Homogentisate solanesyl transferase (HST) catalyzes the prenylation and decarboxylation of homogentisate to form 2-methyl-6-solanesyl-1,4-benzoquinol, the first intermediate in plastoquinone-9 biosynthesis. In vitro, HST from Spinacia oleracea L., Arabidopsis thaliana, and Chlamydomonas reinhardtii were all found to use not only solanesyl diphosphate but also short chain prenyl diphosphates of 10–20 carbon atoms as prenyl donors. Surprisingly, with these donors, prenyl transfer was largely decoupled from decarboxylation, and thus the major products were 6-prenyl-1,4-benzoquinol-2-methylcarboxylates rather than the expected 2-methyl-6-prenyl-1,4-benzoquinolins. The 6-prenyl-1,4-benzoquinol-2-methylcarboxylates were not substrates for HST-catalyzed decarboxylation, and the enzyme kinetics associated with forming these products appeared quite distinct from those for 2-methyl-6-prenyl-1,4-benzoquinolins. Here, prenyl diphosphate binds to HST to form at least two alternative complexes that go on to react differently with homogentisate and prenylate it either with or without it first being decarboxylated. It is supposed that solanesyl diphosphate binds tightly and preferentially in the mode that compels prenylation with decarboxylation.

Plastoquinone-9 (PQ-9) is the major prenylated quinone in chloroplasts. In the thylakoid membrane, it mediates electron flow from photosystem II to the cytochrome b6f complex, and the redox state of the PQ-9 pool regulates the expression of a number of nuclear and plastidial genes as well as the activity of some plastidial enzymes (1). Moreover, PQ-9 is required as a substrate for HST (6).

Test Chemicals for Inhibition Studies—Haloxydine and [2-((R)-1-phenyl-ethylamino)-1-phosphono-ethyl]-phosphonic acid were obtained from Syngenta Ltd. and were synthesized as described previously (12, 13). The purity of haloxydine was 96%, and that of the bisphosphonate was 84% as determined by reverse-phase HPLC and proton NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Heterologous Expression and Preparation of Enzyme-enriched Fractions—The cDNA sequences for the mature homogentisate prenyltransferase proteins from C. reinhardtii CC-1690 (AM285678) and from A. thaliana ecotype Columbia were reconciled into a simple model for the HST mechanism. Here, prenyl diphosphate binds to HST to form at least two alternative complexes that go on to react differently with homogentisate and prenylate it either with or without it first being decarboxylated. It is supposed that solanesyl diphosphate binds tightly and preferentially in the mode that compels prenylation with decarboxylation.
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![Aromatic prenyltransferase reaction in PQ-9 biosynthesis (A) and structure of the herbicide haloxynil (B). PPI, diphosphate.](image)

**FIGURE 1.** Aromatic prenyltransferase reaction in PQ-9 biosynthesis (A) and structure of the herbicide haloxynil (B).

(At3g11945, previously At3g11950) were expressed with an N-terminal glutathione S-transferase (GST) tag in *E. coli* BL21AI as described previously (6). The open reading frame for a 4-hydroxybenzoate prenyltransferase was amplified from the *E. coli* BL21AI genome (ubiA, NC_012947) by using the forward primer 5′-CAC CAT GGA GTG GAG TCT GAC GC-3′ and the reverse primer 5′-TCA GAA ATG CCA GTA ACT CAT TGC CAG-3′. The PCR product was sequenced using standard techniques and cloned into the expression vector pDEST14 (Invitrogen). Membranes of the *E. coli* clones expressing the aromatic prenyltransferase encoding sequences were derived from the standard techniques and cloned into the expression vector pDEST14 (Invitrogen). Membranes of the *E. coli* clones expressing the aromatic prenyltransferase encoding sequences were prepared as described by Ref. 6. GST-tagged proteins were detected on Western blot membranes using mouse GST tag antibodies (Novagen) and goat anti-mouse IgG-peroxidase conjugate antibodies (Qiagen) with Lumi-Light Plus (Roche Applied Science) according to the manufacturer’s instructions.

**Preparation of Chloroplast Envelope Membranes**—Chloroplast envelope membranes were isolated from spinach leaves essentially as described by Ref. 14. Intact chloroplasts were purified from a crude chloroplast preparation by isopycnic centrifugation in a Percoll gradient and subsequently lysed in hypotonically medium. From the lysate, envelope membranes were purified in a three-step sucrose gradient.

**Enzyme Assays**—Homogentisate prenyltransferase activities were measured by determining the incorporation rates of [U-14C]homogentisate and prenyl diphosphates into lipophilic products as described by Ref. 6. [U-14C]Homogentisate (radiochemical purity 85–95%) was synthesized enzymatically from [U-14C]tyrosine as described earlier (6). Unlabeled substrates were purchased from Sigma-Aldrich and American Radiolabeled Chemicals, respectively. *E. coli*-expressed HST from *C. reinhardtii* was assayed for 15 min at 28 °C in a 50-μl reaction volume with 30 μg of membrane proteins, 20 mM magnesium acetate, 100 μM [U-14C]homogentisate (~80 dpm/pmol), and 200 μM prenyl diphosphate (generally farnesyl diphosphate, FPP) in 50 mM Tricine-NaOH buffer, pH 8.5. Spinach envelope membranes (30 μg of total protein) were incubated for 30 min at 28 °C in a 50-μl reaction mixture with 50 mM Bis-Tris propane-HCl, pH 9.0, 50 mM magnesium acetate, 30 μM [U-14C]homogentisate (~520 dpm/pmol), and 200 μM prenyl diphosphate. In general, enzyme reactions were started with the addition of prenyl donor to the reaction mixture. Assays were stopped and extracted with 200 μl of chloroform:methanol (1:1) and 50 μl of 0.9% sodium chloride solution. Labeled products were visualized on silica gel TLC plates (MACHERY-NAGEL) with a bioimager (FLA3000, raytest), and data were processed with the AIDA software (raytest). The amount of radioactivity was quantified by scintillation counting. Kinetic data were graphed and analyzed using GraphPad Prism (GraphPad Software). Error limits around *Km* and *V*max represent the bounds of the 95% confidence limits. Where there were six data points, error bounds for *Ki* values were the upper and lower values derived from the standard errors of the slopes and Y intercepts of the linear regression fits to replots of the estimated values of *V*max or *Km*/*V*max versus inhibitor concentration. With only three data points, the error bounds simply represent the maximum and minimum slopes between any two. 2-Methyquinolin (Alfa Aesar) was oxidized with potassium dichromate in dilute sulfuric acid for co-chromatography analysis.

The *E. coli* 4-hydroxybenzoate prenyltransferase (UbiA) was assayed in 50 mM Bistris propane, pH 7.0, 20 mM magnesium acetate, 30 μM [carboxy-14C]4-hydroxybenzoate (~40 dpm/pmol), 50 μM prenyl diphosphate with 5 μg of membrane proteins in a reaction volume of 50 μl. After a 15-min incubation at 30 °C, lipids were extracted as described above and analyzed on silica gel plates in acetone:petroether (3:7). Specific activities of about 150 pmol min⁻¹ mg⁻¹ protein were determined.

**Mass Spectrometry Analyses of Polar HST Reaction Products**—To generate adequate amounts of polar products for mass spectrometry analysis, *C. reinhardtii* HST was incubated for 30 min at 28 °C in a 2-ml reaction volume of 50 mM Tricine-NaOH buffer, pH 8.5, containing 1.2 mg of membrane proteins, 20 mM magnesium acetate, 200 μM [U-14C]homogentisate (~60 dpm/pmol), and 200 μM geranylgeranyl diphosphate (GGPP). The reaction was stopped and extracted with 1.5 ml of chloroform: methanol (1:1) and 0.5 ml of 0.9% sodium chloride solution. The organic phase was re-extracted with water four times, dried under a stream of nitrogen gas, and redissovled in chloroform. Analysis by TLC using toluene:isoamyl alcohol:acetic acid (80:40:3) as mobile phase confirmed that nearly all of the 14C-labeled product was polar in nature. Further purification was done by HPLC (1100 Series, Agilent Technologies) using a reverse-phase C30 column (250 × 4.6 mm, 5 μm, YMC Co.) and gradient elution between two mobile phases, A and B. Solvent A was methanol:water:formic acid (375:122:2.5), and solvent B was methanol:isopropyl alcohol:formic acid (400:97.5:2.5), B. The gradient was developed as 100% A (initial), 95% A, and 5% B at 2 min, 80% A and 20% B at 5 min, 50% A and 50% B at 12 min, 20% A and 80% B at 35 min, and 10% A and 90% B at 40 min at a flow rate of 0.75 ml/min. Three main radiolabeled fractions containing polar product (as verified by TLC) were collected at 19.5, 21.6, and 22.3 min, respectively, and dried under a stream of nitrogen. High resolution mass spectrometry analysis was performed on samples dissolved in ethyl acetate and injected using an Accela HPLC system coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) and an FSA 610 TR radiodetector (PerkinElmer Life Sciences). The HPLC system was equipped with a reverse-phase C30 column (250 × 3-mm...
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Alternative Products of the HST Reaction—Although the addition of haloxydine inhibited HST and suppressed the appearance of the radiolabeled bands corresponding to MFBQ, the predominant visible effect of adding the inhibitor was on the amount of \(^{14}\text{C}\) label retained near the origin of the TLC plate (Fig. 2A). It appeared that haloxydine also inhibited the formation of hitherto unknown major reaction products that were extracted into chloroform and that, on the basis of their relative immobility in dichloromethane, must be more polar than MFBQ. Further TLC using a toluene:isoamyl alcohol:acetic acid system indicated that these unknown products were more polar than MFBQ. From all three fractions, 6-geranylgeranyl-1,4-benzoquinol-2-methylcarboxylate were detected including the radical anion 439.2853 \(m/z\) (0.25 ppm, \(C_{25}H_{37}O_3\), 6.3 min) as the minor component. Radical anion and other anionic species derived from 6-geranylgeranyl-1,4-benzoquinol-2-methylcarboxylate were detected including the radical anion 440.2928 \(m/z\) (0.47 ppm), the anion 439.2855 \(m/z\) (0.25 ppm), and the radical anion 438.2780 \(m/z\) (0.90 ppm). It is highly likely that these occur as a result of electrochemical redox reactions in the mass spectrometer.

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products were most likely the corresponding 6-prenyl-1,4-benzoquinol-2-methylcarboxylates that would result from where catalyzed prenylation of homogentisate occurring without concomitant decarboxylation (Fig. 3C). This assignment of the major polar products as acids is consistent with their observed lack of TLC movement with dichloromethane as mobile phase as well as the pH dependence of their movement in the toluene:isoamyl alcohol:acetic acid TLC system. Besides the expected 2-methyl-6-prenyl-1,4-benzoquinol and 2-methyl-6-prenyl-1,4-quinone-2-methylcarboxylates were confirmed by major GGPP-derived polar products as 6-geranylgeranyl-1,4-isoamyl alcohol:acetic acid TLC system. The identity of the products formed were carboxylic acids, whereas with the C$_{45}$ prenyl donor, SPP, the major product was MSBQ (Fig. 3B).

**Enzyme Kinetics of HST-catalyzed Product Formation**—Kinetic studies were pursued using only the C. reinhardtii enzyme because the A. thaliana enzyme was more difficult to express in easily assayable amounts. Assay conditions were established to ensure that initial rates could be measured and thus that rates were adequately linear with additions of between 0 and 30 µg of membrane protein and with assay reaction times between 0 and 20 min. It was also established from the outset that haloxydine was neither a slow binding nor a slow dissociating inhibitor and that, on the time scale of the kinetic experiments, it could be treated as being in rapid equilibrium with the enzyme and enzyme substrate complexes.

**FIGURE 3. TLC analyses of C. reinhardtii HST products from assays with various prenyl donors.** Assays were conducted in the presence of 0.1 mM n-dodecyl β-D-maltoside with the exception of sample SPP$_r$ that contained 0.2 mM detergent in the reaction mixture. The chloroform soluble products were separated by TLC using dichloromethane (A) or toluene/isoamyl alcohol:acetic acid (80:40:3) (B) as mobile phase. Besides the expected 2-methyl-6-prenyl-1,4-benzoquinol (marked by 1, 3, 5, and 7) and the respective quinones (marked by 2, 4, 6, and 8), chloroform extracts of assays with prenyl donors up to a chain length of C$_{20}$ contained mainly so far unknown prenylated polar products. * indicates the origin; PPI, diphosphate. C, the structures for the 2-methyl-6-prenyl-1,4-benzoquinol (upper) and the more polar 6-prenyl-1,4-benzoquinol-2-methylcarboxylates (lower) are given with n = 2, n = 3, n = 4, and n = 9 for the respective GPP, FPP, GGPP, and SPP-derived products.

The proportion of non-decarboxylated to decarboxylated prenyl products of the HST reaction changed according to the prenyl donor. With the C$_{10}$ to C$_{20}$ prenyl donors, GPP, FPP, and GGPP, up to 95% of the total prenylated reaction products formed were carboxylic acids, whereas with the C$_{45}$ prenyl donor, SPP, the major product was MSBQ (Fig. 3B).

In control experiments, the ratio of 6-prenyl-1,4-benzoquinol-2-methylcarboxylates to 2-methyl-6-prenyl-1,4-benzoquinol products formed from FPP or GGPP remained unaltered upon further incubation with control E. coli membranes that lacked HST. Neither was any of the methylcarboxylate decarboxylated to 2-methyl-6-prenyl-1,4-benzoquinol when the products from one round of HST reaction were reincubated with fresh HST. Hence, it was confirmed that HST, and not some other component of the E. coli membranes, was solely responsible for the generation of both carboxylated and decarboxylated products and that there was no membrane-associated nonspecific decarboxylase activity capable of converting the carboxylic acid to the respective 2-methyl-6-prenyl-1,4-benzoquinol.
FIGURE 4. Kinetics of inhibition of C. reinhardtii HST by haloxydine. The effects of haloxydine on HST-catalyzed MFBQ formation at 200 μM FPP (A) and at 100 μM homogentisate (B) as well as on total product formation at 200 μM FPP (C) and 100 μM homogentisate (D) are shown.
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**Discussion**

*In vitro*, HST-catalyzed prenylation of homogentisate resulted not only in the formation of the expected 2-methyl-6-prenyl-1,4-benzoquinol products but also in the corresponding 6-prenyl-1,4-benzoquinol-2-methylcarboxylates. Haloxylidine, an anionic (pKₐ 2.15) and credible structural mimic of homogentisate, inhibited the formation of both MFBQ and FBQC consistent with them both being products of HST. Because HST preparations were not able to decarboxylate FBQC, we concluded that FBQC is not made as an exchangeable catalytic intermediate along the pathway of HST-catalyzed formation of MFBQ. Thus the 6-prenyl-1,4-benzoquinol-2-methylcarboxylate and 2-methyl-6-prenyl-1,4-benzoquinol products would appear to be formed via distinct and competing pathways. The scheme of Fig. 3 summarizes our proposal for the order and nature of intermediates along two concurrent pathways of HST-catalyzed prenylation. The essential features are (a) that prenyl diphosphate, in this case FPP, binds to the enzyme to form the (E·FPP) complex; (b) that homogentisate binds to the (E·FPP) complex; and (c) that especially the smaller prenyl donors such as FPP have enough “wobble” room within the enzyme to partition between at least two binding orientations and form alternative productive enzyme complexes, here designated (E·FPP)ₐ and (E·FPP)ₐ. These complexes react differently with homogentisate and go on to prenylate it either with (Enzyme Cycle A) or without (Enzyme Cycle B) it first being decarboxylated. It is supposed that the larger, cognate substrate of HST, SPP, which forms mainly the decarboxylated product (Fig. 3), has less steric freedom to move within the enzyme site and is constrained to predominantly bind in the orientation that takes it down reaction path A and thus to the metabolically productive MSBQ product.

Enzyme Cycle A continues from (E·FPP)ₐ with homogentisate binding to form ((E·FPP)ₐ·H), which then decarboxylates to form a 2-methylquinol complex ((E·FPP·MQ) prior to prenyl transfer to (E·MFBOQ), which then dissociates to release product. Possible alternatives with prenylation preceding decarboxylation and thus having FBQC as an intermediate in MFBQ formation are difficult to reconcile with the observed inability of HST to convert FBQC into MFBQ. Equally, alternatives that have MFBQ and FBQC derived from a common ((E·FPP·H)
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The question is raised therefore as to whether and how the HST reaction is controlled in planta to avoid the production of the kinds of side products observed in vitro or whether they are indeed produced but have yet to be detected. Possibly there is efficient substrate channeling to deliver SPP to HST. Alternatively, and consistent with the ordered mechanism suggested in the scheme of Fig. 6, it could simply be that the $K_m$ for SPP is much lower than for the other prenyl donors so that, effectively, there is little free enzyme and HST exists mainly bound up as the (HST-SPP) complex. In any case, plastoquinones with shorter prenyl side chains have been detected as minor components in higher plants such as plastoquinone-3 in spinach (15).

The observed formation of MFBQ in assays with spinach chloroplast envelopes (Fig. 2) is thus consistent with the occurrence of plastoquinone-3 in this plant species.

Given the large and lipophilic nature of the SPP-derived reaction product it is reasonable to speculate that, as for many enzymes (16), product release or protein conformational changes that precede this rather than a bond-breaking or bond-forming step might be largely rate-limiting in the overall catalytic cycle. At least consistent with this notion is the observation that widely differing catalytic rates were observed according to the product formed.

In vitro, HST appeared to be such a poor catalyst for the production of MSBQ as to raise the question of whether the enzyme has any real role in PQ-9 biosynthesis in plants. However, the flux required in planta is likely very low, and the bleached seedling-lethal phenotype of the A. thaliana pds2 mutant (2), which maps to the same location (At3g11945) as the A. thaliana HST gene (3), clearly demonstrates that the product of this gene is indeed essential for PQ-9 biosynthesis. The biological bleaching effects of haloxydine on plants are also consistent with an essential role for HST; certainly other herbicides known to act at other sites in plastoquinone biosynthesis exhibit similar effects on plants (17).

Acknowledgments—We gratefully acknowledge expert help from Richard Wood (Syngenta Ltd.), Dalila Menguelt (Syngenta Ltd.), and Dr. Peter Howe (Syngenta Ltd.) in the analysis and characterization of chemicals used and products made in the course of this study.

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