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Molecular and Biochemical Characterization of Tomato Farnesyl-Protein Transferase[1]

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The prenylation of membrane-associated proteins involved in the regulation of eukaryotic cell growth and signal transduction is critically important for their subcellular localization and biological activity. In contrast to mammalian cells and yeast, however, the function of protein prenylation in plants is not well understood and only a few prenylated proteins have been identified. We partially purified and characterized farnesyl-protein transferase from tomato (*Lycopersicon esculentum*, LeFTase) to analyze its biochemical and molecular properties. Using Ras- and Gγ-specific peptide substrates and competition assays we showed that tomato protein extracts have both farnesyl-protein transferase and geranylgeranyl-protein transferase 1 activities. Compared with the heterologous synthetic peptide substrates, the plant-specific CaaX sequence of the ANJ1 protein is a less efficient substrate for LeFTase in vitro. LeFTase activity profiles and LeFTase β-subunit protein (LeFTB) levels differ significantly in various tissues and are regulated during fruit development. Partially purified LeFTase requires Zn^{2+} and Mg^{2+} for enzymatic activity and has an apparent molecular mass of 100 kD. Immunoprecipitation experiments using anti-αLeFTB antibodies confirmed that LeFTB is a component of LeFTase but not of tomato geranylgeranyl-protein transferase 1. Based on their conserved biochemical activities, we expect that prenyltransferases are likely integrated with the sterol biosynthesis pathway in the control of plant cell growth.

Prenylation of proteins with the C15 farnesyl and the C20 geranylgeranyl has emerged as an important posttranslational modification. Isoprenylation regulates the biological activity of a select group of proteins. It increases their affinity to intracellular membranes (Omer and Gibbs, 1994) and facilitates their interaction with other regulatory proteins (Marshall, 1993). Several proteins currently known to be isoprenylated belong to the superfamily of Ras-like proteins (Hancock et al., 1989) and include the farnesylated Ras (Casey et al., 1989) and geranylgeranylated Rab proteins (Khosravi-Far et al., 1991). Other proteins modified by prenylation include γ subunits of heterotrimeric G proteins (Mumby et al., 1990), a subset of protein Tyr kinases (Inglese et al., 1992), and nuclear lamins (Wolda and Glomset, 1988). All of the known prenylated proteins have regulatory functions in complex growth signal transduction pathways (Casey, 1994), cell division (Boguski and McCormick, 1993), nuclear envelope assembly (Franke, 1987), and membrane trafficking (Balch, 1990).

In mammalian and yeast cells, protein modification by FPP or GGPP is catalyzed by three prenyltransferases that differ in their isoprenoid substrate and protein targets. FTase attaches farnesyl to a Cys residue in the C-terminal sequence motif CaaX (a = aliphatic, X = usually M, Q, S, C or A; Reiss et al., 1991). GGTase-1 recognizes the same motif but prefers L at the X position (Yokoyama et al., 1995). Although FTase and GGTase-1 have the highest affinities for their respective CaaX substrates, both enzymes show a low level of cross-specificity (Trueblood et al., 1993; Yokoyama et al., 1995). Type 2 geranylgeranyl transferase (RabGGTase) recognizes the C-terminal motifs CXC, CC, or CCXX, which so far have only been found in Rab proteins (Khosravi-Far et al., 1991). Although activities for the three enzymes are present in plants (Randall et al., 1993; Yalovsky et al., 1996) and prenylated proteins have been detected (Swiezewska et al., 1993; Biermann et al., 1994), only a few plant proteins with conserved prenylation motifs have been identified. Among these plant proteins, prenylation has been demonstrated directly for ANJ1, a homolog of the molecular chaperone DNAJ (Zhu et al., 1993a), and three distinct tomato (*Lycopersicon esculentum*) Rab homologs (Loraine et al., 1996; Yalovsky et al., 1996).

FTase and GGTase-1 are heterodimers of an α subunit that is shared between both enzymes and a β subunit that is distinct (Seabra et al., 1991). The genes for both enzymes have been cloned from mammalian and yeast cells. In yeast the α subunit is encoded by *RAM2* (He et al., 1991). Deletion of *RAM2* is lethal, indicating the importance of protein prenylation in eukaryotic cells (He et al., 1991). The β subunits of yeast FTase and GGTase-1 are encoded by *RAM1* and *CDC43*, respectively (Schafer et al., 1990; Finegold et al., 1991; Moores et al., 1991). *RAM1* is required for the farnesylation of the α-mating factor, as well as RAS1 and RAS2 proteins (Powers et al., 1986). *CDC43* is required for cell polarity and budding, both essential functions of the cell cycle progression and growth in yeast (Adams et al., 1990). RabGGTase in the rat brain is a heterodimer of tightly associated α and β subunits (Seabra et al., 1992) that are homologous to the FTase α and β subunits (Armstrong et al., 1993). In yeast the RabGGTase subunits are encoded

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Abbreviations: CAIL, CysAlaIleLeu; CVIM, CysValIleMet; FPP, farnesylpyrophosphate; FTase, farnesyl-protein transferase; GGTase-1 and -2, geranylgeranyl-protein transferase types 1 and 2, respectively; GGPP, geranylgeranylpyrophosphate; IP, immunoprecipitation; LeFTase, tomato LeFTase; LeFTB, FTase β subunit protein; LeGGTase-1, tomato GGTase type 1; MVA, mevalonic acid; TFA, trifluoroacetic acid.

by *BET4* and *BET2*, both of which are essential genes (Jiang et al., 1993; Li et al., 1993). Unlike FTase and GGTase-1, RabGGTase requires the Rab escort protein for activity. The Rab escort protein binds to the Rab protein and delivers it for prenylation to RabGGTase (Andres et al., 1993).

Although progress has been made in understanding the biochemistry and molecular biology of isoprenoid metabolism in plant cells (Bach, 1995; Chappell, 1995a, 1995b), the biochemistry and regulation of prenyltransferases are only poorly understood. Recent work with suspension-cultured tobacco cells (Randall et al., 1993; Morehead et al., 1995) and tomato tissues (Loraine et al., 1996; Yalovsky et al., 1996) established evidence for FTase, GGTase-1, and RabGGTase activities in plant cells. The cloning of genes from pea and tomato with similarity to the α subunit shared between FTase and GGTase-1 (S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, and W. Gruissem, unpublished results) and the β subunit specific for FTase (Yang et al., 1993; S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, and W. Gruissem, unpublished results) indicate that prenyltransferases are conserved in plants. Reported changes in protein isoprenylation during the growth of suspension-cultured tobacco cells (Morehead et al., 1995), together with the implication of isoprenylated proteins in the regulation of cell growth and division (Boguski and McCormick, 1993), reinforce the notion that prenyltransferases are likely to have an important regulatory function in plants as well.

The FPP and GGPP branch points of the sterol biosynthesis pathway shared with prenyltransferases in plants are of particular interest because their flux is dependent on the tight regulation of MVA production by multiple enzyme isoforms (Narita and Gruissem, 1989; Yang et al., 1991; Chye et al., 1992; Enjuto et al., 1994) and because they are also used for synthesis of di- and sesquiterpenes, sterols, polyprenols, and hormones (Chappell, 1995a). In general, integration of key metabolic pathways with regulation of cell division and growth remains one of the fundamental problems in biology. The important discovery of cells recruiting the isoprenoid intermediates FPP and GGPP for the modification of regulatory proteins by three conserved prenyltransferases therefore represents the first critical link between the sterol biosynthesis pathway and control of G1 progression and cell growth. Since the discovery of Ras modification by farnesyl (Casey et al., 1989), FTase became the logical target for the development of peptidomimetic drugs in clinical therapy of oncogenic Ras-dependent growth (James et al., 1993; Kohl et al., 1993). In contrast, understanding the developmental and physiological regulation of prenyltransferases relative to the control of MVA synthesis and the importance of protein prenylation for control of cell function is less advanced.

To gain insight into the regulation of prenyltransferases in plants, we used tomato as a model system to investigate the biochemical specificity of FTase in the prenylation of CaaX target peptides relative to GGTase-1 and to investigate the developmental and spatial regulation of FTase activity. Here we report that FTase and GGTase-1 in tomato represent biochemically and immunologically distinct enzyme activities with different substrate specificities. We established that LeFTase is likely a 100-kD enzyme with biochemical properties similar to those of yeast and mammalian FTases. LeFTase specifically and correctly prenylates a K-RasB CVIM target peptide but is surprisingly less active on the tobacco ANJ1 CAQQ C-terminal target peptide. Our analysis shows that LeFTase activity is regulated during development. The demonstration of distinct FTase and GGTase-1 enzyme activities in plants is consistent with a view that protein prenylation is a fundamental posttranslational modification conserved in all eukaryotic cells.

MATERIALS AND METHODS

All tissues, except fruit, used in prenyltransferase assays were obtained from 4-week-old hydroponically grown tomato (*Lycopersicon esculentum* cv VFNT cherry) plants. Fruit tissue was harvested from soil-grown plants. Seedlings were grown for 6 d in complete darkness or in a 16-/8-h light/dark cycle. After harvesting tissues were frozen immediately in liquid nitrogen.

Peptides

Peptides were synthesized (model 430A, Applied Biosystems) using fluoromethoxycarbonyl chemistry and biotinylated on the resin. Five peptides were synthesized: KRRSCVIM (Ras peptide), biotin-KRRSCVIM (RasB peptide), KRRFCAIL (Gγ peptide), biotin-KRRFCAIL (GγB peptide), and KQRVQCAQQ (ANJ1 peptide). The control peptide MSSKSVLS was purchased from Multiple Peptide Systems (San Diego, CA). All peptides were HPLC-purified and analyzed by MS. Farnesylated Ras peptide was synthesized using 400 μg of Ras peptide dissolved in 100 μL of 50% *n*-propanol, 5 μL of 10% farnesyl bromide, and 25 μL of 0.1 M Na_2CO_3 (Ashby et al., 1992). The reaction mixture was incubated overnight at room temperature. The farnesylated peptide was HPLC-purified and analyzed by MS.

LeFTase and LeGGTase Activity Assays

Isoprenyltransferase activity was determined by quantification of [^{3}H]farnesyl or [^{3}H]geranylgeranyl transferred to RasB or GγB peptides as previously described (Yokoyama et al., 1991). The standard LeFTase and LeGGTase-1 activity assay was carried out in a final volume of 20 μL containing the following compounds: 50 mM Hepes, pH 7, 20 mM $MgCl_2$, 5 μM $ZnCl_2$, 5 mM DTT, 3 mM α,β-methylene-ATP, 15 μM aprotonin, 200 μM leupeptin, 0.74 mM antipain, 130 μM bestatin, 14 μM pepstatin A, 1 mM PMSF, 1 mM Pefabloc, 10 mM benzamidine, 4 μM RasB or GγB, 2.3 μM [^{3}H]FPP (1.4 Ci/mmol, Amersham), or 1.38 μM [^{3}H]GGPP (8 Ci/mmol, Amersham), and 1 to 10 μg of protein extract. The standard incubation time was 40 min at 30°C. After incubation the reaction mixture was chilled on ice and 2 μL of 10% SDS was added. The reaction mixture was then transferred to 10 μL of streptavidin-agarose beads preincubated with 0.5 μL of 1:500 (v/v) farnesol: ethanol, kept on ice for 5 min, and vortexed briefly once every 1 min. The beads were then washed three times with

1 mL of 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1 M NaCl, 0.1% SDS, 1% Triton X-100 (Sigma), 1% sodium deoxycholate, and 0.01% NaN_3 and twice with 1 mL of PBS. The beads were then added to scintillation fluid and the bound ^3H radioactivity was measured in a scintillation counter (model 1900CA, Hewlett-Packard). A negative control assay without peptide substrate was performed in parallel with each assay series and used to determine background activity. The transfer of [^{3}H]farnesyl and [^{3}H]geranylgeranyl was calculated (in pmol/h) from the data and adjusted for background activity.

Preparation of Crude Protein Extracts

All steps were carried out at 4°C. Frozen tomato tissues (except mature green, breaker, and red fruit) were homogenized in 50 mM Hepes, pH 7.5, 250 mM mannitol, 5 μM $ZnCl_2$, 1 mM DTT, and 1 mM PMSF using a mortar and pestle. Mature green, breaker, and red fruit were homogenized in 0.5 M Tris-HCl, pH 11, 250 mM mannitol, 5 μM $ZnCl_2$, 1 mM DTT, and 1 mM PMSF. The resulting extracts were cleared in a microfuge at 10,000 rpm for 15 min. The pH of the supernatants ranged from 6.8 to 7.5. The extracts were stored at −80°C and are referred to here as the "crude extracts."

Partial Purification of LeFTase

Preparation of Tomato Stem Extract

For the partial purification of LeFTase, 1 kg of stem tissue was powdered in liquid nitrogen using a blender (Waring). The liquid nitrogen was allowed to evaporate and 1 L of 50 mM Hepes, pH 7.5, 250 mM mannitol, 5 mM EGTA, 5 mM DTT, 1 mM PMSF, and 0.1% BSA was added. The mixture was further homogenized (model PT10/35, Polytron, Brinkmann Instruments). The resulting extract was filtered through one layer of Miracloth (Calbiochem) and four layers of cheesecloth. The filtrate was centrifuged for 30 min at 16,000*g*.

Ammonium Sulfate Precipitation

The resulting supernatant was adjusted to 30% saturation with solid ammonium sulfate, stirred for 30 min on ice, and centrifuged at 10,000*g* for 15 min to remove precipitated proteins. The supernatant was then adjusted to 50% ammonium sulfate saturation. The precipitated proteins were removed by centrifugation and the resulting pellet was dissolved in 30 mL of 50 mM Tris-HCl, pH 7.5, 5 μM $ZnCl_2$, 1 mM DTT, and 1 mM PMSF (buffer A). The resuspended protein was centrifuged for 1 h at 100,000*g* and the resulting supernatant was dialyzed against buffer A, aliquoted, and stored at −80°C.

Anion-Exchange Chromatography

Ammonium sulfate-fractionated extract (70 mg) was applied at a flow rate of 10 mL/min to a column (Poros PI/M 10/100, PerSeptive Biosystems, Framingham, MA) that was previously equilibrated with buffer A. After the column was loaded it was washed with 4 column volumes of buffer A. Then a 7-column volume gradient was run from 0 to 30% buffer B (buffer A plus 2 M NaCl), followed by a 5-column volume wash with 30% buffer B. LeFTase was eluted by a 5-column volume gradient from 30% buffer B to 70% buffer B, followed by a 10-column volume wash at 70% buffer B. LeFTase eluted between 800 mM and 1 M NaCl. Fractions containing about 74% of the total activity applied were combined, concentrated, and dialyzed against 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 μM $ZnCl_2$, and 1 mM DTT (buffer C).

Gel-Filtration Chromatography

One milliliter of the anion-exchange-purified extract (5 mg/mL) was subjected to a gel-filtration column (1.6/60 cm, Superdex 200 prep grade, Pharmacia). The column was previously equilibrated with buffer C. The flow rate was 0.5 mL/min. The fractions with the highest activity (containing about 41% of the total activity applied) were combined, concentrated, and dialyzed against 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 μM $ZnCl_2$, and 1 mM DTT (buffer D). The combined extract will be referred to as "partially purified LeFTase."

EDTA Treatment of Partially Purified LeFTase

Partially purified LeFTase was dialyzed for 16 h at 4°C in an eight-well microdialysis system (GIBCO-BRL) with prepared dialysis membranes and a 12- to 14-kD exclusion limit (Spectrapor, Spectrum Medical Industries, Los Angeles, CA). The extract was dialyzed against 500 mL of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and 0.2 mM EDTA.

Bacterial Overexpression and Purification of LeFTB

Oligonucleotide primers were constructed for the amplification of a near full-length duplicate of the *LeFTB* clone (S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, and W. Gruissem, unpublished results) and the introduction of 5′ *Eco*RI and a 3′ *Bam*HI restriction sites. The nucleotide sequences were as follows: *LeFTB* 5′ end (AGGATCCATGGAGTCGAGGAAAGTGACG) and *LeFTB* 3′ end (GGAATTCGCAATATCCTAAAC). A PCR amplification using the *LeFTB* 5′ end and *LeFTB* 3′ end as primers and the *LeFTB* cDNA clone as the template generated a 1.8-kb product. This PCR product was subcloned into a PCR1000 vector (Invitrogen, San Diego, CA) and sequenced. The 1.8-kb *Eco*RI/*Bam*HI fragment was then excised from the construct and cloned into the *Escherichia coli* expression vector pGEX-2TK (Pharmacia). The pGEX2TK vector was transformed into *E. coli* strain PRK475. Transformed cells were grown for 3 h at 28°C. Protein expression was then induced by 0.1 mM isopropyl-β-D-thiogalactoside, and the induced cells were allowed to grow overnight. The GST-LeFTB fusion protein was harvested from *E. coli* in the form of inclusion bodies. The inclusion bodies were subjected to SDS-PAGE. The GST-LeFTB fusion protein was excised from the gel, electroeluted, and dialyzed.

Production of LeFTB Antibody and Immunoblot Analysis

The SDS-PAGE-purified GST-LeFTB fusion protein was used as an antigen to raise polyclonal antisera. Rabbits and mice were immunized by subcutaneous injections with 100 μg of GST-LeFTB fusion protein in Ribi Adjuvant (Ribi Immunochem Research, Hamilton, MT). After the primary injection, three boosters were given every 2 weeks. IgG from rabbit serum was purified using Protein A affinity chromatography (Poros A, 4.6/100, PerSeptive Biosystems). For immunoblot analysis tomato extracts were separated by SDS-PAGE and electroblotted onto membranes (Immobilon P, Millipore). Immunoblots were either reacted with rabbit anti-LeFTB IgG (1:1000) or mouse antiLeFTB serum (1:1000), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Boehringer Mannheim). Immunoblots were developed using an enhanced chemiluminescence assay kit (ECL, Amersham).

IP of LeFTase

Rabbit anti-LeFTB IgG (1.2 mg) and rabbit preimmune IgG were covalently linked to 1 mL of Reacti 6× gel (Pierce) according to the manufacturer's protocol. One milligram of antibody was bound in both cases. After the gels were washed they were stored at 4°C in 10 mM Tris-HCl, pH 7.5, containing 0.02% NaN_3. The IP was performed at 4°C using buffer D plus 15 μM aprotonin, 200 μM leupeptin, 0.74 mM antipain, 2 mM Pefabloc, and 20 mM benzamidine (buffer E). Then, IP was performed as follows: partially purified LeFTase in buffer E was added to immobilized rabbit IgG and the reaction mixture was incubated for 2 h at 4°C. The immobilized LeFTase was then pelleted, the supernatant was removed, and the pellet was washed five times with 1 mL of 10 mM Tris-HCL, pH 7.5, 150 mM NaCl, 5 μM $ZnCl_2$, and 0.1% Tween 20 (Sigma). LeFTase activity was measured by adding an aliquot of immobilized LeFTase to the standard enzyme assay.

Identification of [^{3}H]Farnesyl-Modified Ras Peptide

[^{3}H]Farnesyl-modified Ras peptide was determined by reverse-phase HPLC (C18 Vydac semiprep column, Hewlett-Packard series 1050 HPLC). Chromatography was performed as follows: 20 μL of LeFTase assay mixture was added to 100 μL of 30% acetonitrile/1% TFA containing 5 μg of farnesylated Ras peptide standard. The following buffers were used: buffer A: H_2O, 0.1% TFA; buffer B: acetonitrile, 0.083% TFA. Upon injection, a gradient was developed from 35% buffer B to 65% buffer B over 20 min. The column was run at 1 mL/min and chromatography was monitored at 215 nm. Fractions were collected every 20 s starting 2 min after sample injection. Each fraction was added to scintillation fluid and subjected to ^{3}H analysis (Hewlett-Packard model 1900CA).

Reproducibility of Biochemical Assays

Results from enzyme assays shown in Figures 1, 4, and 7 were reproduced with different stem extracts prepared using the identical protocol. Data from single experiments are shown. Results from experiments shown in Figures 2, 3, 5, and 9 were reproduced with the same extract in independent experiments. Data from single experiments are shown. Results presented in Figures 6 and 8 were from single experiments.

RESULTS

Tomato Has Specific FTase and GGTase-1 Activities

We used a modified prenyltransferase assay to investigate FTase and GGTase-1 activities in tomato cell-free extracts using biotinylated peptide substrates. These peptides were designed based on the C-terminal CaaX motifs of p21-K-RasB (CVIM), which functions as a substrate for FTase in rat cells (Reiss et al., 1990), and of the trimeric G protein γ subunit (CAIL), which is prenylated by bovine GGTase-1 (Yokoyama et al., 1991). When these substrates were used FTase and GGTase-1 activities were readily detectable in the plant protein extract (Fig. 1). The activity of FTase was approximately 30-fold higher compared with GGTase-1 and the above substrates. Significantly less prenylation activity was detected for GγB-CAIL in the presence of [^{3}H]FPP, and no prenylation was detected of the RasB-CVIM peptide in the presence of [^{3}H]GGPP. These results are consistent with similar data obtained for mammalian and yeast FTase and GGTase-1 and establish that both activities exist in plants.

As indicated above, we found it necessary to modify the in vitro assay originally developed for the rat FTase (Reiss et al., 1990) because under these conditions [^{3}H]FPP was metabolized into a compound that bound strongly to agarose. Preincubation of the streptavidin-agarose with farnesol reduced nonspecific binding of the [^{3}H]FPP metabolite to background levels (data not shown). Furthermore,

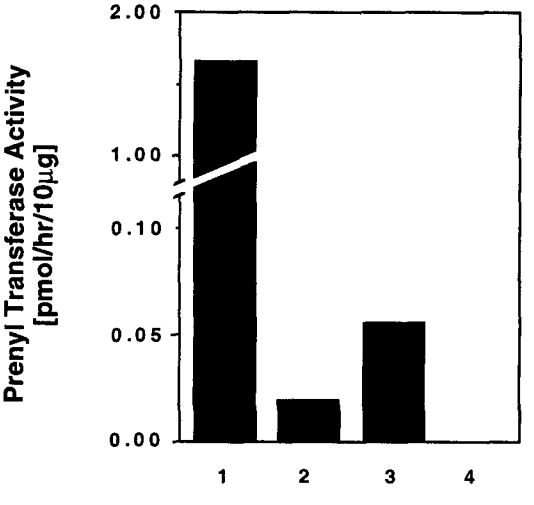

Figure 1. FTase and GGTase activity in tomato. Protein prenyltransferase activity analysis was performed as described in "Materials and Methods." Ten micrograms of ammonium sulfate-fractionated stem extract was used per assay. The following substrate combinations were used in the enzyme activity assays: 1, RasB plus [^{3}H]FPP; 2, RasB plus [^{3}H]GGPP; 3, GγB plus [^{3}H]GGPP; 4, GγB plus [^{3}H]FPP.

[^{3}H]FPP became rapidly depleted from the reaction mixture. TLC experiments revealed that several intermediates and end products were synthesized from [^{3}H]FPP in tomato extract. The synthesis of these compounds was initiated by the removal of PPi from [^{3}H]FPP (data not shown). Addition of the ATP-analog α,β-methylene-ATP stabilized [^{3}H]FPP and at the same time did not significantly inhibit yeast FTase in a control assay (data not shown). The biotinylated substrate peptides were also found to be degraded very rapidly in the tomato extract. A complex mixture of protease inhibitors was necessary to prevent degradation of the biotinylated peptide substrates. Under these conditions, which are described in more detail in "Materials and Methods," prenylation of the substrate peptides was reproducible and stable for FTase and GGTase activities.

To confirm that the detected prenyltransferase activities represented FTase and GGTase activities, we conducted competition experiments with nonbiotinylated Ras and Gγ peptides, as well as with unlabeled FPP and GGPP (Fig. 2). As expected, Ras peptide and FPP competed effectively with the labeling of the RasB peptide substrate. In contrast, Gγ peptide did not compete significantly with the RasB peptide substrate, and GGPP showed only a modest competition with the labeled FPP substrate. These results confirm that FTase activity is present in tomato and that the RasB CVIM peptide is a specific substrate for this enzyme. They also indicate that LeFTase, in contrast to the mammalian FTases (Reiss et al., 1992), does not bind FPP and GGPP with similar affinity in vitro. Labeling of GγB-CAIL with tritiated GGPP effectively competed with nonbiotinylated Gγ peptide and with nontritiated GGPP. In contrast, no competition was observed with nonbiotinylated Ras peptide and only modest competition was observed with nontritiated FPP. These results suggest that prenylation of the Gγ-CAIL substrate must be accomplished by a different prenyltransferase, most likely a GGTase-1 enzyme. They also indicate that the specificity of tomato GGTase is higher for GGPP than for FPP, which is consistent with that reported for mammalian GGTase-1 (Yokoyama et al., 1995). Together, the results of the competition experiments provide direct evidence based on specific substrates that biochemical activities for both FTase and GGTase exist in plants. In analogy to the mammalian and yeast enzymes, we have therefore designated the activities LeFTase and LeGGTase-1.

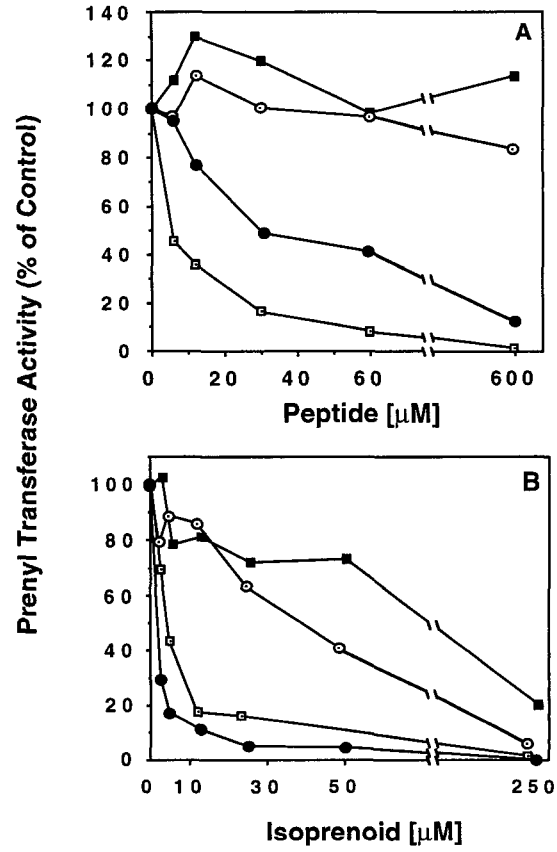

Figure 2. Inhibition of LeFTase and LeGGTase-1 activity by nonbiotinylated peptides and nontritiated isoprenoids. Prenyltransferase activity analysis was performed as described in "Materials and Methods." LeFTase and LeGGTase-1 activities were analyzed with the substrate combinations RasB with [^{3}H]FPP and GγB with [^{3}H]GGPP, respectively. Six micrograms of ammonium sulfate-fractionated stem extract was used per assay. In A, the indicated amounts of nonbiotinylated Ras peptide (□, ○) and Gγ peptide (■, ●) were used to inhibit LeFTase (□, ■) and LeGGTase-1 (○, ●) activity. In B, nontritiated FPP (□, ○) and GGPP (■, ●) were used to inhibit LeFTase (□, ■) and LeGGTase-1 (○, ●) activity.

LeFTase Activity Is Different in Diverse Tissues and during Fruit Development

FTase activity assays using protein extracts from several tomato organs and tissues revealed that LeFTase activity was detectable in all tissues and varied almost 10-fold between the different protein extracts (Fig. 3A). The highest activity was detected in apical bud tissues from young plants and in developing fruit, consistent with tissues and developmental stages in which cells divide and expand. High LeFTase activity was also detected in stem tissues from young plants, although compared with the shoot apex and developing fruit, cell division and expansion are considerably reduced in the stem. In contrast, LeFTase activity was significantly lower in mature leaf and mature green fruit. No significant difference in activity was detected in tissues from root apices and older root segments that were maximally expanded and in which cells had ceased to divide. Furthermore, LeFTase activity in dividing tomato tissue culture cells was surprisingly low compared with stem tissue, for example. Thus, at present there is no clear correlation between LeFTase activity and meristematic activity of plant tissues. Similarly, no significant difference in LeFTase activity was detected between dark-grown and light-grown seedlings, indicating that the enzyme is not strongly light-regulated. Based on control assays (i.e. peptide and FPP stability; data not shown), we do not believe that the apparent differences in LeFTase activity result from the assay conditions or differential inhibition of the degradation of LeFTase in the protein extract.

Detailed analysis of LeFTase activity during fruit development further substantiated tissue-specific and develop-

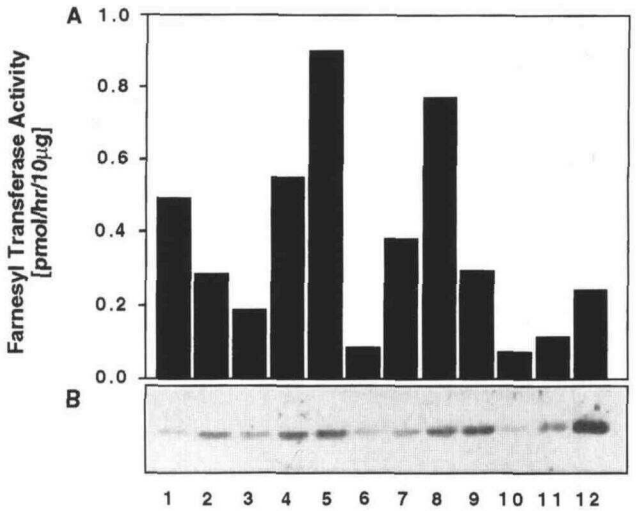

Figure 3. A, LeFTase activity in different tomato organs and tissues. LeFTase activity assays were performed as described in "Materials and Methods." Ten micrograms of crude extract of the following tissues was used in each assay: 1, Callus; 2, dark-grown seedlings; 3, light-grown seedlings; 4, root; 5, stem; 6, leaf; 7, apical bud; 8, fruit (0.5 cm); 9, fruit (1 cm); 10, mature green fruit; 11, breaker fruit; 12, red ripe fruit. B, Immunoblot analysis of LeFTB in tomato. Immunoblot analysis was performed as described in "Materials and Methods." Twenty micrograms of crude extract of the tissues listed in A was used in each lane.

mental regulation of the enzyme (Fig. 3A). LeFTase activity decreased approximately 10-fold between young (5 mm) and mature green fruit. This was followed by an approximately 5-fold increase in LeFTase activity during development of the breaker fruit stage. Increased LeFTase activity levels persisted during fruit development and in red ripe fruit. It is possible that LeFTase activity may be even higher in red fruit, because partial inactivation of the enzyme may occur as a result of the acidic pH during the preparation of the protein extract. We have found in separate control assays that LeFTase has reduced activity below pH 6.5 (see below). The detection of LeFTase activity in ripening fruit is interesting because it suggests an important role for the enzyme during this developmental phase.

To obtain further insights into the distribution of the enzyme, we analyzed LeFTB protein levels from the same tissues and organs (Fig. 3B). The immunoblot analysis confirmed the presence of LeFTB in all tissues, consistent with the detectable enzyme activity. LeFTB levels are high in stem, root, and 0.5- and 1-cm fruit. They are highest in red ripe fruit but are significantly reduced in leaf and mature fruit. Although LeFTB levels and LeFTase activity correlate in some cases (compare A and B in Fig. 3), this pattern is not consistent in all tissues.

LeFTase Requires Zinc for Activity

To further characterize LeFTase we partially purified the enzyme from tomato stem extracts approximately 44.7-fold (Table I). During this purification procedure, LeFTase activity could not be separated from LeGGTase-1 activity (data not shown). Mammalian FTase requires Mg^{2+} and

Table I. *Partial purification of LeFTase from stem tissue*

Purification	Protein	Total Activity	Specific Activity	Purification	Overall Yield
	mg	*pmol h^{-1}*	*pmol $mg^{-1}h^{-1}$*	*fold*	*%*
Ammonium sulfate	451.2	96,560	214	2	100
POROS PI/M	26.8	71,454	2,664	24.9	74
Superdex 200 polyacryl-amide gel	6.1	29,219	4,790	44.7	30.3

Zn^{2+} for full activity, and it has been proposed that FTase is a zinc metalloenzyme (Reiss et al., 1992). To investigate the metal requirement of LeFTase, we extensively dialyzed the partially purified enzyme against EDTA. This treatment resulted in a complete loss of LeFTase activity (Fig. 4). The addition of Zn^{2+} and Mg^{2+} restored full LeFTase activity. Under these conditions LeFTase activity was optimal between pH 6.5 and 7.0 at 30°C (data not shown). This result differs from the mammalian FTase, which has a pH optimum of about 7.5 at 37°C (Reiss et al., 1990). The divalent ion requirements, pH, and temperature optima of LeFTase determined with a specific peptide substrate also differ significantly from those of a putative spinach FTase activity characterized using DTT as a nonspecific substrate (Parmyrd et al., 1996).

A Plant-Specific CaaX Sequence Is Not an Efficient Substrate for LeFTase

The *Atriplex nummularia* ANJ1 protein is a homolog of the bacterial molecular chaperone DNAJ (Zhu et al., 1993b) and is the first identified substrate for plant FTase (Zhu et al., 1993a). We investigated the affinity of the ANJ1 sequence CAQQ as a substrate for LeFTase relative to the RasB substrate CVIM that we used as the standard substrate in the LeFTase assay. As expected, farnesylation of the RasB CVIM biotinylated peptide effectively competed

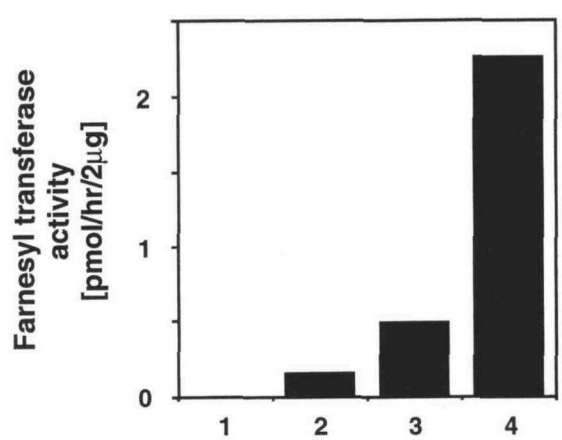

Figure 4. LeFTase requires Zn^{2+} and Mg^{2+} for its catalytic activity. Partially purified LeFTase was treated with EDTA as described in "Materials and Methods." EDTA-treated extract (2 μg) was subjected to LeFTase activity analysis. The following concentrations of $ZnCl_2$ and $MgCl_2$ were used in the LeFTase activity analysis. 1, No $ZnCl_2$, no $MgCl_2$; 2, no $ZnCl_2$, 10 mM $MgCl_2$; 3, 40 μM $ZnCl_2$, no $MgCl_2$; 4, 40 μM $ZnCl_2$, 10 mM $MgCl_2$.

(inhibitor concentration for 50% displacement = 3 μM) with unlabeled substrate (Fig. 5). In contrast, significantly higher concentrations of the ANJ1 CAQQ peptide were required to achieve 50% inhibition (inhibitor concentration for 50% displacement = 100 μM) of RasB peptide farnesylation, indicating that the ANJ1 CAQQ motif has a low affinity for tomato FPTase in vitro. The role of prenylation for ANJ1 cellular function is not known, but if it is important, other regulatory mechanisms may contribute to its efficient in vivo modification.

LeFTase Activity Co-Elutes with a 100-kD Protein Fraction

We used antibodies (αLeFTB) raised against the protein encoded by the gene for the LeFTase β subunit that we cloned from tomato (S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, and W. Gruissem, unpublished results) to confirm that LeFTB is a component of the FTase activity we identified using the CaaX peptide substrates. Partially purified LeFTase was subjected to analytical gel-filtration chromatography and protein fractions were analyzed for the presence of LeFTB (Fig. 6). Most of the LeFTase activity eluted in a single peak at approximately 100 kD, but a significant amount of activity (approximately 10%) eluted at a molecular mass of more than 300 kD (Fig. 6A). It is possible that the high-molecular-mass complex represents aggregated LeFTase, but we cannot exclude at present that it is another form of LeFTase in plants. Immunoblot analysis of proteins in the gel-filtration fractions showed that LeFTB co-eluted with LeFTase activity (Fig. 6B). This result establishes that LeFTB is the component of a 100-kD LeFTase in plants and confirms that LeFTase has a molecular mass of approximately 100 kD, which is similar to that of the mammalian and yeast FTases (Reiss et al., 1990; Gomez et al., 1993).

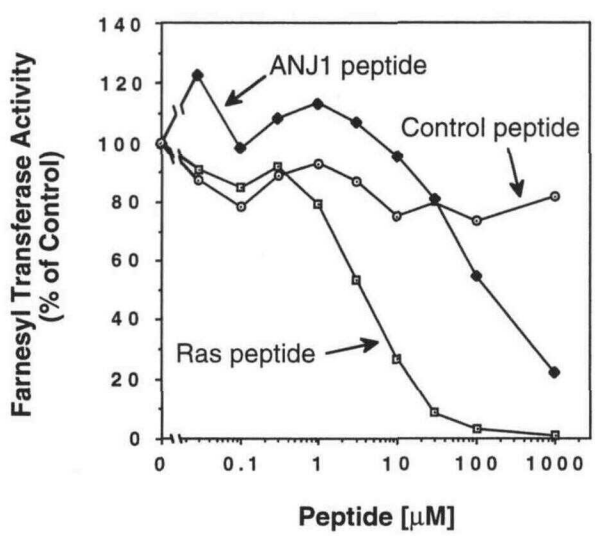

Figure 5. Inhibition of partially purified LeFTase activity by ANJ1 peptide and Ras peptide. LeFTase activity assays were performed as described in "Materials and Methods." Each reaction mixture contained 6.6 μg of partially purified LeFTase. The indicated amounts of ANJ1 peptide, Ras peptide, and control peptide were added.

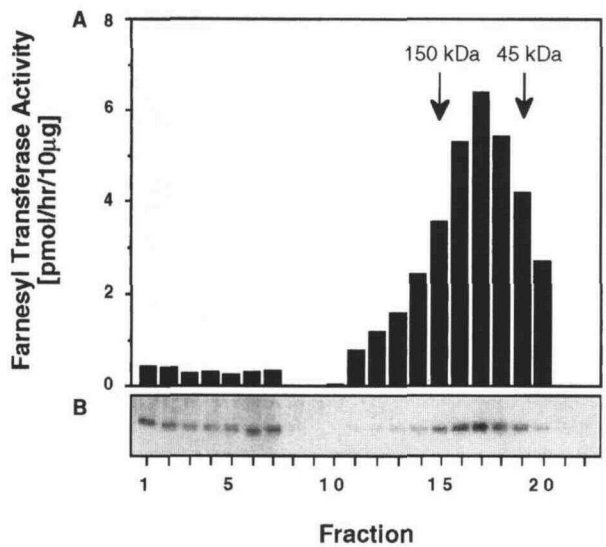

Figure 6. The molecular mass of LeFTase was determined using gel-filtration chromatography. The chromatography was performed as described in "Materials and Methods." Ten microliters of each collected fraction was subjected to LeFTase activity assay (A) and immunoblot analysis (B) as described in "Materials and Methods."

Although unlikely and not consistent with the known FTases, the 100-kD LeFTase could be a homodimer of the 52-kD LeFTB. To exclude this possibility, we assayed a cell-free extract of *E. coli* transformed with *LeFTB* for FTase activity. LeFTB was detectable using the anti-LeFTB antibody, but no FTase activity could be measured in the bacterial extract (data not shown). We therefore concluded that LeFTase consists of at least two different subunits, one of which is encoded by *LeFTB*. In analogy to the mammalian and yeast FTases, the other subunit is likely encoded by *LeFTA*, the gene for the FTase α-subunit that we cloned from tomato (S. Yalovsky and W. Gruissem, unpublished results). This conclusion is consistent with the observation that both *LeFTA* and *LeFTB* are required in yeast to complement a mutation in FTB (RAM1) (S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, and W. Gruissem, unpublished results).

LeFTB Is a Component of LeFTase That Prenylates CaaX Motifs

We took advantage of the αLeFTB antibody to demonstrate directly that LeFTB is a component of LeFTase (Fig. 7). Using purified anti-LeFTB in IP assays, we were able to deplete partially purified LeFTase of more than 80% of the enzyme activity (Fig. 7A) and LeFTB protein (Fig. 7B). In contrast, less than 8% of LeFTase activity was removed from the enzyme preparation using the preimmune antibody. Immunoblot analysis confirmed that depletion of LeFTase activity from the protein extract coincided with the disappearance of a single band for LeFTB (Fig. 7B). Both LeFTase activity and LeFTB were recovered in the anti-LeFTB immunoprecipitate but not in the precipitate with the preimmune serum. Together, these results provide direct immunological evidence that LeFTB is a component of LeFTase.

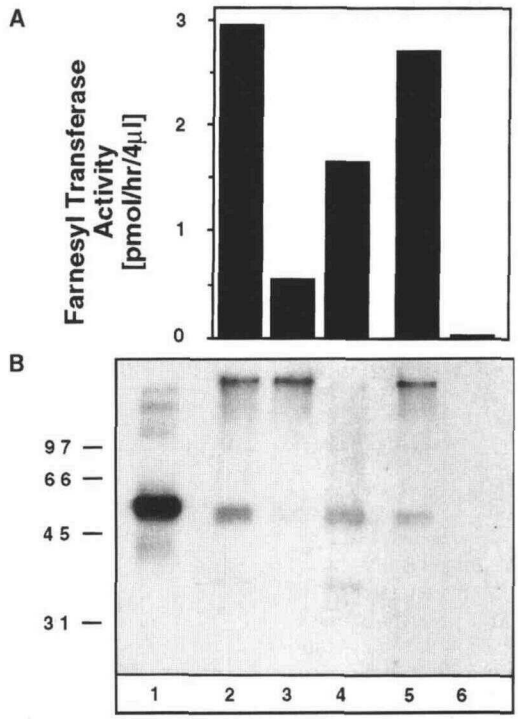

Figure 7. IP of LeFTase. IP was performed as described in "Materials and Methods." The IP mixture contained 20 μg of partially purified LeFTase in 50 μL of buffer F and 50 μL of immobilized αLeFTB or immobilized preimmune IgG. A, Four microliters of the fractions listed below (lanes 2–6) was subjected to LeFTase activity analysis. LeFTase activity assays were performed as described in "Materials and Methods." B, Four microliters of the fractions listed below (lanes 2–6) was subjected to immunoblot analysis as described in "Materials and Methods." Mouse anti-LeFTB serum was used as the primary antibody. Lane 1, Recombinant LeFTB (2.5 ng, standard); lane 2, extract before IP; lane 3, extract following IP using immobilized anti-LeFTB IgG; lane 4, immobilized anti-LeFTB IgG; lane 5, extract following IP using immobilized preimmune IgG; lane 6, immobilized preimmune IgG. The IP was reproducible in several independent experiments. The nature of the high-molecular-mass material that cross-reacts with the mouse anti-LeFTB serum is not known. Molecular weight standards are indicated on the left.

To confirm that the LeFTase activity immunoprecipitated with anti-LeFTB was specific for the farnesylation of the RasB CVIM peptide, we subjected the prenylated peptide to reverse-phase HPLC (Fig. 8). Most of the radioactivity eluted at the position of the internal farnesylated RasB peptide standard (Fig. 8A). This co-migrating radioactivity peak was not detected when the RasB CVIM peptide was omitted from the assays (Fig. 8B). Radioactivity peaks eluting ahead of and trailing the RasB CVIM peptide were detectable in both control and RasB peptide assays and likely represent FPP and farnesol or other farnesyl derivatives, respectively. Thus, the activity that is immunoprecipitated with anti-LeFTB represents LeFTase that specifically farnesylates the RasB CVIM peptide substrate.

LeFTB Is Not a Component of LeGGTase-1

The results shown in Figure 2 provided evidence that both LeFTase and LeGGTase-1 activities are present in tomato. To

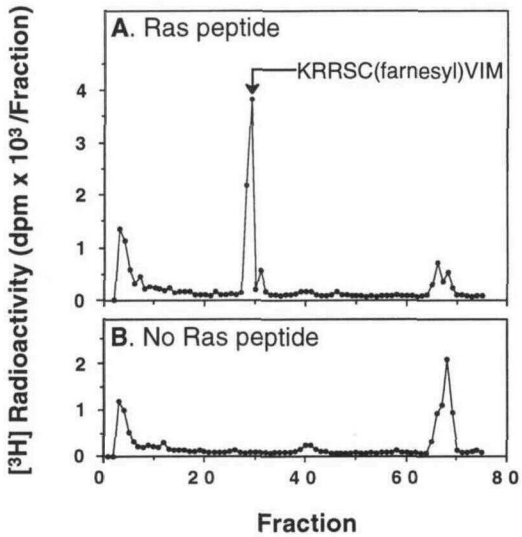

Figure 8. Identification of [^{3}H]FPP-modified Ras peptide. A, A standard LeFTase activity reaction mixture containing immunoprecipitated LeFTase was subjected to reverse-phase HPLC (C_{18}, VyDac) as described in "Materials and Methods." Twenty-second fractions were collected starting 2 min after sample injection. Each fraction was subjected to ^{3}H analysis. B, Negative control. The experiment was performed as described in A with the exception that Ras peptide was omitted from the LeFTase reaction mixture.

substantiate this evidence, we used the anti-LeFTB antibody to distinguish between the two enzyme activities that modify CaaX motifs in the fractionated protein extract (Fig. 9). The experiment showed that after IP of LeFTase activity, most of the LeGGTase-1 activity remained in the supernatant of the protein extract, suggesting that anti-LeFTB does not effi-

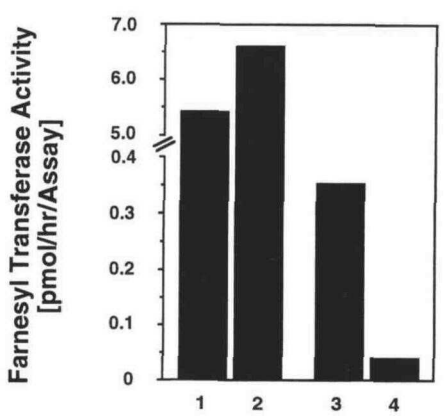

Figure 9. Evidence for LeGGTase-1 activity. IP was performed as described in "Materials and Methods." The IP mixture contained 6.1 mg of partially purified LeFTase in 1 mL of buffer F and 100 μL of immobilized anti-LeFTB IgG. Bar 1, Partially purified LeFTase (6.1 μg) was assayed for activity prior to IP using RasB peptide. Bar 2, Two microliters of immobilized anti-LeFTB IgG was assayed for LeFTase activity following IP using RasB peptide. Bar 3, Partially purified LeFTase containing LeGGTase-1 (see "Results") was assayed for activity using GγB peptide. Bar 4, Two microliters of immobilized anti-LeFTB IgG was assayed for LeGGTase-1 activity following IP using GγB peptide. The enzyme analysis was carried out as described in "Materials and Methods."

ciently recognize this enzyme. Mammalian and yeast FTase and GGTase-1 share a common α subunit, but their respective β subunits are unique (Seabra et al., 1991; Caplin et al., 1994). Although we have not yet cloned the gene for the LeGGTase-1 β subunit, our results strongly suggest that anti-LeFTB does not immunoprecipitate LeGGTase-1. We believe that the low level of apparent LeGGTase-1 activity in the LeFTase immunoprecipitate is due to the ability of LeFTase to modify the Gγ protein CaaX motif with geranylgeranyl rather than representing LeGGTase-1 activity. This view is consistent with results from yeast that in vivo *Saccharomyces cerevisiae* FTase can farnesylate preferred substrates of *S. cerevisiae* GGTase-1 with low efficiency (Trueblood et al., 1993).

DISCUSSION

The multibranched isoprenoid biosynthesis pathway is one of the most important pathways in plants because of the functional requirement for isoprenes in cell growth processes, photosynthesis, plant defense mechanisms, most other metabolic pathways, and pharmaceutically and commercially valuable products (Chappell, 1995a). It is therefore important to understand how this pathway is regulated and how plant cells monitor its activity during cell division, growth, and development to produce adequate supplies of sterols and other isoprene compounds. In general, integration of key metabolic pathways with cell cycle regulation and cell growth remains one of the fundamental problems in biology. The important discovery in 1989 that cells recruit the isoprenes FPP and GGPP for modification of growth-regulatory proteins by prenyltransferases (Hancock et al., 1989; Schafer et al., 1989) established the first critical link among the sterol biosynthesis pathway, control of cell division, and cell growth.

Following this initial discovery, studies in plants reported here and in previous papers (Randall et al., 1993; Yang et al., 1993; Zhu et al., 1993a; Morehead et al., 1995; Loraine et al., 1996; Yalovsky et al., 1996), together with previous work in animals and yeast (Omer and Gibbs, 1994), have now established that most eukaryotic cells utilize prenyltransferases and the sterol intermediates FPP and GGPP for prenylation of proteins (Gibbs, 1991; Clarke, 1992; Cox and Der, 1992; Schafer and Rine, 1992). In mammalian and yeast cells, this novel role of isoprenes appears to be part of an intricate network that links the sterol biosynthesis pathway to regulatory proteins that control cell division, growth, cytoskeletal functions, or membrane traffic in response to external growth factors and hormones (Boguski and McCormick, 1993; Sun and Kamiya, 1994). Although the molecular mechanism of protein prenylation has been clarified, however, the physiological significance of this modification in relationship to sterol biosynthesis regulation and isoprene utilization is still not understood. To facilitate our understanding of this relationship and the function of protein prenyltransferases in plants, we investigated LeFTase and LeGGTase-1 enzyme activities to establish their substrate specificities. In contrast to the mammalian and yeast enzymes, however, very few plant proteins have been identified that can serve as substrates for plant FTase and GGTase-1.

Previous surveys of plant protein extracts showed that tobacco contains activities related to FTase and GGTase-1 (Randall et al., 1993), prompting the authors to propose that both enzymes exist in plants. Our results reported here provide additional biochemical and immunological evidence that both FTase and GGTase-1 represent two distinct enzyme activities. We have termed the tomato GGTase activity LeGGTase-1 because the enzyme activity differs in its biochemical and protein recognition properties from the tomato RabGGTase activity reported recently (Loraine et al., 1996; Yalovsky et al., 1996). Compared with the extensive information now available for FTase, however, relatively little is known about GGTase-1 in plants. Our results establish that LeGGTase-1 utilizes GGPP and prenylates a peptide with the CaaX motif CAIL specific for mammalian and yeast GGTase-1. In addition, LeGGTase-1 activity cannot be efficiently immunoprecipitated with the anti-LeFTB antibody, indicating that LeFTase and LeGGTase-1 do not share a common β subunit. This is similar to GGTase-1 from mammalian and yeast cells, which have different β subunits but share the α subunit with FTase (Seabra et al., 1991; Caplin et al., 1994). Based on this information, we expect that LeFTase and LeGGTase-1 are two distinct enzymes in plants.

We found that LeFTase shares several characteristics with mammalian and yeast FTases. First, LeFTase requires Mg^{2+} and Zn^{2+} for its enzymatic activity, suggesting that the catalytic reaction mechanism is conserved between all eukaryotic FTases. This is different from a recent report in which FTase activity in spinach was apparently not activated by Mg^{2+} (Parmyrd et al., 1996). Second, LeFTase is similar in molecular mass to the yeast and mammalian enzymes. Third, based on the biochemical fractionation and cloning of *LeFTA* and *LeFTB* (S. Yalovsky, K.L. Callan, J.O. Narita, and W. Gruissem, unpublished results), it appears that LeFTase shares a similar subunit composition. The known FTases from mammalian and yeast cells are composed of functionally distinct α and β subunits. The proteins encoded by *LeFTA* and *LeFTB* are most similar to the α and β subunit proteins of the mammalian and yeast enzymes (S. Yalovsky, C.E. Trueblood, K.L. Callan, J.O. Narita, S.M. Jenkins, J. Rine, and W. Gruissem, unpublished results). In addition, we demonstrate here that the 52-kD protein product of *LeFTB* is one subunit of LeFTase. Because of these shared characteristics, we conclude that LeFTase is a heterodimeric enzyme in plants as well.

Finally, LeFTase prenylates the CaaX motif CVIM but discriminates this sequence from the GGTase-1 CaaX motif CAIL. Although this is similar to the specificities of yeast and rat FTase (Reiss et al., 1991; Caplin et al., 1994), additional studies are necessary to understand the substrate recognition of LeFTase. It is interesting that compared with the mammalian RasB motif CVIM LeFTase has a 30-fold lower affinity in vitro for the C-terminal sequence CAQQ of the plant ANJ1 protein, although it was shown to become farnesylated in vitro (Zhu et al., 1993a). Analysis of the farnesylation sequence motif requirement for rat FTase revealed that the enzyme was least active when the a1/a2

positions were occupied by nonpolar aliphatic or aromatic amino acids, with a2 showing a greater effect than a1 (Reiss et al., 1991). Although we have not analyzed peptides with Q in the a2 position, it is possible that this amino acid in the a2 position of ANJ1 motif is the cause of the low peptide affinity for LeFTase.

The biochemical and immunological characterization of LeFTase confirms that enzyme activity and LeFTB are detectable in all plant organs at different developmental stages. This would be expected if LeFTase has an essential function in protein prenylation. Temporal and spatial LeFTase activities and LeFTB levels differ significantly, however, and do not show a consistent correlation with cell division activity or developmental stages characterized by rapid cell growth and elongation. For example, mature breaker fruit, callus tissue, and light-grown seedlings have a comparable low LeFTase activity, but compared with breaker fruit, callus and seedlings are active in cell division. In contrast, the highest levels of LeFTase activity are detectable in stems of established plants, young fruit, and the shoot apex region. The high LeFTase activity in stem tissue is unexpected and may point to a role of the enzyme in prenylation of proteins that differ in their function from the typical FTase substrate proteins, such as Ras and lamins identified in yeast and mammalian cells (Franke, 1987; Chardin, 1988). Until more protein substrates for plant FTases have been identified, however, the temporal and spatial differences in LeFTase activities are difficult to interpret.

Further detailed comparison of LeFTase activity with LeFTB levels at first approximation reveals a complex regulation of the enzyme. For example, LeFTB protein levels are approximately similar in stem tissue from 4-week-old tomato plants and in that of dark-grown seedlings, but LeFTase activity is more than 5 times higher in stem protein extracts. Since analysis of enzyme activity and protein levels was conducted under reproducible experimental conditions with comparable plant extracts, we believe that these differences may reflect a posttranslational regulation of LeFTase activity. Alternatively, we cannot exclude that the protein extracts from dark-grown seedlings and stem, for example, contain different levels of compounds that may interfere with the LeFTase assay. Further immunological characterization and purification of the enzyme is necessary to clarify the mechanism underlying the observed discrepancies. Similarly, analysis of LeFTB protein and mRNA levels also reveals notable differences, suggesting that LeFTase in plants may be subject to translational or posttranslational regulation (S. Yalovsky, K.L. Callan, S.M. Jenkins, and W. Gruissem, unpublished results).

Our analysis of prenyltransferases has established additional biochemical and immunological evidence for distinct LeFTase and LeGGTase-1 activities in plants that utilize FPP and GGPP in the specific modification of different peptide substrates. The confirmation of these enzymes in plants is particularly interesting because the FPP and GGPP branch points of the sterol pathway can also be used for synthesis of ABA (although carotenoids are generally implicated in ABA synthesis) and GAs, in addition to other compounds derived from these two isoprenes (Chappell, 1995b). The molecular and immunological tools that are now available should facilitate our understanding of how the utilization of FPP and GGPP by these enzymes is regulated relative to other enzymes that access these branch points to the control of cellular MVA production.

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