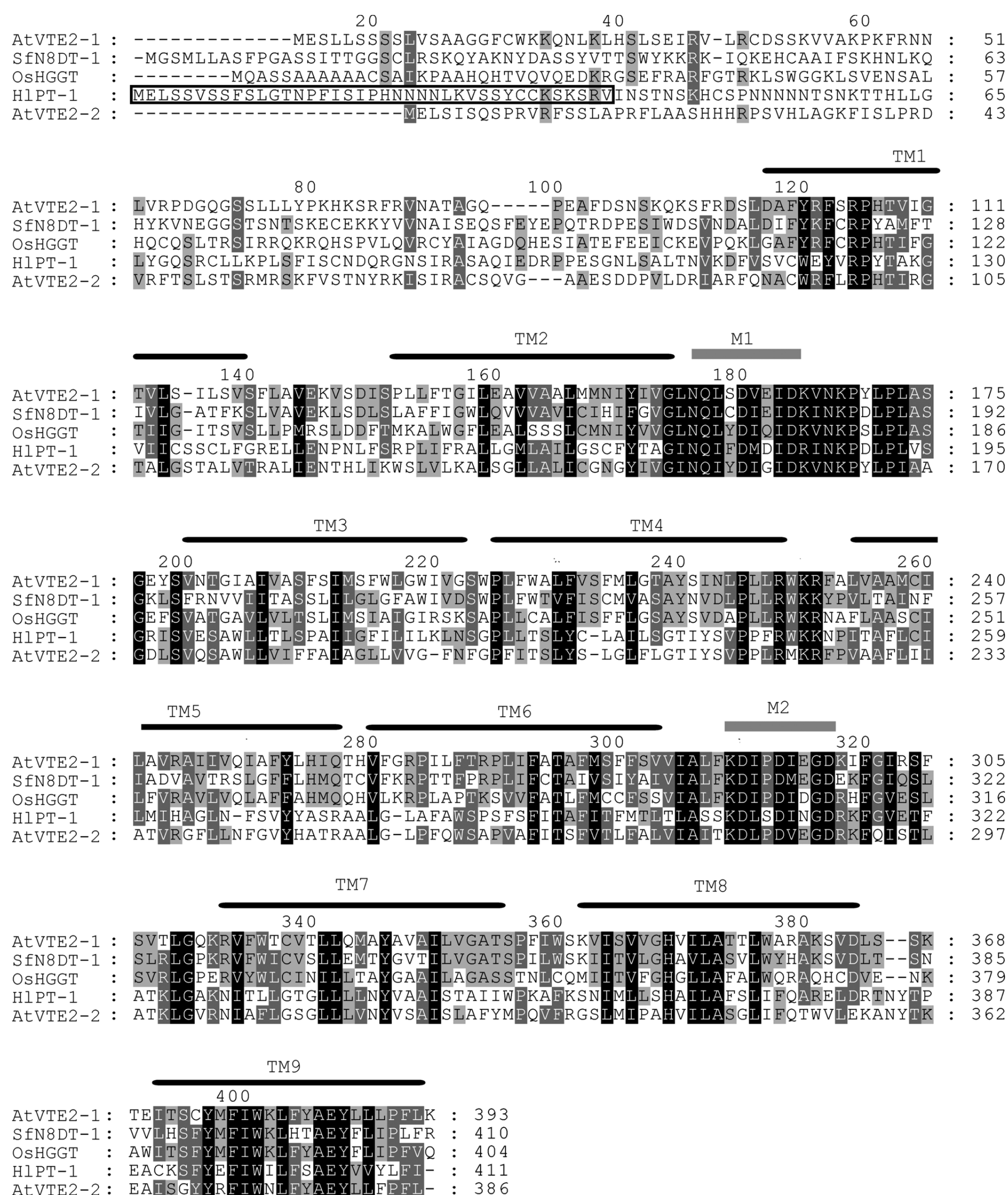

Figure 1. Multiple alignments of the homogentisate prenyltransferase family in plants. Black rectangle at the N-terminus of HlPT-1 was the amino acid sequence used in the heterogous expression analysis for the transit peptide. TM1-9 labeled with black lines show putative transmembrane α-helices. Motif1 (M1) and 2 (M2) are conserved amino acid sequences among aromatic prenyltransferases and are indicated with gray lines: motif 1, NQxxDxxxD; motif 2, KD(I/L)xDx(E/D)GD.

transit peptide of HlPT-1 was fused to GFP under the control of a cauliflower mosaic virus (CaMV) 35S promoter and subsequently introduced into onion peels. In this experiment, the coding 39 amino acids at the N-terminus of HlPT-1 were amplified by PCR with KOD polymerase, specific primers, and *HlPT-1* cDNA as the template. The PCR product was recombined into pDONR221 (Invitrogen) and then into the modified pGWB5 vector (Sasaki et al. 2008), where the recombination reactions were performed according to the manufacturer's protocol (Invitrogen). WxTP-DsRed was used as a positive control for the plastid targeting of the recombinant protein. Ten micrograms of both *HlPT-1-TP-GFP* and *WxTP-DsRed* plasmids were precipitated onto 1.0 μm spherical gold beads (Bio-Rad), and onion peels were bombarded using a particle gun PDS-1000 (Bio-Rad) according to the manufacturer's instructions. After 24 h, GFP and RFP fluorescence in the onion cells were observed using an Axioskop 2 microscope (Zeiss). The GFP fluorescence of the HlPT-1-TP fusion protein mostly matched the red fluorescence of WxTP-DsRed used as a positive control (Figure 3) (Akashi et al. 2009). These results strongly suggest that HlPT-1 was localized to the plastids as other flavonoid prenyltransferases in the legume species (Akashi et al. 2009; Sasaki et al. 2008).

In an evolutional point of view, flavonoid prenyltransferases of plants are thought to have evolved

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

from a primary metabolism, such as vitamin E and plastoquinone biosynthesis, through gene duplication and recruitment (Heide 2009; Yazaki et al. 2009). In the phylogenetic tree of homogentisate and flavonoid prenyltransferases, the hop HlPT-1 appears to be from a common origin with the homogentisate and solanesyltransferase of plastoquinone (AtVTE2-2, GmVTE2-2), but is clustered on a single branch, and not in the legume flavonoid prenyltransferase group (Figure 4). The amino acid identities of HlPT-1 with GmVTE2-2 and AtVTE2-2 were 38–39%, which included the transit polypeptide. This sequence divergence of HlPT-1 may reflect the taxonomical distance of hop from legume plants, or it maybe reflects the difference in the substrate preference. In other words, HlPT-1 recognizes PIVP (phloroglucinol) and naringenin chalcone (chalcone), whereas SfN8DTs and GmG4DT are naringenin- and pterocarpan-specific, respectively. Thus far, flavonoid-specific prenyltransferases have been cloned only from legume plants; however, in order to argue the molecular evolution of their enzyme family, it is necessary to isolate many more orthologues/homologues, especially from non-legume plant species.

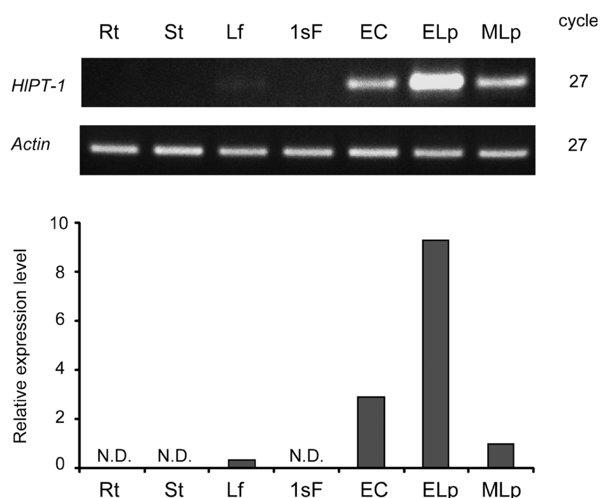

Figure 2. Semi-quantitative RT-PCR analysis for *HlPT-1*gene expression in different tissues of *H. lupulus*. Transcripts levels were normalized with those of actin in the respective tissues. *HlPT-1* expression is shown relative to the expression of lupulin glands with the mature stage of hop cones as 1.0. The values are the averages of three independent experiments. Rt, root; St, stem; Lf, leaf; 1sF, first stage flower; EC, early stage cone; ELp, lupulin glands of early cone stage lupulin gland; MLp, lupulin glands of mature cone stage.

In hop a large number of lupulins are developed at the basis of cone bracts, where seeds are wrapped when pollination is completed. The development of lupulins parallels the increase of the contents of prenylated compounds and the seed maturation as well. Since prenylated compounds show broad antimicrobial activities, particularly to Gramm positive bacteria and also some fungi (Gerhäuser 2005), the prenylated compounds may play a role to protect seeds in hops. Another possibility of physiological roles of prenylated compounds in hops is the protection mechanism against herbivores because the representative compounds of hopes are bitter substances and such bitter compounds can be recognized by insects and functioning as repellant

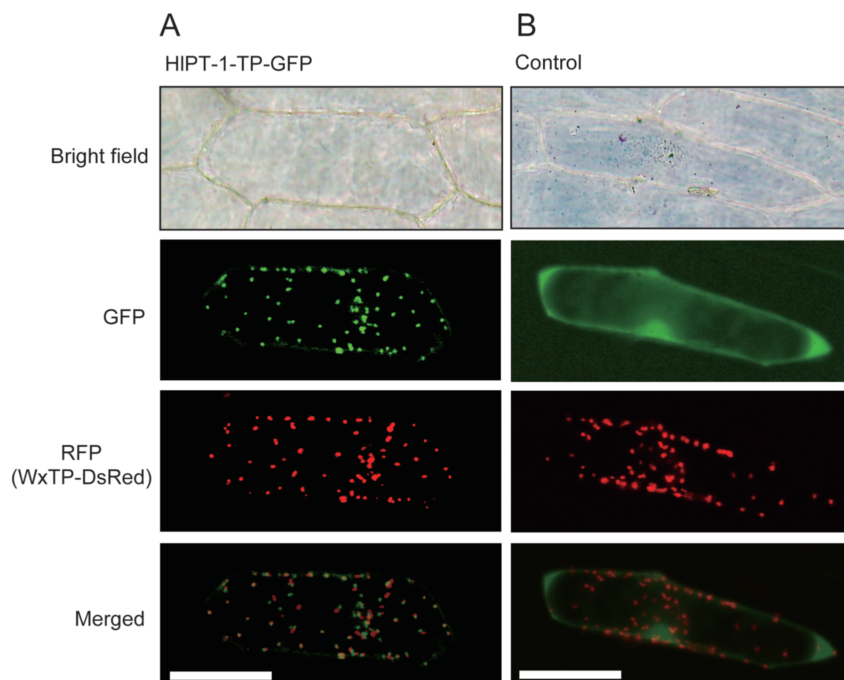

Figure 3. Transient expression of the HlPT-1-TP-GFP fusion protein in onion epidermal peels. (A) HlPT-1-TP-GFP and WxTP-DsRed plasmids were co-transformed into onion epidermal peels by particle bombardment. (B) For the control, pGWB5 (CaMV 35S promoter :: GFP) and WxTP-DsRed plasmids were double transformed into onion epidermal peels. The images were obtained 24 h after bombardment. WxTP-DsRed (red fluorescence) was used as a control for plastid targeting. All scale bars = 100 μm.

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

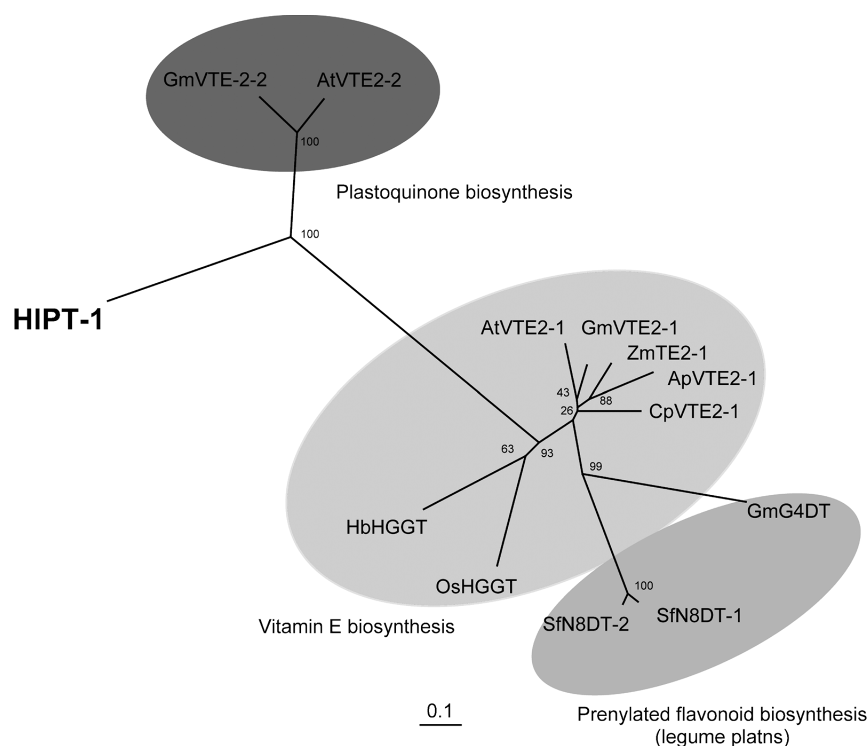

Figure 4. The phylogenetic relationship among HlPT-1 and aromatic prenyltransferases of plants. A rooted phylogram was generated using a ClustalW alignment and a neighbor-joining tree was produced from the results of 1,000 bootstrap replicates. Numbers at the branch points indicate bootstrap fraction (maximum 100). Ap, *Allium porrum*; At, *Arabidopsis thaliana*; Cp, *Cuphea pulcherrima*; Gm, *Glycine max*; Hb, *Hevea brasiliensis*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*; Ta, *Triticum aestivum*; Zm, *Zea mays*. HG phytyltransferases (VTE2-1) and HG geranylgeranyltransferases (HGGT) are involved in vitamin E biosynthesis. HG solanesyltransferases (VTE2-2) are involved in plastoquinone biosynthesis. Accession numbers: ApVTE2-1, DQ231057; AtVTE2-1, AY089963; AtVTE2-2, DQ231060; CpVTE2-1, DQ231058; GmVTE2-1, DQ231059; GmVTE2-2, DQ231061; HbHGGT, BAH10642; HlPT-1, AB543053; HvHGGT, AY222860; OsHGGT, AY222862; SfN8DT-1, AB325579; SfN8DT-2, AB370330; ZmVTE2-1, DQ231055.

(Lee et al. 2009). However, these hypotheses should be evaluated by physiological and ecological studies in future.

In the present study, a prenyltransferase-like gene *HlPT-1* from ca.10,000ESTs of the lupulin-enriched cDNA library was cloned. *HlPT-1* was expressed specifically in the organs where prenylated aromatic compounds were detected, i.e. high in lupulins and low in leaves. Localization of HlPT-1 to plastids was also suggested by GFP fusion protein analysis. Although the enzymatic function of *HlPT-1* should be confirmed, this clone provides valuable information of the amino acid sequence in this enzyme family because this is the first plant aromatic prenyltransferase-like gene from non-legume species. For breweries, HlPT-1 will be a useful marker for the quality test of hop cones or hop cultivars. A biotechnological application is also expected. For example, the expression of HlPT-1 in various hosts may provide prenylated aromatic compounds that show various biological activities that are beneficial for human health.

Acknowledgements

We thank Dr. Tsuyoshi Nakagawa, Shimane University, for the pGWB5 vector; Dr. Toshiaki Mitsui, Niigata University, for the WxTP-DsRed vector; and Dr. Wolf Frommer, Carnegie Institution, for the pDR196 vector. DNA sequencing analysis was conducted with the Life Research Support Center in Akita Prefectural University. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 21310141 to K. Y.).

References

Akashi T, Sasaki K, Aoki T, Ayabe S, Yazaki K (2009) Molecular cloning and characterization of a cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the key prenylation step in the biosynthesis of glyceollin, a soybean phytoalexin. *Plant Physiol* 149: 683–693

Cleemput MV, Cattor Ko, Bosscher KD, Haegeman G, Keukeleire DD, Heyerick A (2009) Hop (*Humulus lupulus*)-derived bitter acids as multipotent bioactive compounds. *J Nat Prod* 72: 1220–1230

Collakova E, DellaPenna D (2003) Homogentisate phytyltransferase activity is limiting for tocopherol biosynthesis in *Arabidopsis*. *Plant Physiol* 131: 632–642

Gerhäuser C (2005) Beer constituents as potential cancer chemopreventive agents. *Eur J Cancer* 41: 1941–1954

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

Gerhäuser C (2005) Broad spectrum anti-infective potential of xanthohumol from hop (*Humulus lupulus* L.) in comparison with activities of other hop constituents and xanthohumol metabolites. *Mol Nutr Food Res* 49: 827–831

Heide L (2009) Prenyl transfer to aromatic substrates: genetics and enzymology. *Curr Opin Chem Biol* 13: 171–179

Keukeleire JD, Ooms G, Heyeric A, Roland-Ruiz I, Bockstaele EV, Keukeleire DD (2003) Formation and accumulation of α-acids, β-acids, desmethylxanthohumol, and xanthohumol during flowering of hops (*Humulus lupulus* L.). *J Agric Food Chem* 51: 4436–4441

Lee Y, Moon SJ, Montell C (2009) Multiple gustatory receptors required for the caffeine response in *Drosophila*. *Proc Natl Acad Sci USA* 106: 4495–4500

Milligan SR, Kalita JC, Pocock V, Kauter VD, Stevens JF, Deinzer ML, Rong H, Keukeleire DD (2000) The endocrine activites of 8-prenylnaringenin and related hop (Humulus lupulus L.) flavonoids. *J Clin Endocrinol Metab* 85: 4912–4915

Miranda CL, Aponso GLM, Stevens JF, Deinzer ML, Buhler DR (2000) Prenylated chalcones and flavanones as inducers of quinone reductase in mouse Hepa 1c1c7 cells. *Cancer Lett* 149: 21–29

Nagel J, Culley LK, Lu Y, Liu E, Matthews PD, Stevens JF, Page JE (2008) EST analysis of hop glandular trichomes identifies an *O*-methyltransferase that catalyzes the biosynthesis of xanthohumol. *Plant Cell* 20: 186–200

Okada Y, Ito K (2001) Cloning and analysis of valerophenone synthase gene expressed specifically in lupulin gland of Hop (*Humulus lupulus* L.). *Biosci Biotechnol Biochem* 65: 150–155

Sasaki K, Mito K, Ohara K, Yamamoto H, Yazaki K (2008) Cloning and characterization of naringenin 8-prenyltransferase, a flavonoid-specific prenyltransferase of *Sophora flavescens*. *Plant Physiol* 146: 1075–1084

Stevens JF, Taylor AW, Nickerson GB, Ivancic M, Henning J, Haunold A, Deinzer ML (2000) Prenylfavonoid variation in *Humulus lupulus*: distribution and taxonomic significance of xanthogalenol and 4'-O-methylxanthohumol. *Phytochem* 53: 759–775

Stevens JF, Page JE (2004) Xanthohumol and related prenylflavonoids from hops and beer: to your good health. *Phytochem* 65: 1317–1330

Wang G, Dixon RA (2009) Heterodimeric geranyl (geranyl) diphosphate synthase from hop (*Humulus lupulus*) and the evolution of monoterpene biosynthesis. *Proc Natl Acad Sci USA* 106: 9914–9919

Wang G, Tian Li, Aziz N, Broun P, Dai X, He J, King A, Zhao PX, Dixon RA (2008) Terpene biosynthesis in glandular trichomes of hop. *Plant Physiol* 148: 1254–1266

Wipf D, Benjdia M, Rikirsch E, Zimmermann S, Tegeder M, Frommer WB (2003) An expression cDNA library for suppression cloning in yeast mutants, complementation of a yeast his4 mutant, and EST analysis from the symbiotic basidiomycete Hebeloma cylindrosporum. *Genome* 46: 177–181

Yazaki K, Kunihisa M, Fujisaki T, Sato F (2002) Geranyl diphosphate: 4-hydroxybenzoate geranyltransferase from *Lithospermum erythrorhizon*. *J Biol Chem* 277: 6240–6246

Yazaki K, Sasaki K, Tsurumaru Y (2009) Prenylation of aromatic compounds, a key diversification of plant secondary metabolites. *Phytochem* 70: 1739–1745

Zanoli P, Zavatti M (2008) Pharmacognositc and pharmacological profile of *Humulus lupulus* L.. *J Ethnopharmacol* 116: 383–396

Zuurbier KWM, Fung SY, Scheffer JJC, Verpoorte R (1995) Formation of aromatic intermediates in the biosynthesis of bitter acids in *Humulus lupulus*. *Phytochem* 38: 77–82

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology