FaQR, Required for the Biosynthesis of the Strawberry Flavor Compound 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone, Encodes an Enone Oxidoreductase

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The flavor of strawberry (*Fragaria × ananassa*) fruit is dominated by an uncommon group of aroma compounds with a 2,5-dimethyl-3(H)-furanone structure. We report the characterization of an enzyme involved in the biosynthesis of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF; Furaneol), the key flavor compound in strawberries. Protein extracts were partially purified, and the observed distribution of enzymatic activity correlated with the presence of a single polypeptide of ∼37 kD. Sequence analysis of two peptide fragments showed total identity with the protein sequence of a strongly ripening-induced, auxin-dependent putative quinone oxidoreductase, *Fragaria × ananassa* quinone oxidoreductase (FaQR). The open reading frame of the *FaQR* cDNA consists of 969 bp encoding a 322–amino acid protein with a calculated molecular mass of 34.3 kD. Laser capture microdissection followed by RNA extraction and amplification demonstrated the presence of *FaQR* mRNA in parenchyma tissue of the strawberry fruit. The FaQR protein was functionally expressed in *Escherichia coli*, and the monomer catalyzed the formation of HDMF. After chemical synthesis and liquid chromatography–tandem mass spectrometry analysis, 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone was confirmed as a substrate of FaQR and the natural precursor of HDMF. This study demonstrates the function of the FaQR enzyme in the biosynthesis of HDMF as enone oxidoreductase and provides a foundation for the improvement of strawberry flavor and the biotechnological production of HDMF.

INTRODUCTION

Strawberry (*Fragaria × ananassa*) constitutes a high-value food crop that is cultivated almost worldwide. The fruit, an aggregate of numerous ovaries, is composed of the seeds (achenes) embedded in the epidermis of the swollen receptacle tissue. Strawberries do not develop properly if seeds have not been formed (Perkins-Veazie, 1995). The phytohormone auxin is regarded as the main signal molecule coordinating the growth and initiation of ripening in strawberry fruit (Perkins-Veazie, 1995). During the early stages of fruit development, the auxins synthesized in the achenes promote fruit growth, but actual strawberry ripening is triggered by the decline in the levels of auxin in the receptacle caused by the cessation of auxin synthesis and transport from the maturing achenes (Perkins-Veazie, 1995). Many metabolic changes take place throughout strawberry fruit growth and ripening, such as the degradation of chlorophyll, the accumulation of anthocyanin, softening, the

metabolism of organic acid and sugars, and the production of flavor compounds.

Along these lines, several recent studies of the molecular aspects of strawberry fruit ripening have been performed, determining the expression patterns of genes encoding already known enzymes involved in cell wall hydrolysis and softening, including pectate lyases (Benítez-Burraco et al., 2003), polygalacturonases (Redondo-Nevado et al., 2001; Aharoni and O'Connell, 2002), pectin methyl esterases (Aharoni and O'Connell, 2002), cellulases (Harpster et al., 1998; Manning, 1998; Trainotti et al., 1999), β-galactosidases (Trainotti et al., 2001), and expansins (Civello et al., 1999; Harrison et al., 2001). In addition, genes corresponding to flavonoid biosynthesis enzymes that catalyze steps in the production of anthocyanins in strawberry have been isolated (Manning, 1998; Moyano et al., 1998; Aharoni et al., 2002), and the genes encoding enzymes of the lignification pathway have been cloned and their expression patterns characterized throughout fruit development and ripening (Aharoni and O'Connell, 2002; Blanco-Portales et al., 2002).

Strawberry fruits are consumed for their pleasant flavor as well as their nutrient content (Hancock, 1999). Strawberry flavor is extremely popular as part of the fruit or as an added flavoring in many manufactured foodstuffs (Hancock, 1999). The volatile components of strawberry fruit formed during ripening have been intensively studied, and >360 volatiles have been identified (Honkanen and Hirvi, 1990; Latrasse, 1991). The relative abundance

of individual volatiles is a fingerprint of a particular cultivar and species. The relationship between the aroma impact volatiles and strawberry varieties has been the subject of numerous investigations (Douillard and Guichard, 1989, 1990; Larsen and Poll, 1992; Larsen et al., 1992). Because volatiles differ in their organoleptic properties, relatively few of these are likely to contribute significantly to flavor. By application of the aroma value concept (i.e., the ratio of concentration to odor threshold), it was shown that only ∼15 odor-active compounds make an important contribution to strawberry flavor (Schieberle and Hofmann, 1997). Studies have demonstrated that strawberry aroma is the result of the combined perception of fruity (ethyl butanoate, ethyl hexanoate, and methyl 2-methylbutanoat) green (Z-3-hexenal), sweaty (butanoic acid and 2-methylbutanoic acid), peach-like (γ-decalactone), and caramel-like [4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF; Furaneol) and 2,5-dimethyl-4-methoxy-3(2H)-furanone (DMMF)] flavor notes (Pyysalo et al., 1979; Larsen et al., 1992). Among these volatiles, HDMF (Figure 1) is the most important because of its high concentration (up to 55 mg/kg strawberry fruit fresh weight) (Larsen et al., 1992) and low odor threshold (10 ppb) (Schwab and Roscher, 1997).

HDMF was first reported as a product of the Maillard reaction (Hodge et al., 1963), and it was then isolated from pineapple (*Ananas comosus*) (Rodin et al., 1965), strawberry (Ohloff, 1969), and a number of different fruits (Schwab and Roscher, 1997). The

methyl ether DMMF, first detected in pineapple (Willhalm et al., 1965), was also identified as an aroma component of a multitude of fruits, such as overripe strawberry, mango (*Mangifera indica*), and arctic bramble (*Rubus arcticus*) (Roscher et al., 1997; Schwab and Roscher, 1997). To date, both furanones have been found only in fruits, being either not present or not detectable in roots, stems, leaves, flowers, or other plant parts (Schwab and Roscher, 1997). In strawberry fruit, HDMF is further metabolized to its β-D-glucopyranoside and subsequently to the malonylated derivative of HDMF glucoside (Figure 1) (Roscher et al., 1996). Enantiomeric analyses have shown that HDMF and DMMF occur as racemates in different fruits (Bruche et al., 1991), and studies of the keto-enol-tautomerism of HDMF have demonstrated its rapid racemization at pH 7.0 (Raab et al., 2003a).

Although exhaustive information regarding strawberry volatile composition is available, few detailed biochemical and genetic studies have been done in relation to aroma biosynthesis. Only recently, it was shown that the *SAAT* (for strawberry alcohol acyl-CoA transferase) and *FaNES* (for *Fragaria* × *ananassa* nerolidol synthase) genes are involved in strawberry fruit ester and terpene formation, respectively (Aharoni et al., 2000, 2004) and that *FaOMT* (for *Fragaria* × *ananassa* O-methyltransferase) encodes an O-methyltransferase responsible for DMMF biosynthesis (Lavid et al., 2002; Wein et al., 2002). The first indications for the enzymatic formation of HDMF in strawberry fruit were provided by studies demonstrating the correlation of fruit ripening stage and HDMF concentration (Sanz et al., 1995). Incorporation experiments showed that, after the application of 15 different water-soluble, radioactively labeled substances, D-[U-^{14}C]fructose-1,6-diphosphate had the highest incorporation rate into the furanone structures (Roscher et al., 1998), followed by D-[U-^{14}C]fructose, D-[U-^{14}C]glucose-6-phosphate, D-[U-^{14}C]glucose, and [2-^{14}C]dihydroxyacetone, in decreasing order. Further incorporation experiments with D-[U-^{13}C]fructose proved the transformation of the complete carbon chain of D-fructose into HDMF (Schwab, 1998), and studies with D-[2-^{2}H]glucose demonstrated the involvement of phosphohexose isomerase in the conversion of D-glucose to the furanones, thus confirming the proposal that D-fructose-6-phosphate is a natural precursor of HDMF and DMMF (Wein et al., 2001). Even though the detailed biogenetic pathway from D-fructose-1,6-diphosphate to HDMF remained unknown, all studies have indicated that HDMF is derived from carbohydrate metabolism.

In this study, we report the isolation and characterization of an enzyme involved in the transformation of D-fructose-1,6-diphosphate to HDMF, one of the key aroma compounds in strawberry fruit. Sequence homology of the isolated enzyme with the protein sequence of a recently sequenced cDNA led to the cloning and characterization of the fruit ripening–induced *Fragaria* × *ananassa* quinone oxidoreductase gene (*FaQR*). On the basis of the observed reaction catalyzed by the heterologously expressed FaQR protein, an unknown intermediate of the HDMF biosynthetic pathway was identified as a natural substrate for the new enzyme. Gene sequence analyses and determination of expression patterns, as well as laser capture microdissection (LCM) followed by RNA extraction and amplification, prompt us to suggest that FaQR is the last enzyme in the biosynthetic pathway leading to HDMF.
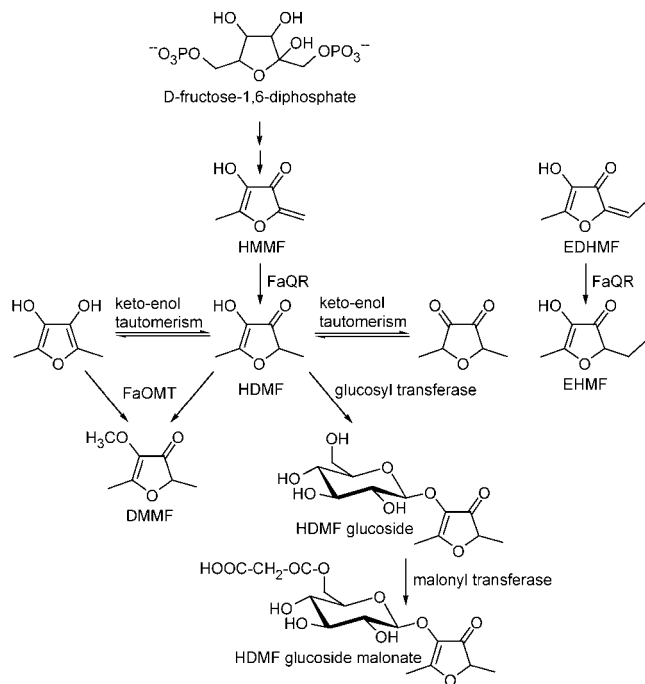

Figure 1. Reactions Catalyzed by FaQR and the Metabolism of HDMF in Strawberry Fruit.

Enzymatic conversion of 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone (HMMF) to 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) and of (2E)-2-ethylidene-4-hydroxy-5-methyl-3(2H)-furanone (EDHMF) to 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone (EHMF).

RESULTS

Development of an Assay for the Determination of HDMF-Forming Activity

A crude protein extract was obtained by homogenizing ripe strawberry fruit with water after the addition of polyvinylpolypyrrolidone in a mixer followed by pH adjustment to 7.0 and two centrifugation steps. Naturally occurring HDMF was removed by dialysis (exclusion limit, 10 kD) against a phosphate buffer solution, pH 7.0. Standard activity assays consisted of the dialyzed crude strawberry protein extract, D-fructose-1,6-diphosphate, as HDMF precursor (Roscher et al., 1998) and the reducing agent NADH, because the conversion involves a reduction step. After 24 h of incubation, a solid-phase extraction was performed and the products eluted by diethyl ether were analyzed by HPLC-UV (285 nm), HPLC–diode array detection (DAD), and HPLC–mass spectrometry (MS). A single compound was detected showing the same retention time, UV spectrum, and liquid chromatography (LC)-MS and product ion spectra as commercially available HDMF (Figure 2). The product ion spectrum of m/z 170 $[M + H + CH_3CN]^+$ displayed a characteristic signal of m/z 129, corresponding to the pseudomolecular ion $[M + H]^+$ of HDMF (Figure 2A). The exclusive formation of HDMF could be demonstrated in incubation experiments after the addition of NADH. However, because small amounts of HDMF were also formed when D-fructose-1,6-diphosphate was incubated with NADH in a buffer solution, indicating a chemical side reaction (Hauck et al., 2002), we tested for evidence of enzymatic HDMF formation. Successive dilutions of the crude protein extract followed by incubation with equal amounts of D-fructose-1,6-diphosphate and NADH revealed a direct correlation between protein content and the amount of HDMF formed that converged on a minimum
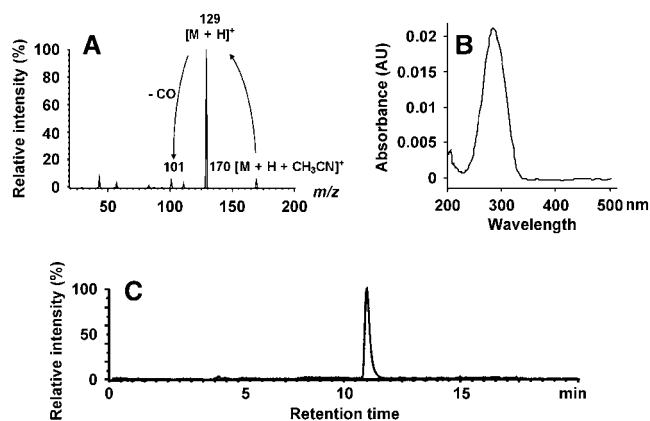

Figure 2. HPLC-DAD-Electrospray Ionization (ESI)-MS/MS Analysis of a Dialyzed Cytosolic Protein Extract Obtained from Ripe Strawberry Fruit after 24 h of Incubation with D-Fructose-1,6-Diphosphate and NADH at 30°C.

Only one compound was detected in the UV chromatogram at 285 nm, and it was identified as HDMF on the basis of its product ion spectrum **(A)**, UV spectrum **(B)**, and retention time **(C)** compared with the synthetic reference. AU, absorbance units.

level corresponding to chemical HDMF formation. Complete inhibition of HDMF formation, apart from the minimum level, was achieved by thermal treatment of the dialyzed protein extract before incubation. Finally, enzymatic activity was confirmed by the demonstration of the formation of enantiomerically enriched HDMF. Recently, it was shown that the rate of HDMF racemization is minimal at slightly acidic pH values (Raab et al., 2003a). HDMF formed in incubation experiments at pH 7.0 and 5.0, at which the enzyme is still active, was analyzed using a newly developed cyclodextrin-modified capillary electrophoresis analysis method (Raab et al., 2003b). A distinct enantiomeric excess of 32% for the (−)-enantiomer was demonstrated at pH 5.0, whereas HDMF formed at pH 7.0 was racemic.

Characterization of the Native HDMF-Forming Activity

A temperature optimum of 37°C and a broad pH optimum peaking at pH 7.0 were determined for the HDMF-forming enzymatic activity. Values greater than pH 8.0 and less than pH 4.0 resulted in the complete inhibition of HDMF synthesis over chemical baseline. At pH 5.0, the extract still showed ∼70% of its activity at pH 7.0. The formation of HDMF displays a two-substrate reaction, in which the kinetics are dependent on the concentrations of D-fructose-1,6-diphosphate as well as NADH. The apparent K_m was determined graphically from double reciprocal plots to be 3.5 mM for D-fructose-1,6-diphosphate and 30 μM for NADH. Although the K_m value for NADH is in the expected order of magnitude, the value for D-fructose-1,6-diphosphate is high for an enzymatically catalyzed reaction (Braunschweig Enzyme Database, Cologne University Bioinformatics Center). Thus, we assumed that D-fructose-1,6-diphosphate first forms an intermediate in a rate-limiting process that is then enzymatically transformed by an oxidoreductase.

HDMF-forming activity was also studied during the course of fruit ripening. Extracts of three different ripening stages—green, pink (turning), and red—were prepared and incubated in a standard assay. HDMF could not be detected after incubation with the green extract. The pink extract (turning stage), however, showed 60% of the activity observed in the red extract from ripe berries, indicating a ripening-induced increase of enzymatic activity similar to the increase in HDMF concentration during ripening (Figure 3A) (Pérez et al., 1996). Incubation experiments with NADPH instead of NADH yielded ∼80% of the amount of HDMF compared with NADH. Addition of Zn^{2+} ions did not increase the enzymatic activity, and the UV spectrum of the enzymatically active recombinant enzyme did not show the typical maximum of flavin-containing proteins at 450 nm (Laskowski et al., 2002).

Purification and Sequencing

The first purification step was accomplished using ultrafiltration membranes with defined exclusion limits of 10, 30, 50, and 100 kD. Activity (79% of total) was recovered in the molecular mass fraction between 30 and 50 kD. However, small amounts of activity (18%) were also detected in the fraction between 10 and 30 kD, indicating a native molecular mass slightly higher than 30 kD for the target protein. The desalted and concentrated
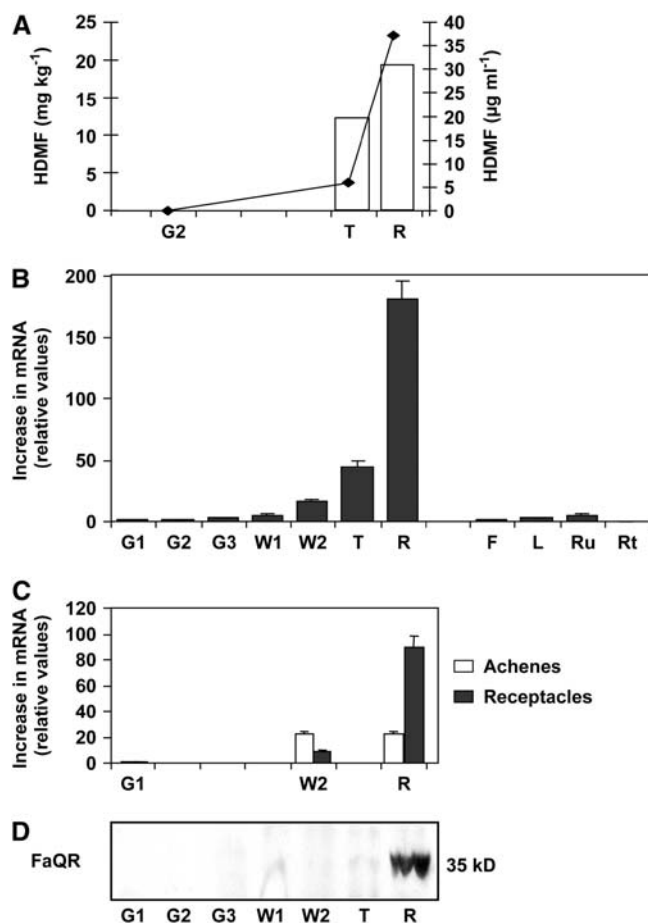
Figure 3. HDMF Content, HDMF-Forming Enzymatic Activity, and Developmental and Spatial Expression Patterns of the *FaQR* Gene in Strawberry.

(A) Concentration of HDMF (mg/kg) in extracts obtained from strawberry fruits of different ripening stages (line), and concentration of HDMF (µg/mL) formed by dialyzed strawberry protein extracts of different ripening stages in incubation experiments with D-fructose-1,6-diphosphate and NADH (bars).

(B) Relative gene expression analysis by QRT-PCR at the different fruit ripening stages (G1, G2, G3, W1, W2, T, and R; see Methods) and in roots (Rt), leaves (L), flowers (F), and runners (Ru). Mean values ± SD of five independent experiments are shown.

(C) Expression studies by QRT-PCR in both strawberry receptacle and achene tissues corresponding to G1, W2, and R stages. Real-time quantification is based on threshold cycle (C_t) values as described in Methods. The relative gene expression was determined using an 18S–26S interspacer gene as an endogenous control gene. The increase in mRNA value was relative to the G1 C_t value of *FaQR*. Mean values ± SD of five independent experiments are shown.

(D) Protein gel blot of proteins extracted from fruits in different growth and ripening stages. The blot was probed with a 1:250 dilution of antiserum raised against strawberry *FaQR*. Each lane contained 100 µg of protein electrophoresed on a 10% SDS-PAGE gel. The size of the hybridizing peptide is indicated in kilodaltons.

molecular mass fraction between 10 and 50 kD was subjected to further purification using gel-permeation chromatography on Sephacryl S300 as well as ion-exchange chromatography on Q-Sepharose FF. Fractions displaying enzymatic activity by both gel-permeation and ion-exchange chromatography were separated by SDS-PAGE. The observed distribution of activity correlated with the presence of a single protein band with a molecular mass of ∼37 kD. The 37-kD band was excised and analyzed for its N-terminal amino acid sequence by automated Edman degradation. Because of N-terminal blocking, the protein was selectively cleaved with cyanogen bromide at Met residues and analyzed by a PAGE method (Schägger and von Jagow, 1987). However, cleavage of the protein, purified under reducing conditions, was incomplete. Only a few peptide fragments were formed by acid-catalyzed hydrolysis as a result of the applied reaction condition (formic acid). Two of these were sequenced by automated Edman degradation, and 7 out of a sequence of 10 as well as 4 out of a sequence of 5 amino acids were unambiguously identified. The obtained sequences showed total identity with the corresponding protein sequence (Figure 4) of a strongly ripening-induced putative gene (*FaQR*) recently isolated from strawberry fruit (GenBank accession number AY048861). Framed amino acids in Figure 4 denote the sequences obtained by automated Edman degradation of the 37-kD native strawberry enzyme. A similar sequence (FvQR) was obtained from *Fragaria vesca* (GenBank accession number AJ001445).

FaQR Has Sequence Similarity to Auxin-Regulated and Quinone Oxidoreductase Genes

The full-length *FaQR* cDNA was isolated, and comparison with the corresponding genomic sequence of the *FaQR* gene revealed the presence of three introns and four exons in this gene. Computer comparisons of the genomic and cDNA nucleotide sequences with other known sequences (GenBank, EMBL, Protein Information Resource [PIR], and SwissProt) revealed a statistically significant identity of this strawberry gene sequence with another gene from *F. vesca* that encodes a ripening-induced protein and with other previously cloned and characterized genes encoding auxin-induced proteins of higher plants. These identities range from 98 to 69% at the amino acid level (Figure 4).

The strawberry *FaQR* cDNA is 1187 bp long and agrees with the estimated size of the mRNA detected in RNA gel blot hybridization experiments, indicating that this cDNA is probably a full-length cDNA. The insert consists of a 969-bp open reading frame that encodes a 322–amino acid long protein with a calculated molecular mass of 34.3 kD and a pI of 6.2.

The predicted amino acid sequence of the deduced FaQR protein contains a typical NAD(P)H binding site (GXXGXXG) (Edwards et al., 1996). Sequence comparison of FaQR with ζ-crystallin and *Escherichia coli* quinone oxidoreductase (QOR) shows that the typical amino acid residues (ζ-crystallin His-200 and His-317), supposedly essential for the binding of the phosphate group in NADP(H), are replaced by Tyr-216 and Arg-312 in FaQR as in QOR (Tyr-192 and Arg-317) (Edwards et al., 1996). The His residues are less flexible than the Tyr and Arg counterparts. The greater adaptability may allow QOR to bind both NADPH and NADH, as was observed for FaQR. The arrows in
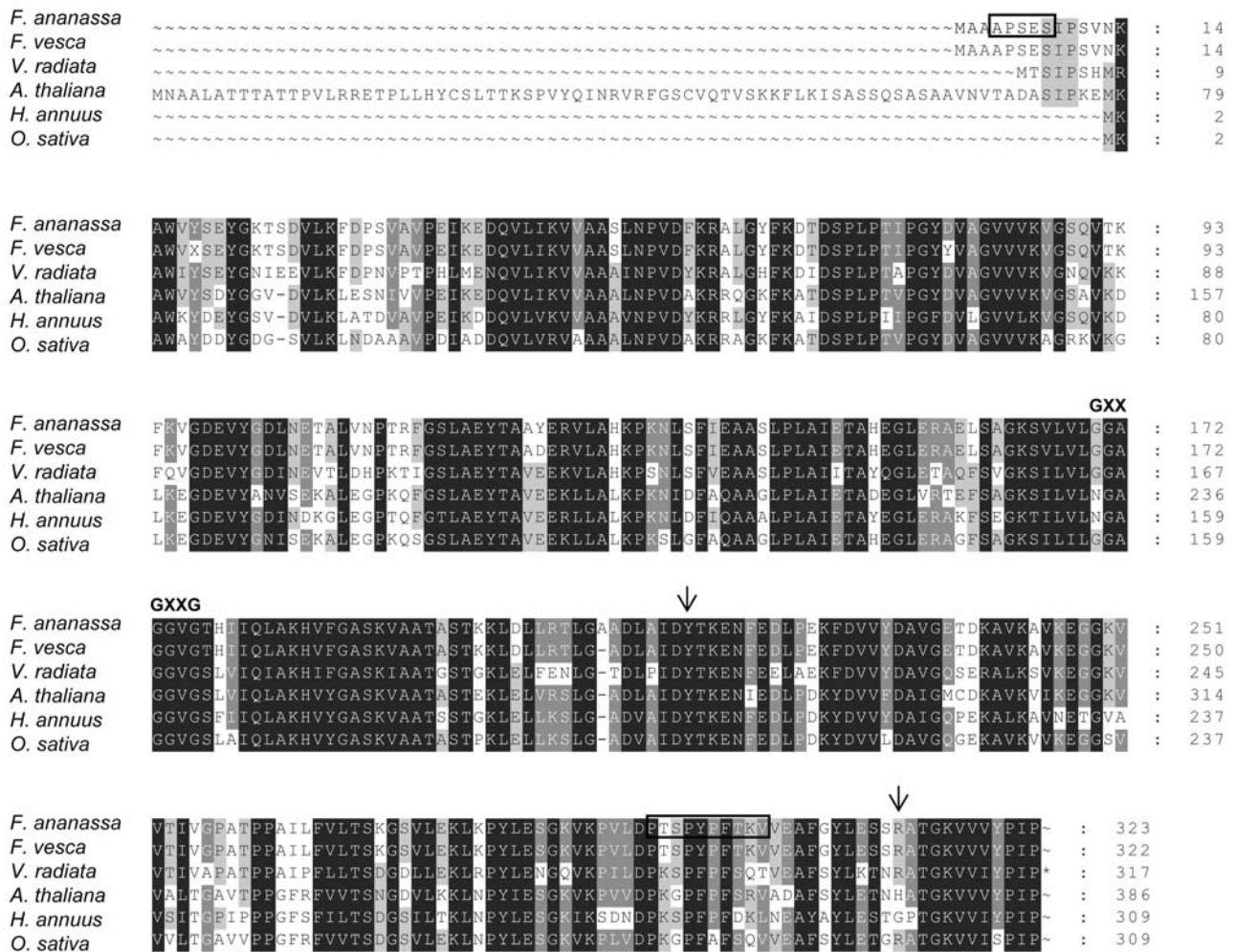
Figure 4. Amino Acid Sequence Comparison of the Predicted Strawberry FaQR Protein and Putative Quinone Oxidoreductases from Higher Plants.

The sequences were aligned using the ClustalW program. Consensus amino acids are shaded. The NAD(P)H binding motif GXXGXXG is marked.

Figure 4 show Tyr-216 and Arg-312, which probably allow the binding of both NADPH and NADH. Although the active-site motif of Zn-containing alcohol dehydrogenases (Edwards et al., 1996) is also partially conserved in the *FaQR* gene, a key His residue located at the active site of all alcohol dehydrogenases is absent, and the sequence contains no Cys residues for the binding of catalytic Zn. These features have also been observed in lens

ζ-crystallin and *E. coli* QOR-related reductases that lack a Zn binding site and do not have alcohol dehydrogenase activity (Edwards et al., 1996), and similar motifs were found in the amino acid sequence of the 2.3R1 polypeptide of *F. vesca* (Nam et al., 1999). The FaQR protein is probably not a flavodoxin quinone reductase, because the 17–amino acid flavodoxin signature sequence [LIV]-[LIVFY]-[FY]-x-[ST]-x-x-[AGC]-x-T-x-x-x-A-x-x-[LIV]

(Laskowski et al., 2002) does not match the protein deduced sequence from the *FaQR* cDNA. Finally, using the National Center for Biotechnology Information Conserved Domain Search program, we compared the deduced FaQR protein with domains included in the database. We found significant sequence similarities with domains corresponding to the NAD(P)H quinone oxidoreductase motif (COG0604) and the Zn binding dehydrogenase motif (pfam 00107). Together, these results strongly suggest that *FaQR* encodes a putative NADH-dependent quinone oxidoreductase.

FaQR Is a Fruit Ripening–Related Gene

The spatial and temporal expression patterns of the *FaQR* gene were studied using quantitative real time (QRT)-PCR. Transcript amount increased throughout ripening, peaking in totally mature, red fruit receptacles (with achenes; R stage) (Figure 3B). *FaQR* is expressed in the late stages of fruit growth and ripening, both in achene and receptacle tissues, with a reduced expression level but similar expression pattern observed in achenes (Figure 3C). A protein gel blot of proteins extracted from different stages of fruit growth and ripening was probed using an antiserum raised against the FaQR protein. A major 35-kD cross-reacting polypeptide present only in red fruit was observed that coincides with the expected size range of the deduced FaQR protein (Figure 3D).

FaQR Expression Correlates Inversely with Auxin Levels

Because some strawberry fruit-specific genes have been shown to be expressed under the control of auxins (Medina-Escobar et al., 1997; Manning, 1998; Moyano et al., 1998; Trainotti et al., 1999; Aharoni et al., 2002; Blanco-Portales et al., 2002), we performed gene expression analysis on deachened green fruit (G2 stage) to determine whether the expression of *FaQR* was also under this hormonal control. QRT-PCR analyses were performed with mRNA isolated from deachened fruits and from deachened fruits treated with the auxin naphthalene acetic acid (NAA). The clear increase in *FaQR* gene expression detected in fruits at 4 d after removing the achenes (65-fold compared with untreated control fruits) was partially reversed by treatment with NAA (11-fold), implying that auxin levels correlate inversely with *FaQR* gene expression.

Identification of *FaQR* Expression in Parenchyma Tissues of Red Fruit

RNA was extracted from parenchyma and from vascular cells individually collected via LCM (Figures 5A to 5E). RNA from the second round of T7-based RNA amplification was analyzed by RT-PCR using specific primers corresponding to the *FaQR* gene. We found that *FaQR* mRNA was amplified exclusively from RNA obtained from the red fruit parenchyma. No amplification from vascular RNA was observed. However, the control interspacer RNA was amplified in RNA from both parenchyma and vascular tissues (Figure 5F). The primers used to amplify the *FaQR* transcript produced the expected 294-bp product from RNA isolated from parenchyma cells. The fact that the primer set used for *FaQR* RT-PCR spans an intron region of the *FaQR* gene,

generating a product of 544 bp using genomic DNA as a template, allowed us to confirm that the RT-PCR product obtained in the amplification reaction derived from parenchyma cell RNA transcripts rather than from contaminating strawberry genomic DNA.

FaQR Protein Shows *o*-Quinone Oxidoreductase Activity and Forms HDMF

FaQR protein was heterologously expressed in *E. coli* as a His-tagged fusion protein. As a result of the results of the similarity search, quinone oxidoreductase activity of the recombinant protein was first determined spectrophotometrically after the addition of 1,2- or 1,4-quinones and NADH. HDMF-forming activity was confirmed with the addition of D-fructose-1,6-diphosphate and NADH. Increases in FaQR level correlate with increases in the specific activity of the preparation. Quinone oxidoreductase activity was also observed in the extracts obtained from *E. coli* containing the empty vector, revealing that native *E. coli* QOR activity was probably not completely separated by His tag affinity chromatography with the Co^{2+}-containing column. Thus, ultrafiltration was applied as an additional purification step to isolate proteins of the molecular mass range from 30 to 50 kD (Figure 6). Because *E. coli* QOR is a dimeric protein with 35-kD subunits, it was completely excluded by the ultrafiltration step, as confirmed by activity assays (Thorn et al., 1995). Although FaQR from strawberry fruit reduced only the artificial substrate 9,10-phenanthrene quinone ($K_m = 35\ \mu M$) out of a number of 1,2- and 1,4-quinones tested, and formed HDMF, the *E. coli* QOR preparation showed a completely different spectrum of enzymatic activity, accepting many 1,2- and 1,4-quinones and not producing HDMF. This observation also confirms the result obtained using the native strawberry enzyme preparation that the monomeric FaQR is catalytically active.

Natural Precursor of HDMF

The observation that HDMF formation from D-fructose-1,6-diphosphate proceeds very slowly, as 1.5 μg of HDMF is produced from 40 mg of D-fructose-1,6-diphosphate within 6 h by 7.5 μg/mL FaQR solution, and the fact that 9,10-phenanthrene quinone, which has not been described as a strawberry fruit constituent, represents a substrate for FaQR, led us to conclude that D-fructose-1,6-diphosphate is not the actual substrate for FaQR. On the basis of structural similarity to 1,2-quinones and alkenals, we hypothesized that HMMF is the natural precursor of HDMF and is converted by the enzyme (Figure 1). To test this notion, we performed the synthesis of this fairly unstable, α,β-unsaturated compound and confirmed its structure after derivatization with 3-mercaptobenzoic acid by LC-MS and ^{1}H- and ^{13}C-NMR (our unpublished data). The in vitro enzymatic transformation of HMMF to HDMF by FaQR within 30 min was observed using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Figure 7). Similarly, HMMF was detected by LC-MS/MS in incubation solutions containing D-fructose-1,6-diphosphate and in strawberry fruit, but only after derivatization with 3-mercaptobenzoic acid to a stable thioether product (Figure 8). Because of the instability of HMMF in aqueous solution, proper V_{max} and K_m
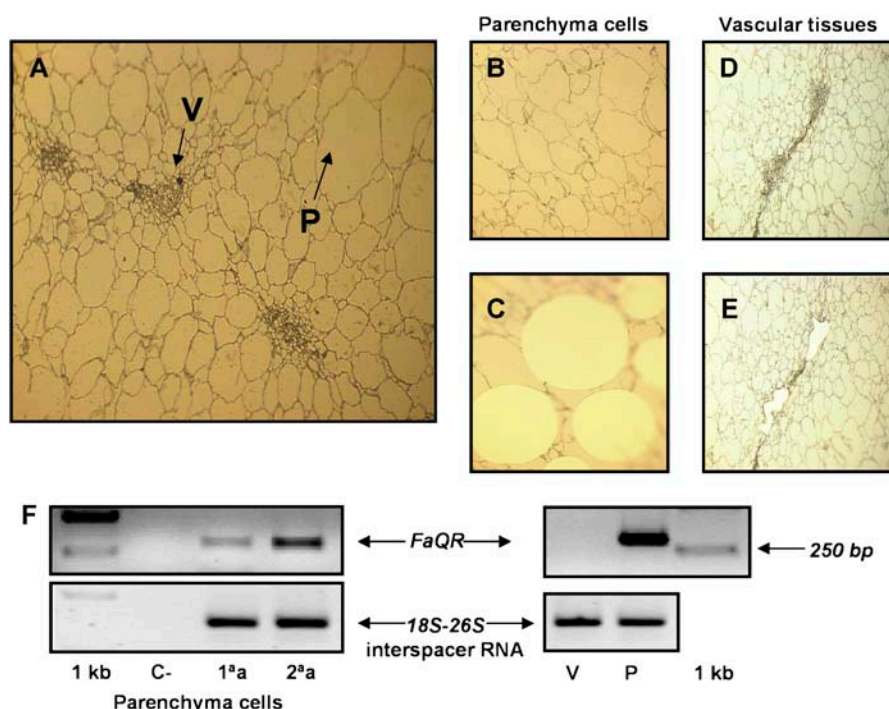
Figure 5. Laser Capture of Parenchyma Cells and Vascular Tissues from Red-Stage Strawberry Fruit.

(A) Tissue section (10 μm) of a red fruit showing vascular tissues (V) and parenchyma cells (P).
(B) and **(C)** Parenchyma cells before laser capture **(B)** and after laser capture **(C)**.
(D) and **(E)** Vascular tissue before laser capture **(D)** and after laser capture **(E)**.
(F) Transcripts of *FaQR* detected by specific RT-PCR analysis from RNA recovered from vascular and parenchyma cells individually isolated by LCM. RT-PCR was performed using specific primers as described in Methods. *FaQR* is a 294-bp specific amplified fragment corresponding to the *FaQR* gene; *18S–26S RNA* is a 150-bp specific fragment of an interspacer control RNA. 1kb, 1-kb DNA ladder; C−, negative control for PCR; 1ªa and 2ªa, RT-PCR analysis of aRNA from parenchyma cells after the first and second rounds of amplification, respectively (see Methods). RT-PCR analysis of aRNA (two rounds of amplification) was from vascular tissue (V) and parenchyma cells (P).

values could not be obtained. However, EDHMF (Figure 1), a substrate analogue, was stable enough, and a K_m of 2.1 ± 0.1 mM and a V_{max} of 56 ± 3.6 nkat/mg could be determined for this compound. These values are similar to those obtained for substrates of other oxidoreductases (Mano et al., 2000, 2002).

Finally, we incubated samples containing FaQR, reducing agent, and D-fructose-1,6-diphosphate in the presence of the trapping reagent 3-mercaptobenzoic acid and without the thiol (Figure 9). As a control, 3-mercaptobenzoic acid was added to a buffered solution containing D-fructose-1,6-diphosphate. In the presence of the thiol, the thioether adduct of HMMF was formed, but addition of FaQR led to significantly reduced levels of the thioether (Figure 9A). By contrast, the highest amounts of HDMF were produced in thiol-free medium, whereas addition of 3-mercaptobenzoic acid reduced the levels of HDMF (Figure 9B). The enzyme is not affected by the applied thiol concentration, as shown by control experiments with 9,10-phenanthrene quinone as substrate. It appears that FaQR shows a higher affinity for HMMF than 3-mercaptobenzoic acid, because the level of the thioether in the sample containing D-fructose-1,6-diphosphate, 3-mercaptobenzoic acid, FaQR, and NADH remained almost constant after 300 min of incubation, whereas the concentration of HDMF still increased.

DISCUSSION

The physiological function of plant quinone oxidoreductases in developmental processes has been little studied, and to date no specific function for this class of proteins has been discovered except for an alkenal/one oxidoreductase (Mano et al., 2002; Dick and Kensler, 2004). Here, we present the isolation and biochemical and molecular characterization of a strawberry fruit ripening–related protein and its corresponding gene, exhibiting identity with plant quinone oxidoreductase enzymes and genes, respectively. Our results suggest the involvement of the enzyme in the biosynthesis of the important strawberry flavor compound HDMF. On the basis of the catalyzed reaction, an intermediate of the biosynthetic pathway was postulated and confirmed by chemical synthesis and detection in strawberry fruit.

It has been proposed that the auxins are the primary hormones controlling strawberry fruit ripening (Perkins-Veazie, 1995). A gradual decline in the supply of auxin from achenes in the later stages of fruit growth (the transition between green and white fruits) has been associated with strawberry fruit ripening (Perkins-Veazie, 1995). Indeed, a direct correlation has been shown between an increase in the expression of strawberry ripening–related genes and a decrease in auxin (Trainotti et al., 1999;
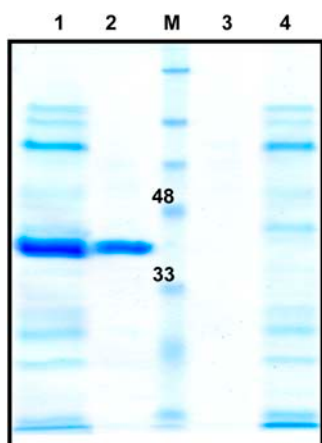
Figure 6. SDS-PAGE Analysis of Recombinant FaQR.

FaQR purified by His tag affinity chromatography (lane 1) and subsequent ultrafiltration to isolate the proteins of the molecular mass range from 30 to 50 kD (lane 2), as described in Methods. M, molecular mass markers in kD. Proteins obtained from *E. coli* cells transformed with the empty vector were also subjected to His tag affinity chromatography (lane 4) and subsequent ultrafiltration (lane 3), which completely removed native *E. coli* quinone oxidoreductase still present in the eluate obtained by His tag affinity chromatography.

Aharoni et al., 2002; Blanco-Portales et al., 2002; Benítez-Burraco et al., 2003). Similarly, strawberry *FaQR* expression correlates inversely with auxin levels. Thus, the high gene expression levels found during strawberry ripening stages and the fact that *FaQR* expression increases in correlation with decreasing auxin levels show clearly that *FaQR* is included in the fruit ripening expression pattern of strawberry.

At the growth stages of fruit development, the expression of the strawberry *FaQR* gene is low, but it increases continuously throughout the fruit ripening stages. The maximum amount of transcript and the maximum enzymatic activity of the native enzyme were found in full-ripe red fruits. Accordingly, the concentrations of HDMF and its derivatives DMMF and HDMF glucoside constantly increase during strawberry ripening (Pérez et al., 1996; Wein et al., 2002). Maximum amounts of furanones are formed at the overripe stage. *FaQR* is expressed in the parenchyma of the fruit and correlates with the detection of high HDMF levels in the fleshy dark-red cortex (Sen et al., 1991). Similarly, in pineapple, a fruit also producing HDMF (Schwab and Roscher, 1997), normal ripening and senescence were characterized by an increase in NADH quinone reductase activity (Soler et al., 1993). *FaQR* mRNA was also detected in the achenes, but its function there remains unknown.

Quinone reductases catalyze the univalent or divalent reduction of quinones (Q) primarily to produce semiquinone radicals or quinols, respectively (Thorn et al., 1995; Ross, 1997; Matvienko et al., 2001). Plant NAD(P)H quinone reductase [NAD(P)H-QR] uses either NADH or NADPH as the electron donor and a hydrophilic quinone as the electron acceptor. NAD(P)H-QRs constitute one of eight families belonging to the superfamily of medium-chain dehydrogenases/reductases (Nordling et al.,

2002). The family containing NAD(P)H-QRs is variable, implying diverse functions in metabolic conversions. NAD(P)H-QR appears to be the only soluble plant cell enzyme that produces fully reduced quinols (Trost et al., 1995). However, a dual mode of reaction, univalent reduction of quinones and divalent reduction of azodicarbonyls, has been shown for the enzyme P1-ZCR, a ζ-crystallin quinone oxidoreductase from *Arabidopsis thaliana* (Mano et al., 2000, 2002). This enzyme (NADPH:2-alkenal α,β-hydrogenase) also catalyzes the reduction of highly reactive alkenals [e.g., (2E)-hexenal] to less reactive *n*-alkanals (e.g., hexanal) (Mano et al., 2002) and thus resembles FaQR. Both enzymes reduce 9,10-phenanthrene quinone (quinone oxidoreductase) and hydrogenate α,β-unsaturated carbonyl compounds (enone oxidoreductase) to their corresponding saturated derivatives. Although not proven, we assume that the reaction of the two enzymes with the *o*-quinone constitutes a promiscuous activity and is similar to the activities of the ancestor proteins (Aharoni et al., 2005). In both cases, the reduction of the acrolein derivatives leads to the formation of aroma-active volatiles. This reaction is reminiscent of the enoyl reductases, which are capable of catalyzing the reduction of the C=C double bond in the enoyl-ACP. α,β-Unsaturated aldehydes and ketones are electrophilic and capable of reacting, via the Michael addition mechanism, with important cellular nucleophiles, which, in turn, leads to macromolecular (protein, DNA) dysfunction and cell death. Because saturated carbonyls lack this reactive moiety, they are often far less toxic. Because of its reactivity, accurate determination of the kinetic data for HMMF could not be performed, but the corresponding data for the substrate analogue
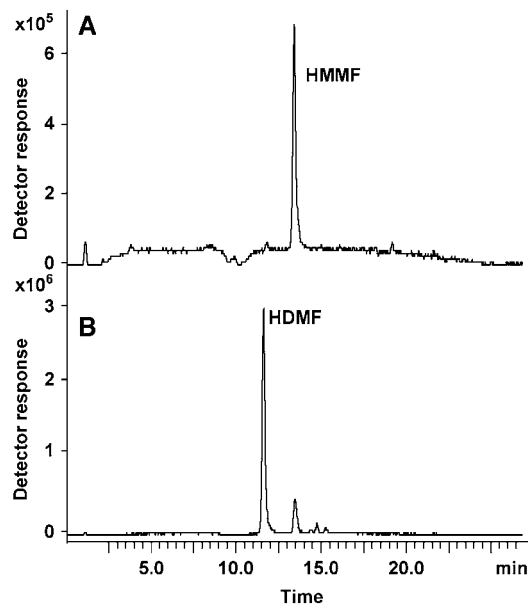

Figure 7. Demonstration of the Formation of HDMF from HMMF by HPLC-ESI-MS/MS.

Chemically synthesized HMMF ($[M + H]^+$; *m/z* 127) **(A)** was transformed by FaQR in the presence of NADH to HDMF ($[M + H]^+$; *m/z* 129) **(B)**. Shown are the ion traces *m/z* 127 to 129 in the positive mode.
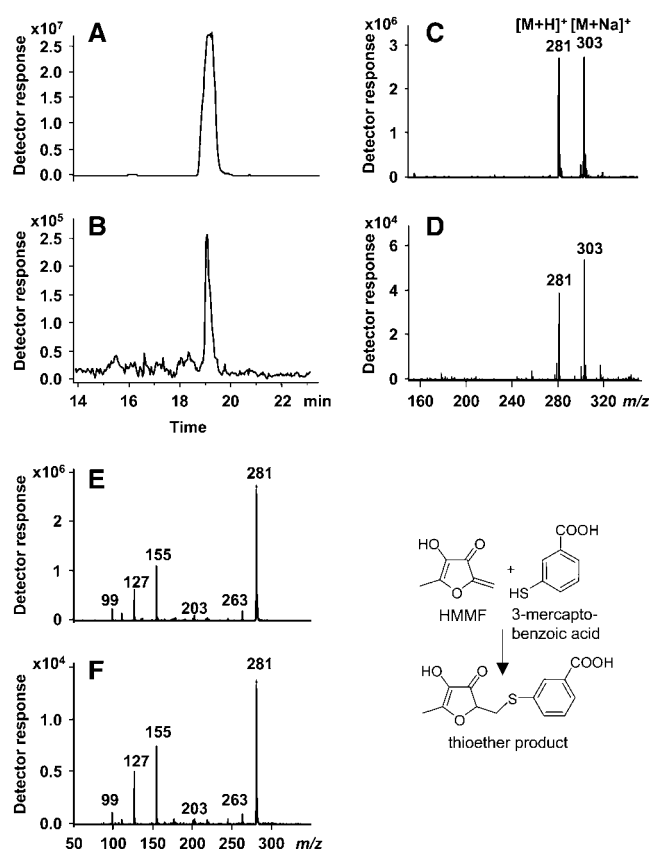
Figure 8. HPLC-ESI-MS/MS Analysis of the Thioether Product Formed from HMMF and 3-Mercaptobenzoic Acid.

(A) and **(B)** Ion traces at m/z 303 of the reference **(A)** and of a diethyl ether extract obtained from strawberry after application of 3-mercaptobenzoic acid (incubation, 72 h at room temperature) followed by solid-phase extraction on XAD-2 **(B)**.
(C) and **(D)** Mass spectra of the reference **(C)** and the compound formed in strawberry eluting at 19 min **(D)**.
(E) and **(F)** Product ion spectra (MS/MS; m/z 281) of the reference **(E)** and the compound formed in strawberry **(F)**.

EDHMF were obtained, indicating that these structures are suitable substrates for FaQR. In addition, we clearly demonstrated the competition of 3-mercaptobenzoic acid and FaQR for the reactive HMMF. The final evidence for the in vivo function of FaQR was provided by the detection of the as yet unknown precursor HMMF (as its thioether derivative) in red ripe strawberry fruit.

In summary, we have isolated and characterized an important protein involved in the biosynthesis of HDMF. Protein sequence analysis allowed the cloning and characterization of the corresponding strawberry gene. An intermediate of the HDMF biosynthetic pathway was deduced from the catalyzed reactions, and the structure of the HDMF precursor was confirmed by chemical synthesis, finally leading to its detection in strawberry fruit. The cDNA corresponds to an mRNA encoding a protein with quinone and enone oxidoreductase enzymatic activities. The

gene expression analyses and LCM studies suggest that the strawberry *FaQR* gene is related to the fruit ripening process and HDMF production. The enone oxidoreductase protein catalyzes the last step in the formation of the important strawberry flavor compound HDMF but acts also on an *o*-quinone. It is assumed but not yet proven that the enzyme's promiscuous activity is similar to the activity of an evolutionarily related enzyme, as has been shown for other proteins (Aharoni et al., 2005).
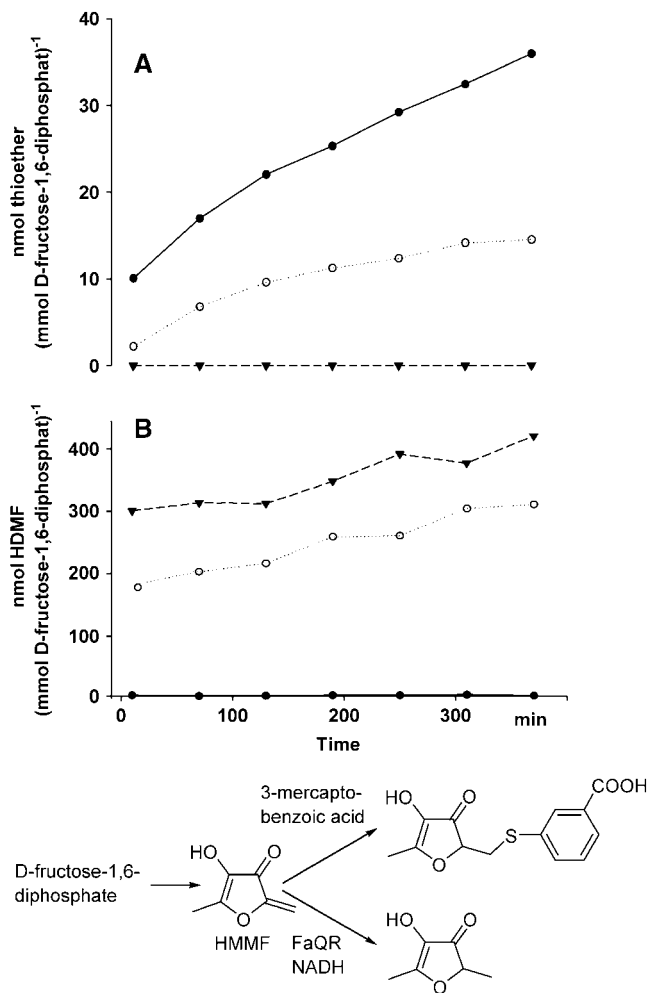

Figure 9. Competition of 3-Mercaptobenzoic Acid and FaQR for HMMF Formed from D-Fructose-1,6-Diphosphate.

(A) A time course experiment showed increasing levels of the thioether when 3-mercaptobenzoic acid was added to D-fructose-1,6-diphosphate (closed circles), whereas the concurrent addition of FaQR and NADH (open circles) led to reduced concentrations. Thioether was not detected when D-fructose-1,6-diphosphate was incubated with only FaQR and NADH (closed triangles).
(B) High concentrations of HDMF were produced when D-fructose-1,6-diphosphate was converted by FaQR in the presence of NADH (closed triangles), whereas concurrent addition of 3-mercaptobenzoic acid (open circles) reduced the concentration of the furanone. FaQR was essential for the formation of significant levels of HDMF (closed circles).

METHODS

Plant Material

Strawberry (*Fragaria* × *ananassa* cv Chandler and Elsanta, octaploid cultivars) fruits were harvested at the following developmental stages: small-sized green fruits (G1), middle-sized green fruits (G2), full-sized green fruits (G3), white fruits with green achenes (W1), white fruits with red achenes (W2), turning stage fruits (T), and full-ripe red fruits (R), immediately frozen in liquid nitrogen, and stored at −80°C.

Preparation of the Clarified Extract

Two hundred grams of red ripe strawberry fruit was homogenized with 20 g of polyvinylpolypyrrolidone and 200 mL of water in a mixer. The pH was adjusted to 7.0 with 1 N NaOH, and the mixture was centrifuged for 30 min at 5000g. The pellet was discarded, and the supernatant was centrifuged a second time for 30 min at 40,000g. The clear solution was dialyzed against a Tris-HCl buffer or a phosphate buffer, pH 7.0 (100 mM) to remove naturally occurring HDMF.

Standard Incubation Assay

One hundred milligrams of D-fructose-1,6-diphosphate and 1 mg of NADH were added to 20 mL of dialyzed cytosolic protein extract. After the addition of 2 mL of 1 M phosphate buffer, pH 7, the solution was adjusted to pH 7.0 using 1 N NaOH or 1 N HCl. The solution was then incubated for ~24 h at 30°C in a shaker. Depending on the experiment, different kinds and amounts of substrates and reduction equivalents were used.

Extraction for HPLC Analysis and HDMF Quantification

The samples were centrifuged and subjected to solid-phase extraction over RP18 cartridges (Supelco; 500 mg/3 mL). After application, the cartridges were rinsed with 2 mL of water and eluted with 3 mL of diethyl ether. Remaining water was removed by freezing the samples to −18°C. The (liquid) diethyl ether was pipetted into a conical 1-mL HPLC vial containing 200 μL of water. The organic solvent was removed under a stream of nitrogen, and HDMF was quantified with an HPLC system equipped with a Basic marathon autosampler (Spark Holland) connected to a Maxistar pump and a variable-wavelength monitor set to 285 nm (both from Knauer). Knauer Eurochrom 2000 software was used for data acquisition and evaluation. The HDMF yields were quantified using a standard curve of commercial HDMF. A Eurospher 100-C18 column (250 mm long × 4 mm i.d.; particle size, 5 μm) (Knauer) was used. A binary gradient starting from 95% A (0.05% formic acid in water) and 5% B (acetonitrile) to 80% A within 10 min, then to 0% A in 30 min, was used at a flow rate of 1.0 mL/min. Injection volume was 20 μL.

Ultrafiltration

The dialyzed extract was separated into fractions containing proteins of different molecular masses (<10 kD, 10 to 30 kD, 30 to 50 kD, 50 to 100 kD, >100 kD) by means of consecutive ultrafiltration with Centricon polycarbonate membranes with exclusion limits of 10, 30, 50, and 100 kD (Amicon). Fractions were analyzed for their HDMF-forming activity using the standard incubation assay.

Chromatography on Q-Sepharose Fast Flow

A Q-Sepharose Fast Flow column (2.6 × 20 cm) was equilibrated with 20 mM phosphate buffer, pH 7.0. After removing naturally occurring HDMF, strawberry extract was applied to the ion-exchange resin and eluted with a linear gradient of 400 mL of 0 to 0.5 M NaCl in 20 mM

phosphate buffer, pH 7.0, at a flow rate of 1.0 mL/min. Fractions (10 mL) were collected and analyzed for HDMF-forming activity.

Size-Exclusion Chromatography on Sephacryl S-300 for Determination of Native Molecular Mass

A Sephacryl S-300 HR fast protein liquid chromatography column (1.6 × 60 cm; Pharmacia) was equilibrated with 20 M phosphate buffer, pH 7.0. Desalted and concentrated protein solutions were applied to the column. Proteins were eluted with 20 mM phosphate buffer, pH 7.0, at a flow rate of 0.2 mL/min. Fractions (2 mL) were collected and tested for activity. Retention times were compared with those of the following gel filtration markers (Amersham Pharmacia Biotech): thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), chymotrypsinogen (25 kD), and ribonuclease A (14 kD). Elution was monitored at 280 nm.

SDS-PAGE

A Bio-Rad Mini-Protean II device was used. Desalted and lyophilized samples were dissolved in 20 μL of sample buffer (Bio-Rad) and 0.4 μL of mercaptoethanol, heated in a boiling-water bath for 3 min, cooled, and applied to Ready Tris-Glycine gels (16.5%; Bio-Rad). Electrode buffer concentrate was purchased from Bio-Rad and diluted before use. Electrophoresis was performed with a voltage of 100 V. Once completed, separation gels were stained in a solution of Coomassie Brilliant Blue R 250 (0.1%).

Internal Sequencing

Desalted and concentrated protein fractions exhibiting activity were resolved on an 8% (w/v) SDS-PAGE gel of 0.75 mm thickness under reducing conditions. The molecular mass of the protein band of interest was determined to be 37 kD. The band was excised from the gel and treated with formic acid and 5% (w/v) cyanogen bromide overnight at room temperature. The eluted peptides were resolved on a gel according to the method of Schägger and von Jagow (1987). After electrotransfer on a polyvinylidene difluoride membrane and staining with Coomassie Brilliant Blue R 250, bands were excised and sequence analysis was performed by automated Edman degradation with a 476A pulsed-liquid-phase protein sequencer (Applied Biosystems). The phenylthiohydantoin derivatives of amino acids were separated and identified with an online phenylthiohydantoin analyzer on a phenylhydantoin-C18 column. The peptide sequences obtained were subjected to homology searches using the public protein sequence databases (SwissProt, PIR, Protein Research Foundation, Nonredundant Protein Sequence Database, GenBank, and EMBL).

Capillary Electrophoresis

Electrophoresis was performed according to Raab et al. (2003b) with a Beckman P/ACE system for methods development and quality control applications equipped with a multiwavelength-variable UV detector. A fused silica capillary with a total length of 40.2 cm, a detection length of 30 cm, and an i.d. of 50 μm was used. Samples were pressure-injected into the anodic end of the capillary for 10 s and separated at 25°C using a constant voltage of 10 kV. The detection wavelength was 280 nm. Ammonium acetate (50 mM), pH 4.0, containing 20 mM heptakis-(2,3-diacetyl-6-sulfato)-β-cyclodextrin was used as a running buffer. The capillary was washed between runs with 0.1 M NaOH (2 min) and running buffer (2 min).

HPLC-ESI-MS/MS Analysis

HPLC-ESI-MS/MS was performed using a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT) and an Applied Biosystems 140b pump. Data acquisition and evaluation were

conducted on a DEC 5000/33 (Digital Equipment) using Finnigan MAT ICIS 8.1 software. HPLC separation with mass spectrometry detection was performed on a Knauer Eurospher-100 C18 column (100 mm long × 2 mm i.d.; particle size, 5 μm) using a binary gradient starting from 95% A (0.05% trifluoroacetic acid in water) and 5% B (acetonitrile) to 80% A within 10 min, then to 0% A in 30 min, at a flow rate of 0.2 mL/min. Mass spectra were acquired in the positive ion mode. For pneumatic assisted ESI, the spray voltage was set to 3.5 kV and the temperature of the heated capillary was 210°C. Nitrogen served both as sheath (70 p.s.i.) and auxiliary gas (10 units). Product ion scanning was performed at a collision gas pressure of 2.0 millitorr and a collision energy of 25 eV, with a total scan duration of 1.0 s for a single spectrum.

HPLC-DAD

For HPLC analysis with DAD, a Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector were used, including Hewlett-Packard chemstation software for data acquisition and evaluation. A Eurosher 100-C18 column (250 mm long × 4 mm i.d.; particle size, 5 μm) (Knauer) was used.

Strawberry Quinone Oxidoreductase cDNA and Gene Cloning

The cloning and isolation of the full-length strawberry cDNA quinone oxidoreductase (*FaQR*) was performed by differential screening of a cDNA subtractive library (red stage versus green stage) according to Medina-Escobar et al. (1997). The isolation of the strawberry quinone oxidoreductase genomic clones was performed by plaque hybridization screening of ~1.5 × 10⁵ plaque-forming units of a strawberry genomic library (*Fragaria × ananassa* cv Chandler) in the phage λ-FIX II (Stratagene), using the ³²P-labeled *FaQR* cDNA previously isolated from strawberry as a probe. Filters were prehybridized and hybridized at 65°C in the following hybridization solution: 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 200 μg/mL salmon sperm, and 0.5% SDS. After hybridization, filters were washed twice for 15 min at room temperature in a 0.2× SSC, 0.5% SDS solution. Afterwards, the filters were washed for 15 min at 65°C in a 0.2× SSC, 0.1% SDS solution. Seventeen positive clones ranging between 12 and 17 kb were isolated, and their inserts were analyzed by restriction mapping and DNA gel blot hybridization using the *FaQR* cDNA as a probe. Suitable clones for DNA sequencing were obtained by subcloning into pBluescript SK– vector, and their DNA inserts were completely sequenced in both strands.

Bioinformatic Analysis of Sequences

Sequences were compared with the GenBank and EMBL nucleic acid databases and the PIR and SwissProt databases using the BESTFIT, DOTPLOT, and PRETTYBOX programs. Additionally, the DNA sequences were analyzed for open reading frames using the GENEBUILDER program (Milanesi et al., 1999). The amino acid sequence was analyzed using programs from the University of Wisconsin Genetic Computer Group package (Devereux et al., 1984). PLACE (Higo et al., 1999) and MATINSPECTOR (Quandt et al., 1995) databases were used as a tool for homology searches of nucleotide sequence motifs.

Auxin Treatments

Achenes of two sets of G2-stage strawberry fruits were carefully removed from the developing fruit using the tip of a scalpel blade. One set of deachened fruits was treated with the synthetic auxin NAA as a lanoline paste, with 1 mM NAA in 1% (v/v) DMSO. The other set of deachened fruits was treated with the same paste but without NAA. Fruit samples were harvested at 0, 24, 48, 72, and 96 h after treatment, immediately frozen in liquid nitrogen, and stored at −80°C. Untreated fruits served as controls.

RNA Isolation and Gene Expression Analysis

Total RNA from a pool of six to seven strawberry fruits at different developmental and ripening stages, subjected to different treatments, and from flowers, runners, leaves, and roots was isolated according to Manning (1991). To investigate the differential expression of *FaQR* during the strawberry ripening process, both RNA gel blot and QRT-PCR analyses were performed.

RNA Gel Blot Analysis

The insert from the *FaQR* cDNA clone (~1.2 kb) was used as a template for generating a radioactive probe. The 26S rRNA was used as a control for uniform RNA loading. The probe was labeled to a specific activity of ~10⁸ cpm/μg using a commercial random priming kit (Amersham Pharmacia Biotech). Twenty micrograms of total RNA per sample was routinely used for RNA gel blot analysis. Filters (Hybond N+; Amersham Pharmacia Biotech) were prehybridized at 65°C for 1 h in 15 mL of hybridization solution (0.25 M NaH₂PO₄, 7% SDS, and 0.1 mM Na₂EDTA). Denatured probes were added to the same hybridization solution, and hybridization was performed at 65°C for 14 to 16 h. Filters were washed (twice) at 65°C for 15 min in 100 mL of 0.2× SSC, 0.1% SDS and then exposed to x-ray film at −80°C for 24 to 48 h.

QRT-PCR Analysis

For QRT-PCR measures of gene expression, the iCycler system (Bio-Rad) was used, using the specific primers 5′-CCAAGGAGAACTTCGAGGA-CCTGC-3′ and 5′-CGGTAGCTCTGGAACTCTCAAGG-3′. The RT reactions contained 1× RT buffer, 10 mM DTT, 1 mM of each deoxynucleotide triphosphate (dNTP), specific 3′ primer (0.2 μM), 1 μg of DNase I–treated RNA, and 40 units of Moloney murine leukemia virus RT. The reaction mixtures were heated at 70°C for 5 min and cooled to room temperature. Afterwards, they were incubated for 5 min at 42°C, for 50 min at 50°C, and then for 15 min at 70°C. The resulting single-stranded cDNAs were amplified by PCR all at once. PCRs consisted of 25 μL of a mixture containing, in 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM of each primer, 3 μL of SYBR Green I (diluted 1:150,000), 3 μL of transcribed cDNA, and 0.5 units of Taq polymerase. The thermal cycling conditions were as follows: 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. A final extension step at 72°C for 5 min was also included. In QRT-PCR analysis, quantification is based on C_t values. The C_t is a measurement taken during the exponential phase of amplification, when limiting reagents and small differences in starting amount have not yet influenced the PCR efficiency. C_t is defined as the cycle at which fluorescence is first detectable above background and is inversely proportional to the log of the initial copy number. In our system, each 10-fold difference in initial copy number produced a 3.2-cycle difference in C_t. Each reaction was done in triplicate, and the corresponding C_t values were determined. These values were then normalized using the C_t value corresponding to a strawberry (housekeeping) interspacer gene. In addition, the efficiency of each QRT-PCR was also calculated. All of these values were used to determine the increases in gene expression according to the following expressions: fold change = $2^{\Delta(\Delta Ct)}$, where $\Delta C_t = C_t$ (target) − C_t (housekeeping gene) and $\Delta(\Delta C_t) = \Delta C_t$ (problem) − ΔC_t (control).

Expression in *Escherichia coli* and Antibody Production

To obtain the construct producing the strawberry protein corresponding to the full-length *FaQR*, two oligonucleotides with the sequence 5′-CTA**ACCATGGCT**GCAGCTCCAAG-3′, containing the start sequence of the *FaQR* cDNA coding region and carrying a *Not*I site, and the sequence 5′-CAAC**CTCGAG**GATGGGATACAC-3′, containing an *Xho*I

site, were used to amplify the entire *FaQR* coding sequence by PCR. The PCR product was digested with *Not*I and *Xho*I restriction enzymes and cloned in-frame into the corresponding sites of the pET29(+) (Novagen) vector, generating the pET29-QR plasmid, which was then introduced into *E. coli* host strain Bl21(DE3). The transformed bacterial cells were grown at 37°C in Luria-Bertani broth containing 50 μg/mL kanamycin until they reached an A_{600} of 0.8 to 1.0. The cells were cooled to 15°C, and 1 mM isopropyl β-D-thiogalactoside was added to induce FaQR synthesis. The bacteria were grown for another 6 h at 37°C and harvested by centrifugation. A pET29(+) plasmid without a DNA insert was used as a parallel control and treated similarly. Preparation of crude extracts from *E. coli* transformants was performed as follows. The cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA), lysozyme was added to a final concentration of 2 mg/mL, and the cells were incubated at 4°C for ~1 h. Cells were disrupted in a French press (1200 p.s.i.) twice. Cell debris were pelleted by centrifugation, and the supernatant was tested for protein induction. For the analysis of total bacterial proteins, aliquots were pelleted in a microcentrifuge, boiled in SDS-lysis buffer (0.1 M Tris-HCl, pH 6.8, 1.6% glycerol, 0.008% bromphenol blue, 4 mM EDTA, 10 mM DTT, and 3% SDS) for 3 min at 95°C, and loaded on a 12% SDS-polyacrylamide gel. Proteins were visualized by Coomassie Brilliant Blue R 250 staining, and their concentrations were determined spectrophotometrically by the Bradford assay (Bradford, 1976). The recombinant protein contained in the supernatant was purified by gel electrophoresis on a 12% SDS-polyacrylamide gel. The band containing the recombinant FaQR protein was carefully excised, and the gel slice was used directly as the antigen source for polyclonal antibody production in white rabbits.

Protein Gel Blot

Proteins were extracted from strawberry fruits at different developmental and ripening stages (Harpster et al., 1998) and were used to test the specificity of the anti-quinone oxidoreductase antibodies and to study the expression pattern of the FaQR protein. A specific band of 35 kD, corresponding to the deduced size of FaQR, was found. Specific detection of FaQR polypeptide was performed with the enhanced chemiluminescence protein gel-blotting kit (Amersham), according to the manufacturer's protocol.

Fixation for LCM

Red strawberry fruits were trimmed to ~2 mm in thickness. Sections were fixed for 24 h at 4°C in a freshly prepared fixative solution containing 75% (v/v) ethanol and 25% (v/v) acetic acid. The fixative solution was vacuum-infiltrated into the sections for 10 min while they were kept on ice. Fixed tissue was dehydrated at 4°C in a graded series of ethanol (3 h each in 75, 85, and 100% and overnight in 100% [v/v]) and followed by an ethanol:xylene series at room temperature (1 h each at 75:25%, 50:50%, 25:75%, 0:100%, and 0:100% [v/v]). Flakes of Paraplast were added to the tissue-embedding medium (100% xylene) at the final step and dissolved until Paraplast saturation. The pieces of tissue were included in liquified Paraplast and kept at 58°C. Samples were positioned in a mold, and sections were cut using a rotary microtome. To stretch the ribbons, sections were floated in water on membrane slides (Molecular Machines and Industries) at 42°C, air-dried, and stored at 4°C under dehydrating conditions. For LCM, slides were deparaffined in xylene for two changes of 5 min and air-dried.

LCM

LCM was performed using a μCut Laser microdissection system (SL Microtest) and reaction tubes with an adhesive lid for single-step collection. Briefly, the slide was placed into position on the LCM microscope stage. The lid of the reaction tube was placed over the tissue during all operations of μCut Laser microdissection (the lid contains a diffusor to improve the image quality). Cells of interest were located, and LCM was performed with the laser beam set to 15 to 20 μm and focused. The power of the laser beam was ~80 to 85 mW. Dissected cells were then removed by lifting the LCM cap using the placement arm.

RNA Extraction and RNA Amplification

RNA from cells was extracted using the Absolutely RNA Nanoprep kit (Stratagene) according to the manufacturer's instructions. Briefly, 100 μL of lysis buffer was applied to the special adherent lid containing laser-captured cells of the LCM microcentrifuge tube. After inverting and vortexing the tube, the lysis buffer was collected and mixed with an equal volume of 70% ethanol. This mixture was transferred to the RNA binding column, and DNase treatment and washings were performed. RNA was eluted in 2 × 10 μL of elution buffer warmed to 60°C and vacuum-concentrated to a volume of 11 μL. cDNA synthesis and RNA amplification were performed using the MessageAmp aRNA kit (Ambion) according to the manufacturer's instructions. Briefly, first-round cDNA synthesis was performed with a T7 oligo(dT) primer. One microliter of T7 oligo(dT) primer was added to 11 μL of RNA and heated to 70°C for 10 min, and after cooling to 42°C, 2 μL of 10× first-strand buffer, 1 μL of ribonuclease inhibitor, 4 μL of dNTP mix, and 1 μL of RT were added. After a 2-h incubation, second-strand synthesis was performed by the addition of 63 μL of nuclease-free water, 10 μL of 10× second-strand buffer, 4 μL of dNTP mix, 2 μL of DNA polymerase, and 1 μL of RNase H. Reactions were incubated for 2 h at 16°C. The cDNA was purified according to the manufacturer's instructions and eluted with 2 × 10 μL of nuclease-free water. For in vitro transcription, 16 μL of double-stranded cDNA was mixed with 4 μL of each dNTP, 4 μL of 10× reaction buffer, and 4 μL of T7 enzyme mix. Reactions were incubated at 37°C for 14 h and then digested with DNase. The aRNA was purified and vacuum-concentrated to 10 μL. To increase the amount of aRNA, a second round of amplification was performed. This procedure is similar to the first round of amplification, but different primers are used. To analyze *FaQR* gene expression, aRNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad), and PCR was performed using the same conditions described above (see QRT-PCR Analysis) except that SYBR Green was omitted.

Expression of FaQR for Enzyme Activity Assays

The pET29-QR plasmid was introduced into *E. coli* host strain Bl21(DE3)-plys. The transformed bacterial cells were grown at 37°C in Luria-Bertani broth containing 50 μg/mL kanamycin until they reached an OD_{600} of 0.4 to 0.6. The cells were cooled to 16°C, and isopropyl β-D-thiogalactoside was added to a concentration of 1 mM. The bacteria were grown for another 16 h at 16°C and harvested by centrifugation at 4000 rpm. An empty pET29(+) plasmid was used as a parallel control and treated in the same way. The cells were solubilized by grinding in a chilled mortar with 2.5 parts glass beads (106 μm; Sigma-Aldrich). The soluble fraction was extracted with an extraction/wash buffer containing 50 mM sodium phosphate and 300 mM sodium chloride, pH 7.0. The suspension was centrifuged, and the resulting supernatant, called the clarified sample, was loaded onto a Co-chelating Sepharose gel (Talon; BD Biosciences). After several wash steps, the poly-His-tagged protein was eluted by an elution buffer containing 50 mM sodium phosphate, 300 mM sodium chloride, and 150 mM imidazol on a gravity flow column (BD Biosciences). For the analysis of total bacterial proteins, aliquots were pelleted in a microcentrifuge, boiled in SDS-lysis buffer (0.1 M Tris-HCl, pH 6.8, 1.6% glycerol, 0.008% bromphenol blue, 4 mM EDTA, 10 mM DTT, and 3% SDS) for 3 min at 95°C, and loaded on a 12% SDS-polyacrylamide gel. Proteins were visualized by Coomassie Brilliant Blue R 250 staining, and their concentrations were determined spectrophotometrically by the Bradford assay (Bradford, 1976). Before enzymatic activity assays, an additional purification step was performed because FaQR extracts

purified by His tag affinity chromatography still contained native quinone oxidoreductase activities of *E. coli* cells. The protein extract eluted from Talon was transferred to an ultrafiltration tube (Amicon Ultra 4 centrifugal filter device) with a nominal molecular mass limit of 50 kD. The flow-through, containing proteins of <50 kD, was transferred to another ultrafiltration tube with a nominal molecular mass limit of 30 kD. The supernatant of the second ultrafiltration step, containing proteins of the molecular mass range from 30 to 50 kD, was devoid of native *E. coli* quinone oxidoreductase activity but still contained recombinant FaQR and was used for enzymatic activity assays.

Determination of HDMF-Forming and Quinone-Reducing Enzymatic Activity

Forty milligrams (98 μmol) of D-fructose-1,6-diphosphate and 2 mg (2.8 μmol) of NADH were added to 15 μg of purified FaQR in 1 mL of phosphate buffer, pH 7.0 (0.1 M). The solution was incubated for 24 h at 30°C in a shaker. Products were analyzed by HPLC-MS/MS. Quinone reductase activity was determined after the addition of different 1,2- and 1,4-quinones (20 nmol) and 90 nmol of NADH to 10 μL of a purified FaQR protein extract (10 μg of protein) dissolved in 1 mL of phosphate buffer, pH 7.0 (0.1 M). Decreasing NADH absorption was determined spectrophotometrically (Safire) at 340 nm for 30 min at 30°C. In addition, synthesized HMMF or EDHMF was incubated under the same conditions (30 min, 30°C) with FaQR protein. Products were analyzed by HPLC-MS/MS. K_m and V_{max} values were determined by fitting the data to the Michaelis-Menten equation with a nonlinear regression program (Hyper32, version 1.00; http://homepage.ntlworld.com/john.easterby/hyper32.html). For the time course experiment showing the competition of FaQR and 3-mercaptobenzoic acid for HMMF, the acid (0.04 mg, 259 nmol) was added to a preincubated (10 min) 1-mL assay solution (4 mg [5.6 μmol] of NADH and 40 mg [98 μmol] of D-fructose-1,6-diphosphate) or to a phosphate-buffered solution, pH 7.0 (1 mL, 0.1 M), containing 40 mg (98 μmol) of D-fructose-1,6-diphosphate, and the mixture was incubated under standard conditions.

Detection of HMMF in Strawberry Fruits

Fifty microliters of a 0.6 M 3-mercaptobenzoic acid solution (70% ethanol) was injected daily into strawberry fruits of the pink ripening stage for a period of 3 d. After storage at room temperature, the fruits were subjected to XAD-2 solid-phase extraction (Roscher et al., 1997), and the diethyl ether and methanol extracts were analyzed for the presence of trapped HMMF using HPLC-MS/MS.

Accession Numbers

Sequence data from this article have been deposited with the GenBank/EMBL data libraries under the accession numbers AY158836, AY048861, AJ001445, AF193789, and AF220491. The accession numbers for the sequence alignment shown in Figure 4 are AY048861 for *Fragaria × ananassa* (*F. ananassa*), AJ001445 for *Fragaria vesca*, U20808 for *Vigna radiata*, AF451799 for *Arabidopsis thaliana*, AF384244 for *Helianthus annuus*, and NM188914 for *Oryza sativa*.

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