the FEBS Journal

Identification and characterization of a *cis,trans*-mixed heptaprenyl diphosphate synthase from *Arabidopsis thaliana*

Kota Kera[1], Seiji Takahashi[1], Tsuyoshi Sutoh[2], Tanetoshi Koyama[2] and Toru Nakayama[1]

1 Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai, Japan
2 Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Sendai, Japan

Keywords
Arabidopsis thaliana; *cis*-prenyltransferase; dolichol; isoprenoid biosynthesis; polyprenol

Correspondence
S. Takahashi, Graduate School of Engineering, Tohoku University, Sendai, Miyagi 980-8579, Japan
Fax: +81 22 795 7271
Tel: +81 22 795 7272
E-mail: takahasi@seika.che.tohoku.ac.jp

(Received 19 November 2011, revised 3 August 2012, accepted 8 August 2012)

doi:10.1111/j.1742-4658.2012.08742.x

In eukaryotes, dolichols (C_{70-120}) play indispensable roles as glycosyl carrier lipids in the biosynthesis of glycoproteins on endoplasmic reticulum. In addition to dolichols, seed plants have other types of *Z*,*E*-mixed polyisoprenoids termed ficaprenol (tri-*trans*,poly-*cis*-polyprenol, C_{45-75}) and betulaprenol (di-*trans*,poly-*cis*-polyprenol, C_{30-45} and $C_{\geq 70}$) in abundance. However, the physiological significance of these polyprenols has not been elucidated because of limited information regarding *cis*-prenyltransferases (cPTs) which catalyze the formation of the structural backbone of *Z*,*E*-mixed polyisoprenoids. In the comprehensive identification and characterization of cPT homologues from *Arabidopsis thaliana*, AtHEPS was identified as a novel *cis*,*trans*-mixed heptaprenyl diphosphate synthase. AtHEPS heterologously expressed in *Escherichia coli* catalyzed the formation of C_{35} polyisoprenoid as a major product, independent of the chain lengths of all-*trans* allylic primer substrates. Kinetic analyses revealed that farnesyl diphosphate was the most favorable for AtHEPS among the allylic substrates tested suggesting that AtHEPS was responsible for the formation of C_{35} betulaprenol. AtHEPS partially suppressed the phenotypes of a yeast cPT mutant deficient in the biosynthesis of dolichols. Moreover, in *A. thaliana* cells, subcellular localization of AtHEPS on the endoplasmic reticulum was shown by using green fluorescent protein fused proteins. However, a cold-stress-inducible expression of *AtHEPS* suggested that AtHEPS and its product might function in response to abiotic stresses rather than in cell maintenance as a glycosyl carrier lipid on the endoplasmic reticulum.

Introduction

Polyisoprenoids, composed of polymers of a five-carbon building unit, isopentenyl diphosphate (IPP), can be subclassified into all-*trans* (*E*) and *cis*,*trans* (*Z*,*E*)-mixed types depending on the configuration of the double bonds in their isoprene units. Most all-*E*-polyisoprenoids with chain lengths ranging from C_{25} to C_{50} play indispensable roles in cell maintenance of all organisms as the hydrophobic side chains of quinones. *Z*,*E*-mixed

Abbreviations
ABA, abscisic acid; AtHEPS, *Arabidopsis thaliana Z*,*E*-mixed heptaprenyl diphosphate synthase; CMC, critical micelle concentration; cPT, *cis*-prenyltransferase; CTAB, cetyl trimethylammonium bromide; DMAPP, dimethylallyl diphosphate; DOC, sodium deoxycholate; *E*,*E*,*E*-GGPP, *E*,*E*,*E*-geranylgeranyl diphophate; *E*,*E*-FPP, *E*,*E*-farnesyl diphosphate; ER, endoplasmic reticulum; GFP, green fluorescent protein; GPP, geranyl diphosphate; GST, glutathione *S*-transferase; IPP, isopentenyl diphosphate; TF, trigger factor; UPPS, undecaprenyl diphosphate synthase; WT, wild-type; *Z*,*E*,*E*-GGPP, *Z*,*E*,*E*-geranylgeranyl diphosphate; *Z*,*E*-DecPP, *Z*,*E*-mixed decaprenyl diphosphate; *Z*,*E*-HepPP, *Z*,*E*-mixed heptaprenyl diphosphate.

polyisoprenoids are also indispensable components for all organisms as glycosyl carrier lipids. In most eubacteria, di-*trans*,poly-*cis*-polyprenols serve as precursors of glycosyl carrier lipids in the biosynthesis of bacterial peptidoglycan, a variety of other cell-wall polysaccharides and *N*-linked glycoproteins [1]. In eukaryotes, other types of *Z,E*-mixed polyisoprenoid alcohols with a saturated α-isoprene unit, whose trivial name is dolichol, play indispensable roles as glycosyl carrier lipids in the biosynthesis of *N*-linked glycoproteins, *O*- and *C*-mannosylated proteins, and glycosylphosphatidylinositol-anchored proteins in the endoplasmic reticulum (ER) and the Golgi apparatus [2]. In yeasts and animal cells, the predominant *Z,E*-mixed polyisoprenoids are dolichols and their derivatives, while their α-isoprene unsaturated form, polyprenol, occurs in small amounts as ~ 1% of total *Z,E*-mixed polyisoprenoids [3,4]. By contrast, in addition to dolichols as glycosyl carrier lipids, seed plants have abundant polyprenols [5–7], although their physiological significance remains to be elucidated.

Z,E-Mixed polyprenols in seed plants contain quite a broad spectrum of chain lengths [5,6,8]. Most prokaryotes possess single *Z,E*-mixed polyisoprenoid as carrier lipids represented by di-*trans*,octa-*cis*-undecaprenyl phosphate (C_{55}) with some shorter variations [9–11]. In yeasts, Drosophila and mammals, dolichols (di-*trans*,poly-*cis*) are reported to accumulate as a mixture of prenologues with a narrow spectrum of chain lengths ranging from four to five isoprene units, with representative chain lengths of $C_{75\text{-}80}$, $C_{80\text{-}85}$ and $C_{95\text{-}100}$, respectively [4,12,13]. In contrast to other species, the diversity of chain lengths of *Z,E*-mixed polyisoprenoids identified in seed plants is extremely broad ranging from C_{30} up to more than C_{650} [5,8]. Moreover, from a structural aspect, *Z,E*-mixed polyisoprenoid alcohol in seed plants can be further subclassified into di-*trans*,poly-*cis*-prenols (trivial name betulaprenols) and tri-*trans*,poly-*cis*-prenols (trivial name ficaprenols), according to the number of *trans* units in the omega terminus (Fig. 1). According to earlier reports [5,6], *Z,E*-mixed polyisoprenoids in seed plants can be divided into four basic groups: (a) dolichols whose chain lengths range from C_{70} to C_{100}, (b) short-chain betulaprenols whose chain lengths range from C_{30} to C_{45}, (c) long-chain betulaprenols whose chain length is longer than C_{70}, and (d) ficaprenols whose chain lengths range from C_{45} to C_{75}.

Research into *Z,E*-mixed polyprenols was initiated from the incidental discovery of short-chain betulaprenols in the early 1960s by Lindgren [14] and a subsequent study [15] where the difficulties in bleaching silver birch wood (*Betula verrucosa*) during the course of cellulose production was noted. These relatively short-chain betulaprenols occur as fatty acid esters and accumulate only in the trunk of trees at high levels at 0.5–1.0% of tissues. Dolichol rather than polyprenol is considered to be utilized as the glycosyl carrier lipid, especially in seed plants [6]. Moreover, it has been shown that the substrate specificities of glycosyl transferases for *Z,E*-mixed polyisoprenoids in yeast depend not only on the α-saturation but also on the chain length, and polyisoprenoids shorter than C_{55} are hardly utilized as a glycosyl carrier lipid *in vitro* [16].

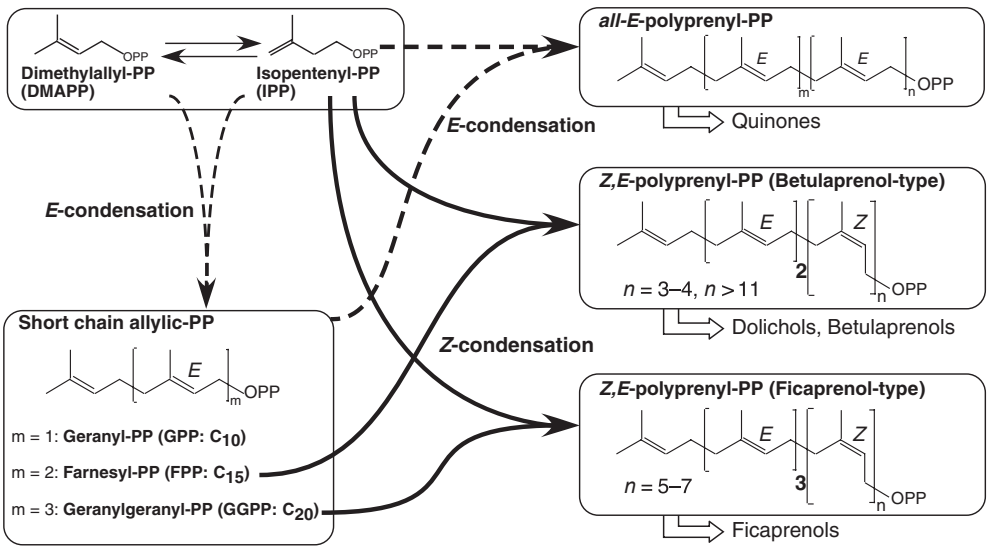

Fig. 1. Summary of the biosynthetic pathways of the basic backbone of polyisoprenoids in plants.

Therefore, in trees, the physiological role of the short-chain betulaprenols is considered to be the provision of mechanical resistance against frost-caused breaks in cold climates [5]. However, to date, this has not been experimentally proved because of the limited information available on the enzymes catalyzing the formation of the basic backbone of *Z,E*-mixed polyisoprenoids, the so-called *cis*-prenyltransferases (cPTs), in seed plants.

cPT catalyzes the sequential *cis*-1,4-condensation of IPP onto the short-chain allylic diphosphates dimethylallyl diphosphate (DMAPP, C_5), geranyl diphosphate (GPP, C_{10}), *E,E*-farnesyl diphosphate (*E,E*-FPP, C_{15}) and *E,E,E*-geranylgeranyl diphosphate (*E,E,E*-GGPP, C_{20}) to form *Z,E*-mixed polyprenyl diphosphate [17] (Fig. 1). Although the entire biosynthetic pathway of dolichol from *Z,E*-mixed polyprenyl diphosphate remains elusive, *Z,E*-mixed polyprenyl diphosphates are proposed to be dephosphorylated to polyprenol followed by reduction of a double bond of the α-isoprene unit to form dolichol [6] in the most widely accepted pathway, which is strengthened by the recent identification of a polyprenol reductase from human [18]. After pioneering research into the molecular cloning, characterization and determination of the crystal structure of undecaprenyl diphosphate synthase (UPPS) from *Micrococcus luteus* B-P 26 [19,20], cPTs have been identified from various organisms [21]. Analyses of the ultimate product chain length from these cPTs *in vitro* revealed that variations in the chain-length distribution among species primarily depend on the enzymatic property of each cPT in cells. However, to date, reports of the identification and characterization of plant cPTs, showing the chain length of the products, were limited as follows: a $C_{75\text{-}95}$ *Z,E*-mixed polyprenyl diphosphate synthase from *Arabidopsis thaliana* [22,23], two cPTs involved in natural rubber biosynthesis from *Hevea brasiliensis* [24] and two cPTs providing prenyl precursors for mono- and sesqui-terpene biosynthesis, di-*cis*-FPP synthase from *Solanum habrochaites* [25] and neryl diphosphate (the *cis* isomer of GPP) synthase from *Solanum lycopersicum* [26]. This limited enzymatic information is not enough to explain the broad spectrum of chain lengths in polyisoprenoids and the occurrence of structurally different polyprenols, ficaprenols and betulaprenols in seed plants. Therefore, we conducted a comprehensive identification and characterization of *A. thaliana* cPTs (S. Takahashi, D. Terauchi, Y. Kharael, K. Kera, T. Nakayama & T. Koyama, in preparation). In total, nine homologous genes of cPTs were found on the *A. thaliana* genome, tentatively termed *AtcPT1* to *AtcPT9* in order of Arabidopsis chromosome based locus identifier (AGI locus) as listed in Table S1. Among them, we succeeded in cloning cDNAs for *AtcPT1* (At2g17570), *AtcPT3* (At2g23410), *AtcPT4* (At5g58770), *AtcPT6* (At5g58782), *AtcPT8* (At5g60500) and *AtcPT9* (At5g60510), in which *AtcPT3* was identical to a cPT previously reported [22,23]. Analyses of the reaction products of cPT reaction with the crude extracts from *Escherichia coli* and *Saccharomyces cerevisiae* overexpressing each AtcPT revealed that AtcPT4 was a medium-chain cPT responsible for the formation of C_{50} ficaprenol, and other cPTs, AtcPT1, AtcPT3, AtcPT6, AtcPT8 and AtcPT9, were long-chain cPTs catalyzing formation of $C_{70\text{-}85}$ polyisoprenoids. Consequently, we had not succeeded in identifying the cPT responsible for short-chain betulaprenols.

In this study, we report the enzymatic characterization of AtcPT5/AtHEPS (At5g58780), a newly characterized AtcPT family, by using partially purified recombinant protein. AtHEPS catalyzed the formation of C_{35} short-chain polyisoprenoids in which the optimal allylic substrate was *E,E*-FPP. Expression of *AtHEPS* predominantly expressed in roots was induced in response to some abiotic stresses, such as cold, and the treatment of abscisic acid (ABA). The results indicated that AtHEPS is a novel *Z,E*-mixed heptaprenyl diphosphate (*Z,E*-HepPP) synthase, which may be responsible for short-chain betulaprenols in *A. thaliana*.

Results

Product analysis of the cPT reaction with crude proteins from *E. coli* and *S. cerevisiae* overexpressing AtcPT5

In order to analyze the prenyltransferase activity, full-length coding cDNA of AtcPT5 fused in-frame with glutathione *S*-transferase (GST) was overexpressed in *E. coli* BL21, and then each lysate was centrifuged to obtain soluble and insoluble fractions. The prenyltransferase assay was carried out with ^{14}C-IPP and *E,E*-FPP as substrates, and the chain length of the products was analyzed by reversed phase TLC. For both soluble and insoluble fractions from *E. coli* overexpressing AtcPT5, additional prenyl products with carbon chain lengths ranging from C_{20} to C_{40} were detected, in which C_{35} prenyl products were the major product by comparison with products from the endogenous activity in the control strain transformed with the empty vector (Fig. 2A).

To characterize AtcPT5 expressed in eukaryotic cells, cPT assays were conducted with crude proteins

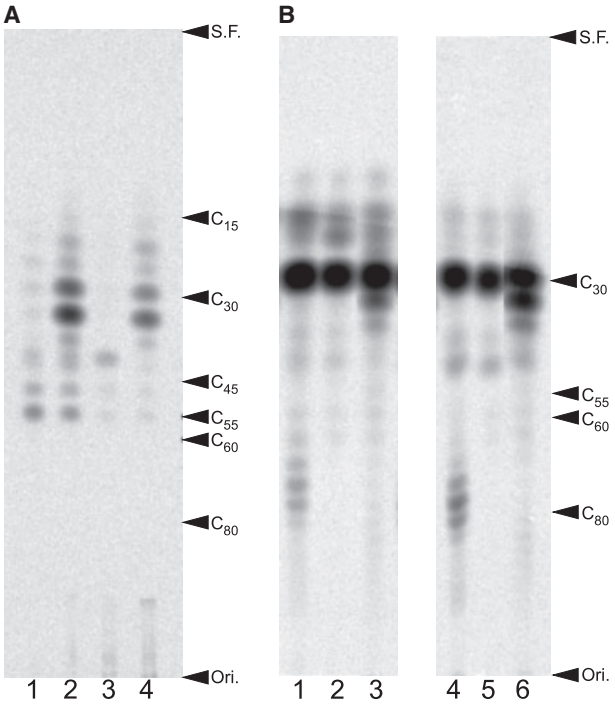

Fig. 2. Product analyses of the prenyltransferase reaction with crude fractions expressing AtHEPS. (A) TLC autoradiograms of polyprenyl alcohols derived from products with crude fractions from *E. coli*. Lane 1, soluble fraction of BL21 harboring pGEX-4T-1; lane 2, soluble fraction of BL21 harboring pGEX-4T-1-AtcPT5; lane 3, insoluble fraction of BL21 harboring pGEX-4T-1; lane 4, insoluble fraction of BL21 harboring pGEX-4T-1-AtcPT5. (B) TLC autoradiograms of polyprenyl alcohols derived from products with crude fractions from *S. cerevisiae*. Lane 1, soluble fraction of SNF9 harboring pJR1133; lane 2, soluble fraction of SNH23-7D harboring pJR1133; lane 3, soluble fraction of SNH23-7D harboring pJR1133-AtcPT5; lane 4, insoluble fraction of SNF9 harboring pJR1133; lane 5, insoluble fraction of SNH23-7D harboring pJR1133; lane 6, insoluble fraction of SNH23-7D harboring pJR1133-AtcPT5. S.F., solvent front; Ori., origin. The numbers on the right indicate the positions of authentic polyisoprenoid alcohol standards *E,E*-farnesol (C_{15}), all-*E*-hexaprenol (C_{30}), all-*E*-nonaprenol (C_{45}), and *Z,E*-mixed polyprenols (C_{55}, C_{60} and C_{80}).

from *S. cerevisiae* expressing AtcPT5, in which a mutant strain of one cPT gene *RER2*, SNH23-7D [27], was applied as a host. This system has been utilized to analyze reaction products from an exogenous cPT [22,28,29] because the activity of the other yeast cPT, Srt1p which produces $C_{95\text{-}105}$ polyisoprenoids [30], is too low to detect until the stationary phase of cell growth. As shown in Fig. 1B, the crude proteins prepared from SNY9, a wild-type (WT) strain for *RER2*, synthesized long-chain polyprenyl products ranging in chain length from C_{65} to C_{85}, whereas corresponding long-chain polyprenyl products were not detected in the reaction products of SNH23-7D. Consistent with the results from the recombinant AtcPT5 expressed in *E. coli*, crude proteins from SNH23-7D/AtcPT5 showed distinct activities producing C_{35} polyisoprenoid. In addition, detectable amounts of radiolabeled products migrated at the position corresponding to $C_{75\text{-}85}$ polyprenols were also observed in the assay with the AtcPT5 overexpressing strain. To investigate the possibility of conversion of C_{35} prenyl diphosphate from AtcPT5 into these additional radiolabeled compounds by the action of enzymes in yeast, the ^{14}C-labeled $C_{30\text{-}35}$ prenyl diphosphates prepared by using the recombinant AtcPT5 expressed in *E. coli* were incubated with the crude extracts from SNH23-7D. Reaction products were extracted with 1-butanol, dephosphorylated and then analyzed by reversed phase TLC. As a consequence, no significant conversion in the radioactive compounds was caused by incubation with the crude extracts from yeast (Fig. S1). Therefore, these additional products observed in the reaction with the crude extracts from SNH23-7D/AtcPT5 were considered to be formed by the prenyl chain elongation reaction of AtcPT5. These results revealed that AtcPT5 expressed in yeast showed a dual-cPT activity producing short- and long-chain prenyl products, although the major product of the recombinant AtcPT5 was C_{35} polyisoprenoid independent of the host species tested. Hence, AtcPT5 was renamed *A. thaliana Z,E*-HepPP synthase (AtHEPS).

Enzymatic characterization of partially purified recombinant AtHEPS

To investigate the precise enzymatic properties of AtHEPS, the recombinant protein was purified from *E. coli* overexpressing AtHEPS. Our first attempt to purify GST-fused AtHEPS by using affinity chromatography from the soluble fraction of *E. coli* was unsuccessful because of the low expression level of AtHEPS as a soluble protein. Solubility of the protein in the *E. coli* expression system was improved by the expression of AtHEPS as a fusion protein with a chaperone protein trigger factor (TF) and His_6-tag under the control of a cold-shock promoter (see also Experimental procedures). The 87 kDa protein corresponding to AtHEPS fused with the tag proteins was purified from the soluble fraction of the crude extracts by using Ni^{2+}-affinity chromatography to semi-homogeneity. Then the tag protein was cleaved by treatment with thrombin (Fig. S2). However, it was difficult to dissociate the 35 kDa AtHEPS protein from the 52 kDa tag fragment completely under non-denaturing conditions because of the strong hydrophobic interaction of the TF.

Therefore, the partially purified AtHEPS containing the cleaved tag fragments was utilized for further characterization.

Since Triton X-100 is known to be required for cPT activity for UPPS from *E. coli* and *M. luteus* B-P 26 [31,32], the effect of various detergents on the cPT activity of AtHEPS was analyzed. In order to determine which type of detergent was necessary for cPT activity, varying concentrations of Triton X-100 as a non-ionic detergent, cetyl trimethylammonium bromide (CTAB) as a cationic detergent and sodium deoxycholate (DOC) as an anionic detergent were tested. The cPT activity was increased significantly in the presence of Triton X-100 in a concentration-dependent manner until 0.02%, which corresponds to a critical micelle concentration (CMC). In contrast, the cPT activity was inhibited drastically in the presence of CTAB and DOC in a concentration-dependent manner until each corresponding CMC, 0.9 mM and 5 mM, respectively (Fig. 3). These results indicated that AtHEPS required a non-ionic detergent until its CMC, but interaction with ionic agents was not favorable. However, the requirement was not strict as ~30% of the activity (with 1% Triton X-100) remained in the absence of detergents.

Divalent cations, such as Mg^{2+}, have also been reported to be essential for the UPPS reaction [31,32]. In order to assess the requirement of AtHEPS for divalent cations, the concentration dependence of Mg^{2+} was analyzed. Although the cPT activity was quite low in the absence of Mg^{2+}, it increased drastically with the addition of Mg^{2+} with a maximum at 1 mM (Fig. 4A). In the presence of other divalent cations, Mn^{2+}, Sn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+} and Co^{2+}, the activity was weak (Fig. 4B).

Structural differences between ficaprenols and betulaprenols in plants suggests the presence of two types of cPTs that prefer either *E,E,E*-GGPP or *E,E*-FPP as substrates, respectively. To investigate the allylic substrate specificity of AtHEPS, the kinetic parameters for IPP and various short-chain allylic diphosphates, DMAPP, GPP, *E,E*-FPP, *E,E,E*-GGPP and *Z,E,E*-geranylgeranyl diphosphate (*Z,E,E*-GGPP), were determined by nonlinear regression analysis. AtHEPS exhibited lower K_m values for *E,E*-FPP and *E,E,E*-GGPP and higher k_{cat} values for *E,E*-FPP and *Z,E,E*-GGPP (Table 1). Among the allylic substrates tested, only DMAPP was not acceptable for AtHEPS. Thus, *E,E*-FPP was the optimal allylic substrate and *E,E,E*-GGPP was also adequate for AtHEPS judging from the k_{cat}/K_m values. AtHEPS exhibited a higher K_m value for IPP compared with the allylic substrates, probably because of the sequential condensations (Table 1).

The product analysis with each allylic substrate by reversed phase TLC revealed that the major products of AtHEPS were *Z,E*-mixed hexaprenyl diphosphate (C_{30}), *Z,E*-HepPP (C_{35}) and *Z,E*-mixed octaprenyl diphosphate (C_{40}) (Fig. 5). Notably, *Z,E*-HepPP was the predominant product, which was independent of the chain length of allylic substrates tested. In conclusion, AtHEPS has been identified as a novel *Z,E*-HepPP synthase in seed plants and may be responsible for the synthesis of short-chain betulaprenols.

Functional complementation of a yeast cPT by AtHEPS

To elucidate the enzymatic function of the *Z,E*-HepPP synthase AtHEPS *in vivo*, functional complementation

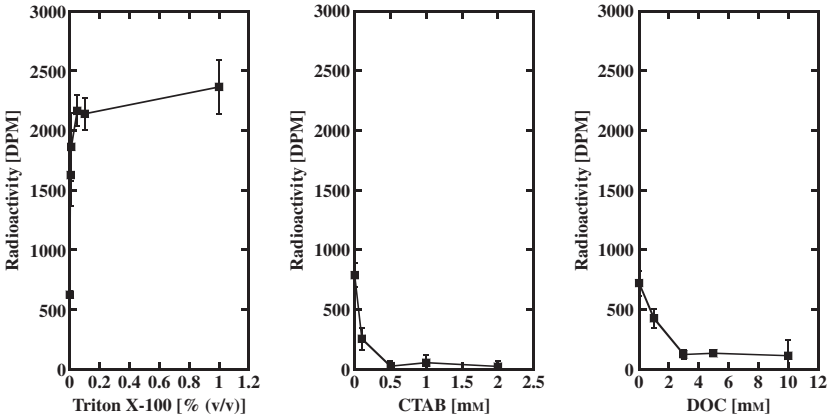

Fig. 3. Effect of detergents on the cPT activity of AtHEPS. In this assay, the indicated amount of detergents Triton X-100, CTAB and DOC were used. After incubation at 30 °C for 12 h, the reaction products were extracted with 1-butanol, mixed with the scintillation mixture and subjected to liquid scintillation counting. Each value is the mean of three separate experiments, and error bars indicate standard deviations.

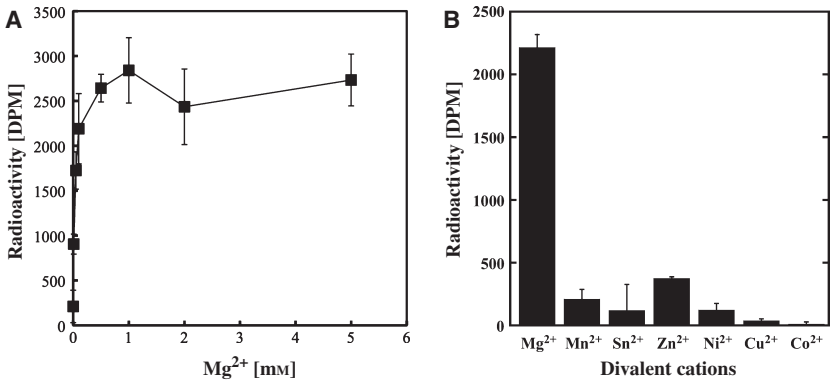

Fig. 4. Effect of divalent metals on the cPT activity. The indicated amount of $MgCl_2$ (A) or 1 mM of divalent cation indicated (B) was added in each reaction. After incubation at 30 °C for 12 h, the reaction products were extracted with 1-butanol, mixed with the scintillation mixture and subjected to liquid scintillation counting. Each value is the mean of three separate experiments and error bars indicate standard deviations.

Table 1. Kinetic parameters of the partially purified AtHEPS. Relative activity gives the relative values of k_{cat}/K_m determined with that for *E,E*-FPP taken to be 100%. n.d., not detected.

Substrate	k_{cat} ($\times 10^{-3}$ s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^{-3}$ $s^{-1}\cdot\mu M^{-1}$)	Relative activity (%)
IPP[a]	14.3 ± 0.5	13.1 ± 2.1	1.13 ± 0.15	–
DMAPP	n.d.	n.d.	n.d.	n.d.
GPP[b]	7.55 ± 0.21	6.07 ± 0.39	1.25 ± 0.06	18.1
E,E-FPP[b]	13.0 ± 0.5	1.88 ± 0.09	6.92 ± 0.55	100
E,E,E-GGPP[b]	6.64 ± 0.15	1.07 ± 0.05	6.23 ± 0.16	90.0
Z,E,E-GGPP[b]	13.7 ± 0.8	4.78 ± 0.57	2.90 ± 0.22	41.9

[a] For the assay with 10 μM *E,E*-FPP. [b] For the assay with 100 μM IPP.

of the cPT mutant of *S. cerevisiae* by the expression of AtHEPS was conducted. The mutant strain SNH23-7D containing a mutation in a yeast cPT, *RER2*, displays some phenotypes caused by dolichol deficiency such as slow and temperature-sensitive growth and defects in protein glycosylation [27]. As indicated in Fig. 6, the mutant strain SNH23-7D had slow growth at 23 °C compared with the WT strain SNY9 and hardly grew at 37 °C. Expression of AtHEPS could partially suppress the slow growth phenotype under the permissive temperature of 23 °C and the temperature-sensitive phenotype under the restricted temperature of 37 °C. These results suggested that AtHEPS could act as a cPT instead of Rer2p *in vivo*, and their products could function as a precursor of glycosyl carrier lipid in *S. cerevisiae*.

Subcellular localization of AtHEPS in plant cells

Because Rer2p localizes in the ER [27], AtHEPS was also expected to localize in the ER. Subcellular localization of AtHEPS in plant cells was analyzed by the fluorescent imaging of AtHEPS fused in-frame with synthetic green fluorescent protein sGFP(S65T) [33] expressed in T87 Arabidopsis cultured cells [34]. In the sGFP-expressing cells, the fluorescent signal was observed in the nucleus and cytosol, which was a typical pattern of GFP localization (Fig. 7A), while the fluorescence of AtHEPS-sGFP was observed as a network pattern, which was similar to a typical pattern of some ER-localized proteins (Fig. S3A). Subcellular localization of AtHEPS on the ER was also suggested by the specific detection of AtHEPS-sGFP in the microsomal fraction extracted from the T87 cells by western blotting using a GFP antibody (Fig. S3B). To confirm the localization of AtHEPS on the ER, mCherry, a red fluorescent protein, fused to an N-terminal ER signal peptide and a C-terminal ER-retention signal HDEL (SP-mCherry-HDEL) was co-expressed with AtHEPS-sGFP in the T87 cells. In contrast to the chlorophyll autofluorescence observed in sGFP-expressing cells (Fig. 7B), the red fluorescence emitted from mCherry-HDEL in the co-transformed cells showed a typical ER network pattern (Fig. 7E). Superimposition of the fluorescence images revealed that the green fluorescence signal from AtHEPS-sGFP (Fig. 7D) was mostly co-localized with red fluorescence signal from mCherry-HDEL (Fig. 7F).

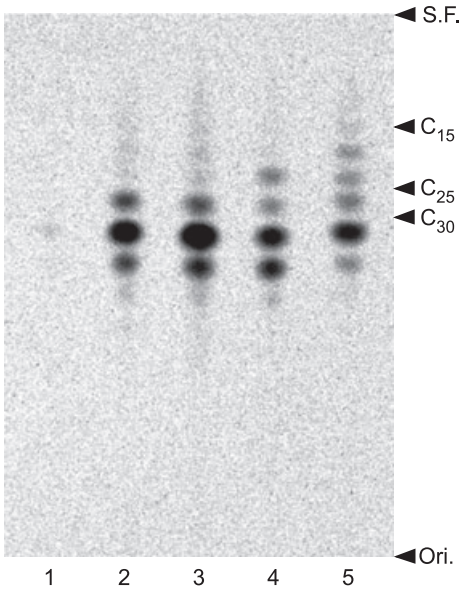

Fig. 5. TLC autoradiograms of polyprenyl alcohols derived from products with the partially purified AtHEPS. Prenyl alcohols were analyzed by reversed phase TLC (LKC-18) with a solvent system of acetone/water (19 : 1). As primer substrates, DMAPP (lane 1), GPP (lane 2), *E,E*-FPP (lane 3), *E,E,E*-GGPP (lane 4) and *Z,E,E*-GGPP (lane 5) were utilized. S.F., solvent front; Ori., origin. The numbers on the right indicate the positions of authentic polyisoprenoid alcohol standards.

These results indicated that AtHEPS functioned in the ER of Arabidopsis cells. Thus, detection of the activity of the recombinant AtHEPS in both membrane and soluble fractions of *E. coli* and yeast (Fig. 2) may show mislocalization of the protein in a heterologous expression system.

Induction of *AtHEPS* in response to cold and abscisic acid treatment

Although the physiological significance of short-chain betulaprenols has not yet been elucidated, short-chain betulaprenols in silver birch are supposed to provide the trunk with mechanical resistance against frost-caused breaks in cold climates [5]. To investigate the involvement of AtHEPS in response to such environmental stimuli in *A. thaliana*, the detailed expression pattern of *AtHEPS* in root tissues under cold stress and ABA treatments was investigated, because *AtHEPS* was shown to be predominantly expressed in roots (Table S1). The expression level of *AtHEPS* oscillated slightly under the long-day light conditions (Fig. 8, Control and Mock). After the ABA treatment, *AtHEPS* was induced rapidly within 2 h and then decreased to basal levels. In contrast, *AtHEPS* was induced by cold treatment in a time-dependent manner until 16 h after the treatment was initiated, where the magnitude of the induction was higher than that by the ABA treatment. These results strongly suggest that AtHEPS and its products may have a role in response to cold stress in root.

Discussion

To date, quite limited information regarding the cPTs responsible for the occurrence of *Z,E*-mixed polyisoprenoids in seed plants is known despite the very long history of identifications of orchestral polyisoprenoids from various plants [5,6,8]. Some fundamental issues remain to be answered. First, it has not been uncovered whether *Z,E*-mixed polyisoprenoids in plants with broad diversity in their carbon chain length are biosynthesized by many kinds of cPTs having a specific product chain length preference or are accumulated as intermediates *in vivo* by some limited kinds of cPTs. Next, no critical evidence has been provided to show the physiological significance of *Z,E*-mixed polyprenols which may not play roles as glycosyl carrier lipids on the ER. To address these questions, the comprehensive identification and functional analysis of cPTs in *A. thaliana* was conducted (S. Takahashi, D. Terauchi, Y. Kharel, K. Kera,

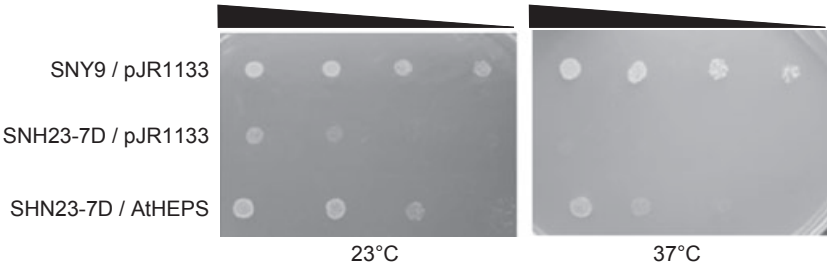

Fig. 6. Functional complementation of *rer2-2* mutant, SNH23-7D, by AtHEPS. SNY9 harboring pJR1133 (SNY9/pJR1133), SNH23-7D harboring pJR1133 (SNH23-7D/pJR1133) and SNH23-7D harboring pJR1133-AtcPT5 (SNH23-7D/AtHEPS) were cultured on the minimum medium at 23 or 37 °C. Black slopes indicate the gradation of culture applied on the plates.

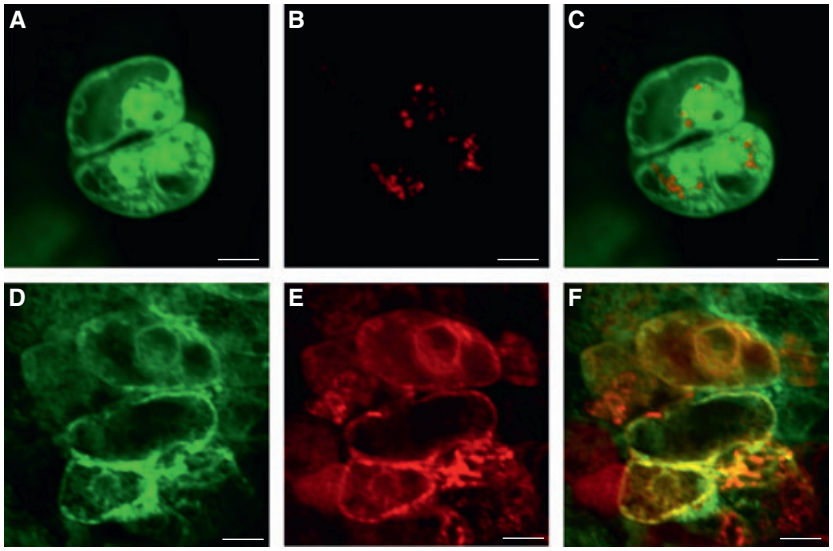

Fig. 7. Co-localizations of AtHEPS-sGFP with ER-targeted mCherry-HDEL in Arabidopsis cells. Confocal laser microscope images of Arabidopsis T87 cultured cells expressing sGFP (A, B, C) and AtHEPS-sGFP with SP-mCherry-HDEL (D, E, F). Green fluorescence signals (A, D) and red fluorescence signals (B, E) were obtained using filter sets for GFP and RFP, respectively, and then superimposed to create merged images (C, F). Fluorescence signals shown in (B) were chlorophyll autofluorescences, confirmed by bright field observations (data not shown). Bar, 10 μm.

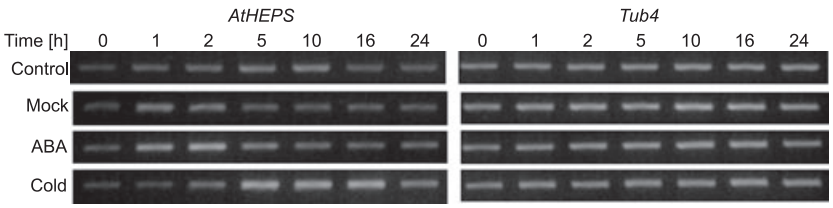

Fig. 8. Semi-quantitative RT-PCR analyses of *AtHEPS* in Arabidopsis roots under the cold stress and ABA treatments. *AtHEPS* and a β-tubulin, *Tub4*, were amplified by RT-PCR from total RNAs isolated from Arabidopsis seedling treatmented with cold (4 °C) and 100 μM ABA in MS liquid medium. As controls, total RNAs were also isolated from seedlings grown on normal MS agar media (Control) and those transferred to MS liquid media (Mock). Amplified cDNAs were analyzed by agarose gel electrophoresis.

T. Nakayama & T. Koyama, in preparation; also summarized in Table S1). Analyses of the reaction products in the cPT assay with the crude proteins from *E. coli* and *S. cerevisiae* overexpressing each AtCPT revealed that AtcPT1, AtcPT3, AtcPT6, AtcPT8 and AtcPT9 did not show any cPT activity when they were expressed in *E. coli* but these enzymes expressed in yeast showed long-chain cPT activities catalyzing the formation of $C_{70\text{-}85}$ polyisoprenoids. AtcPT4 expressed in *E. coli* showed significant prenyltransferase activity producing $C_{50\text{-}60}$ polyisoprenoids, whereas AtcPT4 expressed in yeast showed a dual-cPT activity producing middle-chain ($C_{50\text{-}60}$) and long-chain ($C_{85\text{-}95}$ as major products) prenyl products. Taken together, AtcPT4 was considered to be a medium-chain cPT responsible for the formation of C_{50} ficaprenol in plastid, and other AtcPTs analyzed were considered to be responsible for long-chain dolichols or polyprenols in *A. thaliana*. Each *AtcPT* showed tissue-specific expressions, which was also shown recently in a review by Surmacz and Swiezewska [35]. In addition, some *AtcPT*s were induced in response to abiotic stimuli, such as cold, high salinity, dehydration, light and treatment with ABA. These results suggested that some *Z,E*-mixed polyisoprenoids participate in the stress response in plants. Among them, the full-length cDNA of *AtcPT5*/*AtHEPS*, one of the stress-responsive cPTs, was not identified. Now we report that AtHEPS is a *Z,E*-HepPP synthase (Fig. 5) functioning in the ER membrane in seed plants (Fig. 7). Kinetic parameters of AtHEPS for various allylic substrates indicated that the optimal allylic substrate was *E,E*-FPP with the highest k_{cat}/K_m value (Table 1), suggesting that the major product of AtHEPS *in vivo*

might be di-*trans*,tetra-*cis*-heptaprenyl diphosphate. Therefore, this is the first report of the identification and characterization of a medium-chain cPT which may be responsible for the formation of short-chain betulaprenol, the first *Z,E*-mixed polyprenol identified from nature [14,15].

With respect to product chain length, cPTs identified to date can be classified into three subfamilies: short-chain (C_{10-20}), medium-chain (C_{50-55}) and long-chain (C_{70-120}) cPTs [21]. The short-chain cPTs are represented by *Z,E*-FPP (C_{15}) synthases identified from *Mycobacterium* species, *Corynebacterium* species and *Themobifida fusca* [36–38], with a related enzyme *Z,E*, *E*-GGPP (C_{20}) synthase from *Mycobacterium* species [38]. Medium-chain *cis*-prenyltransferases are represented by UPPS from eubacteria [19,31] and archaea [39], which catalyzes the *cis* condensation of eight molecules of IPP with *E,E*-FPP and *E,E,E*-GGPP, respectively. *Z,E*-mixed decaprenyl diphosphate (*Z,E*-DecPP, C_{50}) synthases from *Mycobacterium* species and *Themobifida fusca* [40,41] are also in this subfamily. Most cPTs in eukaryotes catalyzing the synthesis of the basic backbone of dolichols are categorized as long-chain cPTs, in which Rer2p [27] and Srt1p [30] from *S. cerevisiae* and HDS from human [29] are included. In terms of the distributions of cPTs among species, eubacteria and archaea have short- and medium-chain cPTs, while eukaryotes have long-chain cPTs [21]. However, seed plants have all subfamilies of cPTs, neryl diphosphate and *Z,Z*-FPP synthases from *Solanaceae* as short-chain cPTs [25,26], AtcPT4 as a medium-chain cPT and AtcPT1, AtcPT3/ACPT/DPS [22,23], AtcPT6, AtcPT8 and AtcPT9 as long-chain cPTs (Table S1). In this study, a new class of enzyme, *Z,E*-HepPP synthase, is added into the plant cPT family.

Phylogenic analysis of the amino acid sequences of identified cPTs and AtHEPS (Fig. S4) showed that cPTs could be divided into three major clades: (a) short-chain cPTs from Actinobacteria, (b) prokaryotic medium-chain cPTs including a portion of AtcPTs and (c) eukaryotic long-chain cPTs. AtHEPS was included in clade (b) as well as AtcPT4 suggesting that these are considered to be an evolutionary derivative of medium-chain cPTs in prokaryotes. Recently, a cPT homologue Mvan_3822 from *Mycobacterium vanbaalenii* was identified as a bifunctional cPT catalyzing the formation of *Z,E*-DecPP and *Z,E*-HepPP *in vitro*, in which the product chain length was altered depending on the types of allylic primer substrates [38]. The high similarity of Mvan_3822 with Rv2361c, a *Z,E*-mixed DecPP synthase from *Mycobacterium tuberculosis* (76% identity), also suggests the possibility of the occurrence of a *Z,E*-HepPP synthase via genetic alterations of *Z,E*-DecPP synthase. While Mvan_3822 accepts DMAPP, GPP, *E*, *E*-FPP and *E,E,E*-GGPP as primer substrates, AtHEPS did not accept DMAPP (Table 1). In addition, the ultimate chain length of the prenyl products from AtHEPS was independent of the type of allylic substrate (Fig. 5). These results indicate that AtHEPS works specifically to provide *Z,E*-HepPP by strict regulation of the product chain length. It is proposed that the ultimate product chain length of AtHEPS may be regulated by the spatial recognition of the chain length of products within the enzyme, not by the number of IPP condensation cycles controlled kinetically. The precise mechanism responsible for the regulation of the ultimate chain length of the product by medium-chain cPTs remains to be elucidated. However, the catalytic mechanism for short-chain cPTs has been uncovered by site-directed mutational analyses targeting characteristic amino acid residues among short-chain cPTs [37,42]. Detailed comparisons of the primary structure of AtHEPS with those of other medium-chain cPTs is expected to reveal important residues that regulate the ultimate chain length of the products.

It should be noted that the AtHEPS expressed in *E. coli* showed activity as a *Z,E*-HepPP synthase *in vitro*, whereas that expressed in yeast showed a dual-cPT activity producing medium- and long-chain prenyl products (Fig. 2). Since no long-chain cPTs from *A. thaliana* [22], *S. cerevisiae* [27] and human [29] showed cPT activities *in vitro* when expressed in *E. coli*, these long-chain cPTs are considered to require eukaryotic protein partners and/or post-translational modifications, such as glycosylations on the ER, to demonstrate catalytic activities. These unknown eukaryotic factors in the host strain may affect AtHEPS causing alteration of the ultimate chain length of the products. Therefore, AtHEPS is a suitable target to use for elucidating the regulation mechanisms of medium- and long-chain cPTs from eukaryotes.

In this study, detailed enzymatic characterization of the partially purified AtHEPS expressed in *E. coli* revealed requirements of non-ionic detergent, such as Triton X-100 (Fig. 3) and Mg^{2+} (Fig. 4). Comparatively, Baba and Allen [32] reported that UPPS from *M. luteus* had only 12% of its cPT activity without Triton X-100 compared with that in the presence of 0.5% Triton X-100. Moreover, not only Triton X-100 but also egg lecithin and phospholipid extracts from *M. luteus* activated UPPS over the entire concentration range studied, showing 11-, 14- and 10-fold activation at concentrations of 1%, 2 mM and 5.6 mM, respectively. The ionic detergents cardiolipin and DOC were less effective, showing only 4.2- and 2.4-fold activation at concentrations of 0.1 mM and 0.1%,

respectively [32]. These results indicate that a hydrophobic environment like a biological membrane is important for cPT activity, but ionic interaction with the enzyme or the substrates may adversely impact the activity, consistent with the inhibitory effects on AtHEPS (Fig. 3).

Based on the crystal structure analysis of UPPS from *E. coli*, the allylic diphosphate can bind to UPPS without Mg^{2+}, but binding of IPP requires Mg^{2+} [43]. The activity of AtHEPS was also markedly activated in the presence of Mg^{2+} (Fig. 4), suggesting that AtHEPS requires Mg^{2+} for binding of IPP similar to UPPS. The optimal concentration of Mg^{2+} for AtHEPS (1 mM) was almost the same as UPPSs from *E. coli* [31,43] and *M. luteus* [32] and *Z*-FPP synthase from *Mycobacterium tuberculosis* [36], indicating that the involvement of Mg^{2+} in the catalytic mechanism of cPTs may be conserved among cPTs in all organisms.

The functional complementation of the mutant phenotypes of *rer2* (Fig. 6) suggested that the products from AtHEPS could act as a glycosyl carrier lipid at least in yeast. However, the major product of AtHEPS, *Z,E*-HepPP, may only minimally function as a precursor of glycosyl carrier lipid based on the fact that the glycosyl transferases for *Z,E*-mixed polyisoprenoids in yeast hardly utilize polyisoprenoids shorter than C_{55} as acceptor lipids *in vitro* [16]. Therefore, the minor products from AtHEPS, $C_{65\text{-}85}$ *Z,E*-mixed polyisoprenoids, might be supplied for the formation of glycosyl carrier lipids in yeast, resulting in the partial complementation of the phenotypes by AtHEPS. Although no direct evidence has been supplied in this study, the major product of AtHEPS on the ER in *A. thaliana* cells is considered to be *Z,E*-HepPP according to the *in vitro* results. In addition, AtHEPS, predominantly expressed in root, was induced by cold stress and ABA treatment (Fig. 8). Taken together, products from AtHEPS and their derivative, probably short-chain betulaprenol, might function in response to abiotic stresses rather than in cell maintenance through the biosynthesis of glycoprotein on the ER. Interestingly, some reports have proposed that *Z,E*-mixed polyisoprenoids in mammals and seed plants can act as donors of the isoprenoid group during protein prenylations [44–49], as an inducer of apoptosis via reactive oxygen species formation [50,51], as modulators of membrane fluidity [52] and as a shield against reactive oxygen species [53]. In addition, polyprenols are considered to be correlated with environmental responses because polyprenols in some plants accumulate with aging [5,54] and in response to light irradiation [55] and infections by fungus [56] and virus [57]. However, no direct evidence of the function of polyprenols has been indicated. To address the questions of the physiological significance of polyprenols in seed plants, AtHEPS and other AtcPTs could be powerful tools. The analysis of knockout mutants and transgenic *Arabidopsis* seedlings overexpressing AtHEPS is currently ongoing in our group.

Experimental procedures

Materials

A full-length cDNA for AtHEPS (At5g58780) and Arabidopsis T87 cultured cells were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The expression vector pJR1133 was kindly provided by A. Ferrer (University of Barcelona, Spain). The *S. cerevisiae* strain SNY9 (*MAT*α, Δ*mf*α*1::ADE2*, Δ*mf*α*2::TRP1*, Δ*mf*α*1::HIS3*, *ura3*, *trp1*, *ade2*, *his3*, *lys2*, *leu2*, *lys2*) and SNH23-7D strain (*MAT*α, *rer2-2* Δ*mf*α*1::ADE2*, Δ*mf*α*2::TRP1*, Δ*mf*α*1::HIS3*, *ura3*, *trp1*, *ade2*, *his3*, *lys2*, *leu2*, *lys2*) were kindly provided by A. Nakano (RIKEN, Japan). The sGFP(S65T) [33] expression binary vector based on pBE2113Not [58] was kindly provided by Y. Niwa (University of Shizuoka, Japan). The Gateway plant vinary vector pGWB502Ω [59] was kindly provided by T. Nakagawa (Shimane University). [1-^{14}C]IPP (specific activity, 59 Ci·mol^{-1}) was purchased from GE Healthcare (Buckinghamshire, UK). Non-labeled IPP, GPP, *E,E*-FPP, *E,E,E*-GGPP and *Z,E,E*-GGPP were synthesized according to Davisson *et al.* [60]. All other chemicals were analytical grade.

Expression and partial purification of recombinant AtHEPS in *E. coli*

The full-length AtHEPS CDS was amplified by PCR (AtcPT5-S, 5′-GCCGGATCCATGTTGTCTATTCTCTC-3′; AtcPT5-A, 5′-ACAGGATCCAGCAGAGGATCAAACC-3′; *Bam*HI sites are underlined). The PCR product was subcloned into the *Bam*HI site of pGEX-4T-1 (GE Healthcare), pCold TF (Takara Bio, Ohtsu, Japan). *E. coli* BL21 was transformed with the resulting constructs, pGEX-4T-1-AtcPT5 and pCold TF-AtcPT5, and cultured to mid-stationary phase in LB medium containing 50 μg·mL^{-1} ampicillin at 37 °C with vigorous aeration. Overexpression of the protein in strain pGEX-4T-1-AtcPT5/BL21 was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM and then incubated at 18 °C for 24 h. Overexpression of the protein in pCold TF-AtcPT5/BL21 was induced by cold shock supplemented with 1 mM IPTG and then incubating at 15 °C for 24 h. The harvested cells were lysed by lysozyme (Sigma-Aldrich, St Louis, MO, USA), disrupted by sonications, and divided

into supernatant (soluble fraction) and pellet (insoluble fraction) by centrifugation at 7903 ***g*** for 15 min at 4 °C. The expressed TF and His_6-tag fusion proteins were affinity purified on HisTrap HP (GE Healthcare) from the soluble fraction of pCold TF-AtcPT5/BL21. The column was washed with 20 mM phosphate buffer containing 20 mM imidazole and fusion proteins were eluted in 1 mL of 20 mM phosphate buffer containing 200 mM imidazole and then dialyzed with 100 mM Tris/HCl (pH 7.5). The tag sequence was cleaved from AtHEPS by the treatment of thrombin (40 unit·mL^{-1}) (GE Healthcare) at 10 °C for 2 h. The cleavage reaction was stopped by the addition of Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland), and then thrombin was removed by HiTrap Benzamidine FF (GE Healthcare). The partially purified proteins were separated by SDS/PAGE. Protein concentrations were measured by Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard.

Heterologous expression of AtHEPS in *S. cerevisiae*

The full-length AtHEPS CDS was amplified by PCR (AtcPT5-S and AtcPT5-A) and subcloned in the *Bam*HI site of pJR1133, which contains the *URA3* marker [22]. *S. cerevisiae* SNH23-7D was transformed with pJR1133-AtcPT5 by the lithium acetate procedure [61] and selected on minimal medium containing 0.17% (w/v) yeast nitrogen base without amino acids, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose, 60 μg·mL^{-1} leucine and 30 μg·mL^{-1} lysine, at 23 °C. For controls, SNH23-7D and SNY9 were transformed with pJR1133 and selected respectively. Ura^+ transformants were cultured in minimal medium until D_{600} reached 1.5. Cell lysates from these strains were prepared according to the procedure described previously [29] and then centrifuged at 300 ***g*** for 5 min to remove unbroken cells. Supernatants were further centrifuged at 13 000 ***g*** for 10 min and then divided into supernatant (soluble fraction) and pellet (insoluble fraction).

Prenyltransferase assay and product analysis

The assay mixture for the crude fraction of *E. coli* contained 100 mM Tris/HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM *E,E*-FPP, 41.5 μM IPP, 8.5 μM [1-^{14}C]IPP (specific activity, 59 Ci·mol^{-1}) and 50 μg proteins in a final volume of 100 μL. The assay mixture for the crude fraction of *S. cerevisiae* contained 25 mM sodium phosphate buffer (pH 7.4), 4 mM $MgCl_2$, 20 mM KF, 20 mM β-mercaptoethanol, 14 μM *E,E*-FPP, 50 μM [1-^{14}C] IPP (specific activity, 59 Ci·mol^{-1}) and 100 μg proteins in a final volume of 100 μL. After incubation at 30 °C for 1 h for *E. coli* crude fractions or 2 h for *S. cerevisiae* crude fractions, the mixture was washed with 1 mL of diethylether after the addition of 200 μL of saturated NaCl solution. The reaction products were extracted from the aqueous phase with 1 mL of water-saturated 1-butanol, and then the radioactivity in 1-butanol extracts was measured with a liquid scintillation counter (Packard 1600TR, PerkinElmer, Waltham, MA, USA). For the product analysis, radioactive prenyl diphosphate products were hydrolyzed to corresponding alcohols according to the method described earlier [62]. In brief, the reaction solution contained 40 mM acetate buffer (pH 5.6), 0.1% (v/v) Triton X-100, 40% (v/v) methanol and potato acid phosphatase (Sigma) in a final volume of 1 mL. The mixture was incubated at 37 °C for 12 h. The resulting alcohols were extracted with pentane and analyzed by reversed phase TLC (LKC-18; Whatman, Maidstone, Kent, UK) with a solvent system of acetone/water (39 : 1). Positions of authentic standards were visualized with iodine vapor, and distribution of radioactivity was analyzed with a Bio-image analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

The assay mixture for the partially purified AtHEPS contained 100 mM Tris/HCl (pH 7.5), 1 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.1% (v/v) Triton X-100, 10 μM FPP, 50 μM [1-^{14}C]IPP (specific activity, 5 Ci·mol^{-1}) and 0.42 μg proteins in a final volume of 100 μL. After incubation at 30 °C for 12 h, the reaction products were extracted with 1-butanol, mixed with scintillation mixture and subjected to liquid scintillation counting as mentioned above.

Kinetics study

The standard assay mixture in kinetics studies with the partially purified AtHEPS contained 100 mM Tris/HCl (pH 7.5), 1 mM $MgCl_2$, 20 mM β-mercaptoethanol, 0.1% (v/v) Triton X-100, 1 μg protein and substrates, [1-^{14}C]IPP (specific activity, 10 Ci·mol^{-1}) and allylic diphosphates, in a final volume of 50 μL. The concentration of allylic diphosphate or homoallylic diphosphate IPP was varied, while the counter substrate IPP or allelic diphosphate was kept constant, respectively. The mixture was incubated at 30 °C for 2 h and then reaction products were extracted with water-saturated 1-butanol and the radioactivity in butanol extract was measured with a liquid scintillation counter (Packard 1600TR). The k_{cat} value was determined from the amounts of radioactivity incorporated into 1-butanol-extractable products when [1-^{14}C]IPP and the indicated allylic substrate were used. Kinetics parameters were calculated by nonlinear regression analysis using ENZYMEKINETICS software, version 1.5 (Trinity Software, Campton, NH, USA).

Stable transformation of Arabidopsis cultured cells and GFP imaging

To construct a plant binary plasmid for expression of AtHEPS fused in-frame with sGFP(S65T) at C-terminus, the

full-length AtHEPS CDS was amplified by PCR using a set of primers (AtcPT5-S, 5′-GCCGGATCCATGTTGTCT ATTCTCTC-3′; AtcPT5-A3 for GFP, 5′-CAGAGGATCC AACCCGACAGC-3′; *Bam*HI site underlined). The resulting fragment was subcloned in the *Bam*HI site of binary vector pBE2113Not harboring sGFP(S65T). The chimeric gene encoding SP-mCherry-HDEL was prepared based on the construct reported earlier [63]. The full-length mCherry CDS was amplified by PCR using a set of primers: ERSP-mCherry-Fw, 5′-CACCATGGCCAGACTCACAAGCA TCATTGCCCTCTTCGCAGTGGCTCTGCTGGTTGCA GATGCGTACGCCTACCGCACCATGGTGAGCAAGG GCG-3′ (a nucleotide sequence encoding the signal peptide of pumpkin 2S albumin underlined); mCherry-HDEL-Rv, 5′-GGTACCTCAAAGCTCATCGTGGTGGTGGTGGTG GTGCCCCCCCCCAGATCTCTTGTACAGCTCGTCCA TG-3′ (a nucleotide sequence encoding a peptide including ER-retention signal HDEL underlined). The resulting fragment was subcloned into Gateway entry vector pENTR/D-TOPO (Life Technologies, Carlsbad, CA, USA). The chimeric gene was transferred from the entry clone to Gateway binary vector pGWB502Ω by LR reaction as described by the manufacturer's instructions (Life Technologies). *A. thaliana* T87 cultured cells [34] were transformed by co-cultivation with *Agrobacterium tumefaciens* GV3101 (pMP90) carrying the resulting construct, as described previously [64]. For fluorescence imaging, the transgenic T87 cell lines were observed under a BX61 microscope equipped with a confocal scanning system (Fluoview FV1000; Olympus, Tokyo, Japan) using filter sets for GFP (excitation at 473 nm, emission at 510 nm) and red fluorescent protein (RFP) (excitation at 559 nm, emission at 583 nm).

Immunodetection of sGFP in subcellular fractions of T87 cells

The transgenic T87 cultured cells expressing AtHEPS-sGFP or sGFP were homogenized by mortar and pestle under liquid nitrogen with 400 μL of the extraction buffer containing 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 3 mM DTT, 5% (w/v) glycerol and Complete Protease Inhibitor Cocktail. Cell lysates were centrifuged at 15 000 rpm for 30 min, and the resulting supernatants were additionally centrifuged at 107 000 ***g*** for 40 min. Resulting supernatants were collected as soluble cytosol fractions. The deposits were washed with the extraction buffer and resuspended with 50 μL extraction buffer as the microsomal fraction. These fractions were separated by SDS/PAGE (13%) and blotted onto poly(vinylidene difluoride) membrane. Immunodetection was performed with the horseradish-peroxidase-conjugated GFP antibody (Nacalai Tesque, Kyoto, Japan) with an enhancer solution Can Get Signal Buffer II (Toyobo, Osaka, Japan), detected by ECL-Plus western blotting detection reagents (GE Healthcare).

Semi-quantitative RT-PCR

A. thaliana (Col-0) seedlings grown on MS plate under long-day conditions at 22 °C for 23 days were transferred to a liquid MS medium (Mock), a liquid MS medium containing 100 μM ABA (ABA) and a liquid MS medium prechilled and kept at 4 °C (Cold). After the treatments for 1, 2, 5, 10, 16 and 24 h, the seedlings were collected. Total RNA was extracted from roots by the method based on the procedure of Extract-A-Plant RNA Isolation Kit (Clontech, Mountain View, CA, USA). Transcripts in each sample were quantified by semi-quantitative RT-PCR, using first-strand cDNAs, synthesized from total RNA by PrimeScript II 1st strand cDNA synthase Kit (Takara Bio) using an oligo (dT) primer, as templates with a primer set for *AtHEPS* (AtcPT5S2, forward primer 5′-agatccaatatttccaaa gag-3′; AtcPT5A3, reverse primer 5′-agaatgatctctgactgtgg-3′) and for a β-tubulin *TUB4* (At5g44340) (TUB4S, forward primer 5′-ttgctgtcttcgtttccctgg-3′; TUB4A, reverse primer 5′-gagggtgccattgacaacatc-3′). The resulting PCR products were analyzed by agarose gel electrophoresis.

Acknowledgements

We are grateful to Dr A. Nakano and Dr M. Sato (RIKEN, Japan) for kindly providing the yeast strain SNH23-7D, Dr A. Ferrer (University of Barcelona, Spain) for kindly providing the vector pJR1133, Dr Y. Niwa (University of Shizuoka, Japan) for kindly providing the plasmid harboring sGFP(S65T) and Dr T. Nakagawa (Shimane University, Japan) for providing the plant binary vector pGWB502Ω. We are also grateful to Dr R. Yokoyama and Dr K. Nishitani (Tohoku University, Japan) for kindly supporting operations of the confocal scanning system. This work was supported in part by Grants-in-Aid for Scientific Research from JSPS, Japan Society for the Promotion of Science, and by the Foundations of the Sumitomo Foundation.

References

1 Bouhss A, Trunkfield AE, Bugg TDH & Mengin-Lecreulx D (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiol Rev* **32**, 208–233.
2 Helenius A & Aebi M (2004) Roles of N-linked glycans in the endoplasmic reticulum. *Annu Rev Biochem* **73**, 1019–1049.
3 Chojnacki T & Dallner G (1988) The biological role of dolichol. *Biochem J* **251**, 1–9.
4 Krag SS (1998) The importance of being dolichol. *Biochem Biophys Res Commun* **243**, 1–5.
5 Swiezewska E, Sasak W, Mankowski T, Jankowski W, Vogtman T, Krajewska I, Hertel J, Skoczylas E &

Chojnacki T (1994) The search for plant polyprenols. *Acta Biochim Pol* **41**, 221–260.
6 Swiezewska E & Danikiewicz W (2005) Polyisoprenoids: structure, biosynthesis and function. *Prog Lipid Res* **44**, 235–258.
7 Chouda M & Jankowski W (2005) The occurrence of polyprenols in seeds and leaves of woody plants. *Acta Biochim Pol* **52**, 243–253.
8 Rezanka T & Votruba J (2001) Chromatography of long chain alcohols (polyprenols) from animal and plant sources. *J Chromatogr A* **936**, 95–110.
9 Ishii K, Sagami H & Ogura K (1986) A novel prenyltransferase from *Paracoccus denitrificans*. *Biochem J* **233**, 773–777.
10 Kaur D, Brennan PJ & Crick DC (2004) Decaprenyl diphosphate synthesis in *Mycobacterium tuberculosis*. *J Bacteriol* **186**, 7564–7570.
11 Mahapatra S, Yagi T, Belisle JT, Espinosa BJ, Hill PJ, McNeil MR, Brennan PJ & Crick DC (2005) Mycobacterial lipid II is composed of a complex mixture of modified muramyl and peptide moieties linked to decaprenyl phosphate. *J Bacteriol* **187**, 2747–2757.
12 Burgos J, Hemming FW, Pennock JF & Morton RA (1963) Dolichol: a naturally-occurring C100 isoprenoid alcohol. *Biochem J* **88**, 470–482.
13 Rupar CA & Carroll KK (1978) Occurrence of dolichol in human tissues. *Lipids* **13**, 291–293.
14 Lindgren BO (1965) Homologous aliphatic C_{30}–C_{45} terpenols in birch wood. *Acta Chem Scand* **19**, 1317–1326.
15 Wellburn AR & Hemming FW (1966) Polyprenols of wood and leaf tissue of the silver birch, *Betula verrucosa*. *Nature* **212**, 1364–1366.
16 Palamarczyk G, Lehle L, Mankowski T, Chojnacki T & Tanner W (1980) Specificity of solubilized yeast glycosyl transferases for polyprenyl derivatives. *Eur J Biochem/FEBS* **105**, 517–523.
17 Ogura K & Koyama T (1998) Enzymatic aspects of isoprenoid chain elongation. *Chem Rev* **98**, 1263–1276.
18 Cantagrel V, Lefeber DJ, Ng BG, Guan Z, Silhavy JL, Bielas SL, Lehle L, Hombauer H, Adamowicz M, Swiezewska E *et al.* (2010) SRD5A3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder. *Cell* **142**, 203–217.
19 Shimizu N, Koyama T & Ogura K (1998) Molecular cloning, expression, and purification of undecaprenyl diphosphate synthase. No sequence similarity between E- and Z-prenyl diphosphate synthases. *J Biol Chem* **273**, 19476–19481.
20 Fujihashi M, Zhang YW, Higuchi Y, Li XY, Koyama T & Miki K (2001) Crystal structure of *cis*-prenyl chain elongating enzyme, undecaprenyl diphosphate synthase. *Proc Natl Acad Sci USA* **98**, 4337–4342.
21 Takahashi S & Koyama T (2006) Structure and function of *cis*-prenyl chain elongating enzymes. *Chem Rec* **6**, 194–205.
22 Cunillera N, Arro M, Fores O, Manzano D & Ferrer A (2000) Characterization of dehydrodolichyl diphosphate synthase of *Arabidopsis thaliana*, a key enzyme in dolichol biosynthesis. *FEBS Lett* **477**, 170–174.
23 Oh SK, Han KH, Ryu SB & Kang H (2000) Molecular cloning, expression, and functional analysis of a *cis*-prenyltransferase from *Arabidopsis thaliana*. Implications in rubber biosynthesis. *J Biol Chem* **275**, 18482–18488.
24 Asawatreratanakul K, Zhang YW, Wititsuwannakul D, Wititsuwannakul R, Takahashi S, Rattanapittayaporn A & Koyama T (2003) Molecular cloning, expression and characterization of cDNA encoding *cis*-prenyltransferases from *Hevea brasiliensis*. A key factor participating in natural rubber biosynthesis. *Eur J Biochem* **270**, 4671–4680.
25 Sallaud C, Rontein D, Onillon S, Jabes F, Duffe P, Giacalone C, Thoraval S, Escoffier C, Herbette G, Leonhardt N *et al.* (2009) A novel pathway for sesquiterpene biosynthesis from *Z,Z*-farnesyl pyrophosphate in the wild tomato *Solanum habrochaites*. *Plant Cell* **21**, 301–317.
26 Schilmiller AL, Schauvinhold I, Larson M, Xu R, Charbonneau AL, Schmidt A, Wilkerson C, Last RL & Pichersky E (2009) Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. *Proc Natl Acad Sci USA* **106**, 10865–10870.
27 Sato M, Sato K, Nishikawa S, Hirata A, Kato J & Nakano A (1999) The yeast RER2 gene, identified by endoplasmic reticulum protein localization mutations, encodes *cis*-prenyltransferase, a key enzyme in dolichol synthesis. *Mol Cell Biol* **19**, 471–483.
28 Kharel Y, Takahashi S, Yamashita S & Koyama T (2006) Manipulation of prenyl chain length determination mechanism of *cis*-prenyltransferases. *FEBS J* **273**, 647–657.
29 Endo S, Zhang YW, Takahashi S & Koyama T (2003) Identification of human dehydrodolichyl diphosphate synthase gene. *Biochim Biophys Acta* **1625**, 291–295.
30 Sato M, Fujisaki S, Sato K, Nishimura Y & Nakano A (2001) Yeast *Saccharomyces cerevisiae* has two *cis*-prenyltransferases with different properties and localizations. Implication for their distinct physiological roles in dolichol synthesis. *Genes Cells* **6**, 495–506.
31 Apfel CM, Takacs B, Fountoulakis M, Stieger M & Keck W (1999) Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential uppS gene. *J Bacteriol* **181**, 483–492.
32 Baba T & Allen CM (1980) Prenyl transferases from *Micrococcus luteus*: characterization of undecaprenyl

pyrophosphate synthetase. *Arch Biochem Biophys* **200**, 474–484.
33 Chiu W-l, Niwa Y, Zeng W, Hirano T, Kobayashi H & Sheen J (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* **6**, 325–330.
34 Axelos M, Curie C, Mazzolini L, Bardet C & Lescure B (1992) A protocol for transient gene-expression in *Arabidopsis-thaliana* protoplasts isolated from cell-suspension cultures. *Plant Physiol Biochem* **30**, 123–128.
35 Surmacz L & Swiezewska E (2011) Polyisoprenoids – secondary metabolites or physiologically important superlipids? *Biochem Biophys Res Commun* **407**, 627–632.
36 Schulbach MC, Mahapatra S, Macchia M, Barontini S, Papi C, Minutolo F, Bertini S, Brennan PJ & Crick DC (2001) Purification, enzymatic characterization, and inhibition of the *Z*-farnesyl diphosphate synthase from *Mycobacterium tuberculosis*. *J Biol Chem* **276**, 11624–11630.
37 Ambo T, Noike M, Kurokawa H & Koyama T (2008) Cloning and functional analysis of novel short-chain *cis*-prenyltransferases. *Biochem Biophys Res Commun* **375**, 536–540.
38 Sato T, Takizawa K, Orito Y, Kudo H & Hoshino T (2010) Insight into C35 terpene biosyntheses by nonpathogenic mycobacterium species: functional analyses of three z-prenyltransferases and identification of dehydroheptaprenylcyclines. *ChemBioChem* **11**, 1874–1881.
39 Hemmi H, Yamashita S, Shimoyama T, Nakayama T & Nishino T (2001) Cloning, expression, and characterization of *cis*-polyprenyl diphosphate synthase from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*. *J Bacteriol* **183**, 401–404.
40 Schulbach MC, Brennan PJ & Crick DC (2000) Identification of a short (C15) chain *Z*-isoprenyl diphosphate synthase and a homologous long (C50) chain isoprenyl diphosphate synthase in *Mycobacterium tuberculosis*. *J Biol Chem* **275**, 22876–22881.
41 Ambo T, Noike M, Kurokawa H & Koyama T (2009) Cloning and functional analysis of *cis*-prenyltransferase from *Thermobifida fusca*. *J Biosci Bioeng* **107**, 620–622.
42 Dhiman RK, Schulbach MC, Mahapatra S, Baulard AR, Vissa V, Brennan PJ & Crick DC (2004) Identification of a novel class of omega,E,E-farnesyl diphosphate synthase from *Mycobacterium tuberculosis*. *J Lipid Res* **45**, 1140–1147.
43 Guo RT, Ko TP, Chen AP, Kuo CJ, Wang AH & Liang PH (2005) Crystal structures of undecaprenyl pyrophosphate synthase in complex with magnesium, isopentenyl pyrophosphate, and farnesyl thiopyrophosphate: roles of the metal ion and conserved residues in catalysis. *J Biol Chem* **280**, 20762–20774.
44 Thelin A, Low P, Chojnacki T & Dallner G (1991) Covalent binding of dolichyl phosphate to proteins in rat liver. *Eur J Biochem/FEBS* **195**, 755–761.
45 Hjertman M, Wejde J, Dricu A, Carlberg M, Griffiths WJ, Sjovall J & Larsson O (1997) Evidence for protein dolichylation. *FEBS Lett* **416**, 235–238.
46 Parmryd I, Andersson B & Dallner G (1999) Protein prenylation in spinach chloroplasts. *Proc Natl Acad Sci USA* **96**, 10074–10079.
47 Shipton CA, Parmryd I, Swiezewska E, Andersson B & Dallner G (1995) Isoprenylation of plant proteins *in vivo*. Isoprenylated proteins are abundant in the mitochondria and nuclei of spinach. *J Biol Chem* **270**, 566–572.
48 Swiezewska E, Thelin A, Dallner G, Andersson B & Ernster L (1993) Occurrence of prenylated proteins in plant cells. *Biochem Biophys Res Commun* **192**, 161–166.
49 Gutkowska M, Bienkowski T, Hung VS, Wanke M, Hertel J, Danikiewicz W & Swiezewska E (2004) Proteins are polyisoprenylated in *Arabidopsis thaliana*. *Biochem Biophys Res Commun* **322**, 998–1004.
50 Yasugi E, Nakata K, Yokoyama Y, Kano K, Dohi T & Oshima M (1998) Dihydroheptaprenyl and dihydrodecaprenyl monophosphates induce apoptosis mediated by activation of caspase-3-like protease. *Biochim Biophys Acta* **1389**, 132–140.
51 Yokoyama Y, Nohara K, Okubo T, Kano I, Akagawa K & Kano K (2007) Generation of reactive oxygen species is an early event in dolichyl phosphate-induced apoptosis. *J Cell Biochem* **100**, 349–361.
52 Valtersson C, van Duyn G, Verkleij AJ, Chojnacki T, de Kruijff B & Dallner G (1985) The influence of dolichol, dolichol esters, and dolichyl phosphate on phospholipid polymorphism and fluidity in model membranes. *J Biol Chem* **260**, 2742–2751.
53 Bergamini E (2003) Dolichol: an essential part in the antioxidant machinery of cell membranes? *Biogerontology* **4**, 337–339.
54 Tateyama S, Wititsuwannakul R, Wititsuwannakul D, Sagami H & Ogura K (1999) Dolichols of rubber plant, ginkgo and pine. *Phytochemistry* **51**, 11–15.
55 Bajda A, Chojnacki T, Hertel J, Swiezewska E, Wojcik J, Kaczkowska A, Marczewski A, Bojarczuk T, Karolewski P & Oleksyn J (2005) Light conditions alter accumulation of long chain polyprenols in leaves of trees and shrubs throughout the vegetation season. *Acta Biochim Pol* **52**, 233–241.
56 Monaco P, Previtera L & Belardini M (1983) Polyprenols from the leaves of *Quercus ilex* infected by *Microsphaera alphitoides*. *J Nat Prod* **46**, 174–177.
57 Bajda A, Konopka-Postupolska D, Krzymowska M, Hennig J, Skorupinska-Tudek K, Surmacz L, Wójcik J, Matysiak Z, Chojnacki T, Skorzynska-Polit E *et al.* (2009) Role of polyisoprenoids in tobacco resistance against biotic stresses. *Physiol Plant* **135**, 351–364.

58 Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S *et al.* (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* **37**, 49–59.
59 Nakagawa T, Suzuki T, Murata S, Nakamura S, Hino T, Maeo K, Tabata R, Kawai T, Tanaka K, Niwa Y *et al.* (2007) Improved gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci Biotechnol Biochem* **71**, 2095–2100.
60 Davisson VJ, Woodside AB & Poulter CD (1985) Synthesis of allylic and homoallylic isoprenoid pyrophosphates. *Methods Enzymol* **110**, 130–144.
61 Gietz D, St Jean A, Woods RA & Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**, 1425.
62 Koyama T, Fujii H & Ogura K (1985) Enzymatic hydrolysis of polyprenyl pyrophosphates. *Methods Enzymol* **110**, 153–155.
63 Mitsuhashi N, Shimada T, Mano S, Nishimura M & Hara-Nishimura I (2000) Characterization of organelles in the vacuolar-sorting pathway by visualization with GFP in tobacco BY-2 cells. *Plant Cell Physiol* **41**, 993–1001.
64 Suzuki H, Takahashi S, Watanabe R, Fukushima Y, Fujita N, Noguchi A, Yokoyama R, Nishitani K, Nishino T & Nakayama T (2006) An isoflavone conjugate-hydrolyzing beta-glucosidase from the roots of soybean (Glycine max) seedlings: purification, gene cloning, phylogenetics, and cellular localization. *J Biol Chem* **281**, 30251–30259.

Supporting information

The following supplementary material is available:
Fig. S1. TLC analysis of the C30-35 radioactive poly-isoprenoids incubated with the crude extracts from SHN23-7D.
Fig. S2. SDS/PAGE analysis of overexpression and purification of the recombinant AtHEPS.
Fig. S3. Subcellular localizations of AtHEPS-sGFP in Arabidopsis cells.
Fig. S4. Molecular phylogenetic tree of the amino acid sequences of the identified cPTs.
Table S1. Summary of AtcPTs.

This supplementary material can be found in the online version of this article.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be reorganized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.