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Plant Farnesyltransferase Can Restore Yeast Ras Signaling and Mating

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Farnesyltransferase (FTase) is a heterodimeric enzyme that modifies a group of proteins, including Ras, in mammals and yeasts. Plant FTase α and β subunits were cloned from tomato and expressed in the yeast *Saccharomyces cerevisiae* to assess their functional conservation in farnesylating Ras and a-factor proteins, which are important for cell growth and mating. The tomato FTase β subunit (LeFTB) alone was unable to complement the growth defect of *ram1*Δ mutant yeast strains in which the chromosomal FTase β subunit gene was deleted, but coexpression of *LeFTB* with the plant α subunit gene (*LeFTA*) restored normal growth, Ras membrane association, and mating. LeFTB contains a novel 66-amino-acid sequence domain whose deletion reduces the efficiency of tomato FTase to restore normal growth to yeast *ram1*Δ strains. Coexpression of LeFTA and LeFTB in either yeast or insect cells yielded a functional enzyme that correctly farnesylated CaaX-motif-containing peptides. Despite their low degree of sequence homology, yeast and plant FTases shared similar in vivo and in vitro substrate specificities, demonstrating that this enzymatic modification of proteins with intermediates from the isoprenoid biosynthesis pathway is conserved in evolutionarily divergent eukaryotes.

Modification of various proteins by the C_{15} isoprene farnesyl or the C_{20} isoprene geranylgeranyl groups facilitates their adherence to membranes and, for some proteins that have been studied in detail, is also required for their function (34, 44, 54). Protein prenylation was discovered almost 2 decades ago as a modification of peptide mating pheromones produced by fungi (28, 43). During the last 8 years, a growing number of proteins have been discovered that are prenylated. Interestingly, many of these proteins play key roles in the regulation of cell division, cell growth, and signal transduction processes. These proteins include normal and oncogenic forms of Ras, the yeast **a**-factor mating pheromone, certain γ subunits of heterotrimeric G proteins, proteins in the vertebrate visual system, hepatitis delta virus large antigen, and type I inositol triphosphate 5′-phosphatase (1, 2, 11, 16, 18, 23, 25, 26, 30, 36, 45, 52).

All known farnesylated proteins have a common C-terminal sequence motif known as the CaaX box (C, cysteine; a, usually an aliphatic amino acid; X, any amino acid). Yeast and mammalian protein farnesyltransferases (FTases) attach the farnesyl group from farnesyl pyrophosphate via a thioether linkage to the cysteines of CaaX sequences in which X is serine, methionine, cysteine, alanine, or glutamine (44). In most cases, farnesylation is followed by proteolytic removal of the C-terminal three amino acids and methylation of the free carboxyl group of cysteine (2, 15, 22).

In mammals and the yeast *Saccharomyces cerevisiae*, protein FTase is a heterodimeric enzyme composed of an α and a β subunit (the yeast subunits are designated Ram2p and Ram1p, respectively) (21, 24, 40, 41, 45). A second protein prenyltransferase, geranylgeranyltransferase type I (GGTase-I), uses geranylgeranyldiphosphate (GGPP) to modify proteins which contain a CaaX box in which X is usually leucine (44). GGTase-I is a heterodimeric enzyme that shares a common α subunit with FTase but has a distinct β subunit (31, 48).

Farnesylpyrophosphate (FPP) is an early intermediate of the mevalonate pathway and the last common precursor to most of its products (20). Thus, protein farnesylation might have a role in coordinating cell division and growth with prenol biosynthesis in eukaryotes. It is therefore important to establish whether all eukaryotic organisms contain a conserved FTase and whether FTases from different species have similar roles and substrate specificities.

FTases have been studied in mammals and yeast, but much less is known about FTases in plants. Protein farnesylation activity has been detected in protein extracts from cultured cells and tissues (38, 47) and in at least one case has been correlated with cell division (33). Several plant genes encoding proteins containing a CaaX box have been cloned based on farnesylation of the proteins by tobacco tissue culture cell extracts (5). Only one of these proteins has homologs in the data base: ANJ1 is homologous to the bacterial chaperone DnaJ and the yeast Ydj1 proteins. The farnesylation of ANJ1 is crucial for membrane binding and function at high temperatures (56). A gene encoding a putative FTase β subunit has been cloned from pea (53), but evidence that this gene encodes a farnesyltransferase subunit was based solely on homology. Recently, the plant FTase was shown to be biochemically similar to the mammalian FTase and distinct from GGTase-I activity (47).

Here we report the cloning of both the α and the β subunits of FTase from tomato (*Lycopersicon esculentum*). We demonstrate that the tomato FTase α and β subunit genes (*LeFTA* and *LeFTB*, respectively) encode a protein farnesyltransferase that is able to fully complement yeast mutants in which *RAM1* was deleted to restore Ras signaling and mating. The plant FTase expressed from *LeFTA* and *LeFTB* in yeast or insect cells correctly farnesylated CaaX-box-containing peptides, and the two subunits could be reconstituted in vitro into an active heterodimeric enzyme. These results show that FTase is functionally similar in widely divergent eukaryotes, indicating that the enzyme establishes an evolutionarily conserved link between isoprene biosynthesis and protein prenylation.

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
CTY716	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2*	C. Trueblood
JRY3387	*MAT***a** *ade2 trp1 ura3 lys2 his4 his3 ram1*Δ::*HIS3*	Segregant from cross JRY3092 × JRY3097 (51)
JRY5269	*MAT*α *his4 leu2 trp1 ura3*	C. Fox, Rine laboratory
JRY254	*MAT***a** *lys1*	Rine laboratory
JRY3443	*MAT*α *sst2 trp1 his⁻ ura⁻ can1*	M. Ashby, Rine laboratory (49)
PHY500	*MAT*α *ade2 lys2 trp1 leu2 ura3 RAM1*	
SYY100	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pSYYFTB2 [high-copy-number *TRP1*, *LeFTB*])	Transformant of CTY716
SYY101	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pRH98-3 [high-copy-number *URA3*]), pSYYFTB2 [high-copy-number *TRP1*, *LeFTB*])	Transformant of CTY716
SYY102	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pSYYFTA1 [high-copy-number *URA3*, *LeFTA*])	Transformant of CTY716
SYY105	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pSYYFTA1 [high-copy-number *URA3*, *LeFTA*]), pSYFTB2 [high-copy-number *TRP1*, *LeFTB*])	Transformant of CTY716
SYY120	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pSYYFTBd66-3 [high-copy-number *TRP1*, *LeFTB* Δ*66*])	Transformant of CTY716
SYY121	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pRH98-3 [high-copy-number URA3]), pSYYFTBd66-3 [high-copy-number *TRP1*, *LeFTB* Δ*66*])	Transformant of CTY716
SYY125	*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2* (pSYYFTA1 [high-copy-number *URA3*, *LeFTA*]), pSYYFTBd66-3 [high-copy-number *TRP1*, *LeFTB* Δ*66*])	Transformant of CTY716
JRY3399	*MAT***a** *ade2 trp1 ura3 lys2 his4 his3 ram1*Δ::*HIS3* (pSEY8 [high-copy-number *URA3*])	Transformant of JRY3387
JRY3401	*MAT***a** *ade2 trp1 ura3 lys2 his4 his3 ram1*Δ::*HIS3* (pJR856 [high-copy-number *URA3*, *RAM1*])	Transformant of JRY3387
JRY3455	*MAT***a** *ade2 trp1 ura3 lys2 his4 his3 ram1*Δ::*HIS3* (pSEY8 [high-copy-number *URA3*]), pJR1136 [high-copy-number *TRP1*])	Transformant of JRY3387
SYY111	*MAT***a** *ade2 trp1 ura3 lys2 his4 his3 ram1*Δ::*HIS3* (pRH98-3 [high-copy-number *URA3*]), pSYYFTB2 [high-copy-number *TRP1*, *LeFTB*])	Transformant of JRY3387
SYY115	*MAT***a** *ade2 trp1 ura3 lys2 his4 his3 ram1*Δ::*HIS3* (pSYYFTA1 [high-copy-number *URA3*, *LeFTA*]), pSYYFTB2 [high-copy-number *TRP1*, *LeFTB*])	Transformant of JRY3387

MATERIALS AND METHODS

Yeast strains. Yeast strains used in this study are listed in Table 1. CTY716 was derived from PHY500 (49) by replacement of the *RAM1* gene with *ram1*Δ::*ADE2*, an allele in which a 1,633-bp *Hpa*I fragment carrying RAM1 is deleted and replaced with a 2,241-bp *Bgl*II fragment of *ADE2* from plasmid pASZ11 (50). Construction of strain JRY3387, which has a *ram1*Δ::*HIS3* allele in which a *Pst*I-*Hpa*I fragment carrying *RAM1* is deleted and replaced with a *Pst*I-*Sma*I fragment carrying the *HIS3* gene, was described previously (51). To create the additional strains listed in Table 1, CTY716 and JRY3387 were transformed by the lithium acetate transformation protocol (27) with the plasmids indicated in Table 1.

Cloning of *LeFTA*. Two degenerate oligonucleotide primers designed according to the region conserved between the yeast and mammalian FTase α subunits, 5′-AACGGATCCAARAAYTAYCARATYTGGCAYCA-3′ (corresponding to amino acids 119 to 126) and 5′-TCATCGATTGRTTCCANGCNGARTTRTT-3′ (corresponding to amino acids 190 to 196), were used in a touchdown PCR with annealing temperatures ranging from 52 to 38°C (the temperature was reduced by 2°C after each two cycles, followed by 30 cycles at 38°C) to amplify a 250-bp fragment from a tomato young-fruit cDNA library. The resulting fragment was used as a probe to screen the same library for *LeFTA*. The young-fruit cDNA library was made from mRNA from 3- to 8-mm-diameter fruits of greenhouse-grown tomato plants (*L. esculentum* cv. VFNT Cherry LA1221) by using a Stratagene Uni-ZAP XR kit.

Cloning of *LeFTB*. cDNA was prepared by reverse transcription of tomato young-fruit poly(A)$^+$ RNA with a degenerate oligonucleotide primer specific for FTase β of yeast and mammals, 5′-AGCGCTAGCANCCGTCNACNAGTTTGTT-3′ (corresponding to amino acids 260 to 268). The resulting cDNA was amplified by PCR with two degenerate oligonucleotide primers, 5′-GCGAAGCTTTGGNGGNGGNCCNGGNCA-3′ (corresponding to amino acids 109 to 114) and 5′-CCGCTGCAGAANGTGTANCCNCCGTGNGC-3′ (corresponding to amino acids 214 to 222), at an annealing temperature of 45°C. A 380-bp fragment was amplified. This fragment was used as a probe to clone a partial 1.3-kb 3′-end cDNA of *LeFTB* from a tomato young-fruit cDNA library. A third oligonucleotide primer was made according to the sequence of this clone (5′-GGACTAGTGCACATCTTTCTCTATTAATTG-3′ [corresponding to amino acids 139 to 145]) and was used in a PCR with M13 Reverse primer (Stratagene). A 550-bp fragment corresponding to the 5′ end of *LeFTB* was amplified in this reaction. This fragment was then used as a probe to screen a tomato young-fruit cDNA library in order to clone the full-length *LeFTB* sequence.

Construction of yeast expression vectors. The yeast high-copy-number expression plasmid pJR1136 was constructed by ligating a 3.4-kb *Hin*dIII-*Xba*I fragment of pG-3 (46) into an altered YEplac112 (19), in which the polylinker region from the *Bam*HI site through the *Eco*RI site had been destroyed. pRH98-3 was constructed (by Randolph Hampton, currently of the Department of Biology, University of California, San Diego) by ligating a *Hin*dIII-*Xba*I fragment of pG-1 (46) into an altered YEplac195 (19) in which the *Bam*HI site had been destroyed. Both pJR1136 and pRH98-3 are 2μm-based high-copy-number plasmids that have the glyceraldehyde-3-phosphate dehydrogenase gene (*GPD*) promoter and the phosphoglycerate kinase gene (*PGK*) terminator separated by restriction sites. pRH98-3 has the *URA3* selectable marker, and pJR1136 has the *TRP1* selectable marker. pSYYFTA1 was made by ligating a *Bam*HI-*Xho*I fragment of *LeFTA* into the *Bam*HI-*Sal*I sites on pRH98-3. Plasmid pSYYFTB2 was prepared in a similar manner by ligating a *Bam*HI-*Xho*I fragment of *LeFTB* into the *Bam*HI-*Sal*I sites of pJR1136. pJR856 was constructed (by W. Schafer, currently of the Department of Biology, University of California, San Diego) by subcloning a 4.8-kb *Sal*I-*Bam*HI fragment containing *RAM1* (4) into the polylinker of the high-copy-number *URA3* plasmid pSEY8 (17).

Determination of intracellular localization of Ras1p and Ras2p in yeast cells. Yeast strains CTY716 (*ram1*Δ), PHY500 (*RAM1*), SYY101 (*LeFTB*, p*URA3*), and SYY105 (*LeFTA LeFTB*) (Table 1) (see Fig. 2) were grown at 25°C in minimal medium supplemented with the appropriate nutrients. At mid-log phase, cells were harvested, lysed by vortexing with glass beads, and fractionated into soluble and insoluble cell fractions as previously described (51). A fraction of each cell extract was removed before fractionation to be used as total cell extract. Volumes of all fractions were kept equal during the experiment. Following fractionation, equal volumes of all fractions were electrophoretically separated on a 10% polyacrylamide gel (29) and proteins were then transferred to a nitrocellulose membrane. Ras1p and Ras2p were identified by incubating the membrane with v-H-Ras monoclonal antibody (Ab) Ab-1 (Oncogene Science). Sheep anti-rat horseradish peroxidase (HRP)-conjugated Ab (Amersham) was used as a secondary Ab. Blots were developed with an ECL kit (Amersham). The average time of exposure to film was 10 to 15 min. Quantitations were carried out by laser scanning of films with a Presonal densitometer (Molecular Dynamics).

Pheromone diffusion (halo) assays. Halo assays were carried out essentially as previously described (3). One microliter of a slurry of *MAT***a** cells (approximately 10^7 cells) was applied to a lawn of *MAT*α *sst2* cells (approximately 2×10^6 cells) (12); this was followed by incubation at 25°C for 3 days. When the **a** factor mating pheromone is processed and exported, it arrests growth of *MAT*α *sst2* cells, thereby producing a zone of growth inhibition (halo). The size of the halo is proportional to the amount of **a** factor produced.

Construction of baculovirus containing *LeFTA* and *LeFTB*. *Bam*HI-*Nsi*I fragments of *LeFTA* and *LeFTB* were cloned into *Bam*HI-*Pst*I sites on pVL1393 (Invitrogen) to make pBacFTA1 and pBacFTB1, respectively. These two constructs were then recombined with linear virus in *Sf*9 cells according to standard procedures (35). Viral stocks were then amplified by standard methods (35). Typically, 50-ml cell cultures were grown to a density of 10^6 cells/ml. Cells were then infected with virus at a multiplicity of infection of 2. For coexpression of

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LeFTA and LeFTB, viral stocks containing *LeFTA* and *LeFTB* were used to coinfect cells, each at a multiplicity of infection (MOI) of 2.

Preparation of protein extracts from yeast and baculovirus-infected *Sf9* cells. (i) Yeast cells. Cultures of yeast strains CTY716 (*ram1*Δ), PHY500 (*RAM1*), and SYY105 (*LeFTA LeFTB* [LeFTA/B]) were grown at 25°C in minimal medium, supplemented with the appropriate nutrients, to mid-log phase. Protein extracts were prepared from these cultures as described above for the Ras protein localization experiments.

(ii) *Sf9* cells. Viral stocks containing wild-type virus, *LeFTA*, and *LeFTB* were prepared and amplified (see above). These stocks were used to independently infect or coinfect *Sf9* cells cultures, and protein extracts were prepared. Cells were harvested at 48 h postinfection, washed once by gentle resuspension in 10 ml of lysis buffer (50 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, 70 μM $ZnSO_4$, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), and then resuspended in 1 ml of lysis buffer. Cells were lysed by vortexing them with 0.45-mm-diameter glass beads for 30 s six consecutive times, cooling the suspension on ice for 1 min between each vortexing round. The resulting cell suspension was then centrifuged for 10 min at 10,000 × *g*. The supernatant was collected and subjected to a second centrifugation step of 30 min at 100,000 × *g*. The supernatant was collected, aliquoted, frozen, and kept at −80°C until used.

In vitro farnesylation assays. Protein extracts were incubated with CaaX-box-containing peptides in prenylation reactions as previously described (51). Reaction mixtures contained 50 μg of protein extract, 50 μM peptide, and 1 μM [^{3}H]FPP. Reaction product analysis was carried out on silica gel 60 thin-layer chromatography (TLC) plates (Merck) that were developed in solvent (pyridine-isoamyl alcohol-ethanol-water-acetic acid [70:70:20:20:5, vol/vol/vol/vol/vol]), sprayed with EN^{3}Hance spray (New England Nuclear), and exposed to X-ray film for 18 to 48 h.

Purification of α-LeFTB Ab. Abs against glutathione *S*-transferase (GST)::LeFTB were raised in rabbits as previously described (47). Immunoglobulin G (IgG) Abs were first purified from the serum by two successive precipitations at 30 and 50% NH_4SO_4, respectively. The precipitated IgGs were resuspended in phosphate-buffered saline and dialyzed for 12 h against the same buffer. The dialyzed Ab suspension was purified from anti-*Escherichia coli* and anti-GST protein Abs by successively passing it through two Reacti-Gel columns (Pierce) that were cross-linked with either *E. coli* protein extract or purified GST protein, respectively. Both cross-linking of proteins to the Reacti-Gel resin and purification of Abs were carried out according to the manufacturer's instructions (Pierce). The GST protein that was cross-linked to the Reacti-Gel resin was produced in bacteria by inducing protein expression in 1 liter of *E. coli* XL-1Blue cells harboring the plasmid pGEX2T (Pharmacia) with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. GST protein was purified over a gluthatione-Sepharose column according to the manufacturer's instructions (Pharmacia). Fractions containing purified GST were pooled and dialyzed-concentrated against PBS in a Collodion apparatus (Schleicher & Schuell). The resulting GST protein fraction was approximately 95% pure as judged from Coomassie blue-stained polyacrylamide gels (data not shown).

Probing of baculovirus-infected *Sf9* cell protein extracts with purified α-LeFTB Ab. Protein extracts were prepared as described above. Ten micrograms of each extract was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (29). Proteins were then electrotransferred onto a nitrocellulose membrane, and the membrane was then incubated with affinity-purified α-LeFTB Ab at a dilution of 1:10,000. HRP-conjugated goat anti-rabbit IgG (Bio-Rad; blotting grade) was used as the secondary Ab. Blots were developed with an ECL kit (Amersham). The average exposure time was a few seconds.

Construction of the cDNA for the LeFTBΔ66 protein and its subcloning into yeast expression vectors. A fragment of *LeFTB* was amplified by PCR with pBSFTB as a template and two primers, M13 Reverse (Stratagene) and SYFTBd66P (5′-GACTCCGGATGACAGCCCTAGTTGTTCATG-3′), together with PFU DNA polymerase (Stratagene). Thirty cycles consisting of the following reaction conditions were used: 1 min at 94°C, 2 min at 52°C, and 3 min at 72°C. A 1.1-kb fragment amplified in the reaction was gel purified, digested with *Bam*HI and *Stu*I, and subcloned into the appropriate sites in *Bam*HI- and *Stu*I-digested pBSFTB. The resulting construct (pSYFTBΔ66-1) had a 198-bp in-frame deletion in the LeFTB cDNA. The mutation caused the deletion of 66 amino acids between serine 300 and proline 367 and the introduction of three new amino acids, Ser-Gly-Val, that were not present in the original protein. The entire PCR-amplified fragment and the ligation sites were sequenced to verify that no mutations were created by the PCR reaction. pSYFTBΔ66-1 was digested with *Bam*HI and *Xho*I, and the resulting 1.6-kb fragment was ligated into *Bam*HI-*Sal*I sites in either pRH98-3 or pJR1136 to create pSYYFTBd66-1 and pSYYFTBd66-3, respectively.

Growth curve analysis of *ram1*Δ yeast strains complemented with LeFTA, LeFTA/LeFTB, or LeFTA/LeFTBΔ66. Twenty-five-milliliter cultures were grown in minimal medium supplemented with the appropriate nutrients at either 25 or 37°C. The optical densities were determined at a wavelength of 600 nm at various times.

Nucleotide sequence accession numbers. The sequences determined for *LeFTA* and *LeFTB* were deposited in GenBank under accession numbers U83707 and U83708, respectively.

RESULTS

***LeFTA* and *LeFTB* encode conserved subunits of tomato FTase.** The genes coding for the tomato FTase α and β subunits (*LeFTA* and *LeFTB*, respectively) were cloned from a tomato young-fruit cDNA library by PCR with degenerate oligonucleotide primers (Fig. 1). Both LeFTA and LeFTB share approximately 45% amino acid sequence identity with their mammalian homologs but exhibit only 30% identity with yeast FTase subunits encoded by *RAM2* and *RAM1*. The most striking difference is the insertion of a novel sequence domain in the plant β subunit (Fig. 1B). This sequence domain in FTBs from pea and tomato consists of 66 amino acids starting at position 335 and interrupts a highly conserved region proposed to participate in the binding of both the CaaX peptide and the prenyl substrate (55). Sequence domains at positions 264 (**EA HGGYTFC**), 311 (**NKLVDGCYSFW**), and 436 (**DKPGK**g**RD**y **YHTCYCLSGL**) are also highly conserved among FTase β subunits and may participate in the active site of the enzyme. (Boldfaced uppercase characters indicate consensus among all species, capital characters indicate consensus between the plant and mammalian proteins only, and lowercase characters indicate lack of consensus.) In the published sequence of the pea FTase β subunit protein (PsFTB), an entire domain of 30 amino acids from amino acid 46 to 73 is absent (53). PCR and sequence analysis of the *PsFTB* gene revealed that it contains this sequence, which is colinear with the sequence in LeFTB (33a). Thus, the tomato *LeFTB* gene represents a full-length sequence of the FTase β subunit from plants.

In contrast to the β subunits, FTase α subunits from yeast, plants, and mammals do not share significant homology except for repeats of the sequence motif NYxxWxxR (where x is an undefined amino acid) that were previously noted and proposed to have a role in protein-protein interactions or formation of a pocket for FPP or GGPP binding (8). The conservation of specific sequence motifs in both FTase α and β subunits from three evolutionarily divergent eukaryotes may be of functional significance because earlier models assigned catalytic function and CaaX peptide binding to FTB only (42).

LeFTase restores growth of the yeast *ram1*Δ mutant by a conserved mechanism. The function of the FTase encoded by *LeFTA* and *LeFTB* was tested in *S. cerevisiae ram1*Δ mutant strains, which lack the β subunit of yeast FTase. At 25°C, *ram1*Δ strains have a slow rate of cell division, and at temperatures above 34°C they fail to grow (51). Coexpression of *LeFTA* and *LeFTB* restored growth of *ram1*Δ yeast cells at 34 and 37°C to levels near those of wild-type (wt) *RAM1* cells, and cells formed larger colonies at 25 and 30°C, indicative of an increased rate of cell division (Fig. 2, *LeFTA LeFTB*). Farnesylation of Ydj1p, a DnaJ homolog, and of Ras proteins is required for growth at 37°C (9, 51). It appears that coexpression of *LeFTA* and *LeFTB* is sufficient to produce functional Ydj1p and Ras proteins, as well as any other unknown farnesylated proteins that may be required for growth. *LeFTB* alone could not complement the growth defects of the *ram1*Δ strain at 25, 30, 34, or 37°C, indicating that LeFTB and the yeast FTase α subunit expressed from the chromosomal *RAM2* gene did not assemble into an active FTase (Fig. 2, *LeFTB*, *LeFTB URA3*, *LeFTB* 5FOA).

The growth defect of *ram1*Δ strains below 34°C is mainly due to the reduced prenylation of Ras1p and Ras2p and the consequent reduction of Ras proteins in the membrane. In *ram1*Δ cells, less than 10% of the total Ras1p and Ras2p is found in the membrane fraction, and GGTase-I is responsible for the membrane localization of this small amount of Ras, which keeps the *ram1*Δ strains alive (51). To test whether restoration

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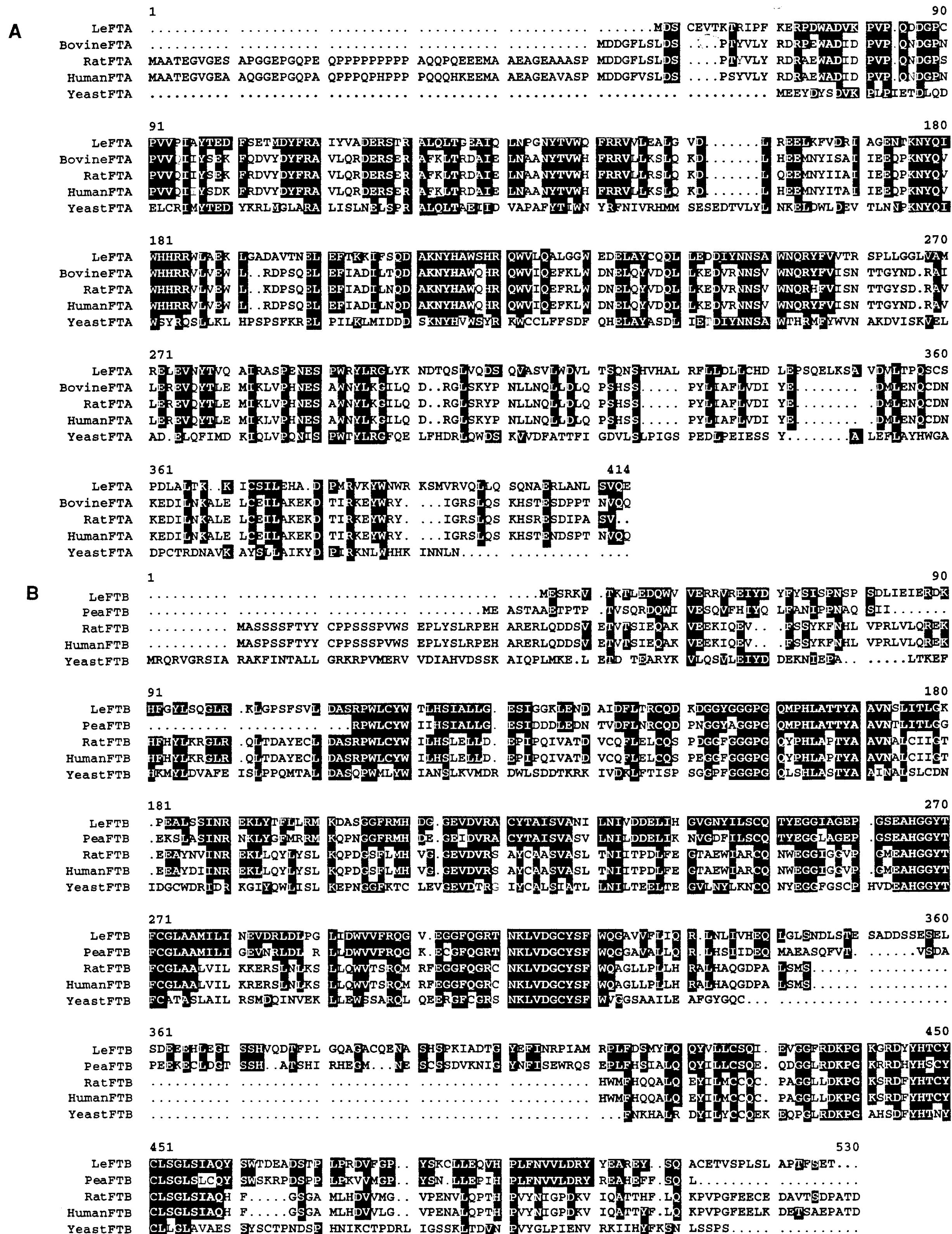

FIG. 1. Amino acid sequence alignments of protein farnesyl transferase α and β subunits. FTase α (A) and FTase β (B) sequence alignments were established by using the best-fit algorithm (GCG; University of Wisconsin). Amino acid sequence identities between LeFTA and LeFTB and their homologs from plants, yeast, and mammals are indicated by black boxes. Dots denote gaps formed by the alignment algorithm. *LeFTA* was isolated as a 1.5-kb clone with a 1,050-bp open reading frame. *LeFTB* was isolated as a 1.8-kb clone with a 1,443-bp open reading frame. Low-stringency genomic DNA analysis indicates that both *LeFTA* and *LeFTB* exist in a single-copy form in the tomato genome. RNA blot analysis suggests that both *LeFTA* and *LeFTB* clones represent the full-length cDNAs (data not shown). The GenBank accession numbers for the aligned sequences of the farnesyltransferase α subunit are as follows: LeFTA, U83707; bovine FTA, M74083; rat FTA, M81225 and M81450, human FTA, L00634; and yeast FTA, M88584. The GenBank accession numbers for the aligned sequences of the farnesyltransferase β subunit are as follows: LeFTB, U83708; pea FTB, L08664; rat FTB, M69056; human FTB, L10414; and yeast FTB, M22753.

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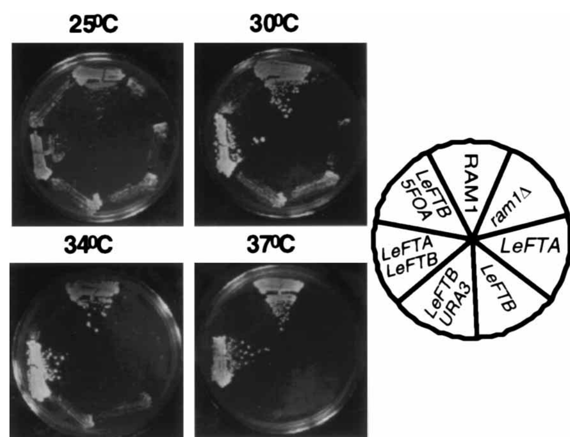

FIG. 2. Restoration of growth in a yeast *ram1*Δ strain by LeFTase. Restoration of growth at 37°C by *LeFTA* and *LeFTB* occurs following their transformation into a *ram1*Δ yeast strain. *S. cerevisiae* CTY716 (*MAT*α *ade2 lys2 his3 trp1 leu2 ura3 ram1*Δ::*ADE2*) (*ram1*Δ) was transformed with plasmids carrying the *LeFTA* or *LeFTB* gene (pSYYFTA1 and pSYYFTB2, respectively) to produce strains SYY102 (*LeFTA*), SYY100 (*LeFTB*), SYY101 (*LeFTB URA3*), and SYY105 (*LeFTA LeFTB*) (see Materials and Methods and Table 1). To verify that the growth phenotype of SYY105 was plasmid dependent, SYY105 was grown on 5-fluoro-orotic acid (5FOA) to select for cells that had lost the *LeFTA URA3* plasmid (pSYYFTA1) (*LeFTB* 5FOA). *ura3* strains can grow on media containing 5FOA, but *URA3* strains cannot (6, 7). All strains were grown at either 25, 30, 34, or 37°C, together with a congenic *RAM1* strain, PHY500 (*MAT*α *ade2 lys2 trp1 leu2 ura3*) (RAM1), on rich (YPD) medium plates (4).

of *ram1*Δ cell growth by LeFTase occurs via a conserved mechanism which involves Ras protein farnesylation and localization to the membrane, the subcellular localization of Ras was determined. As expected from the growth results shown in Fig. 2, expression of *LeFTB* alone did not affect Ras membrane localization (Fig. 3, *LeFTB*+p*URA3* and *ram1*Δ). However, in *ram1*Δ cells coexpressing *LeFTA* and *LeFTB*, greater than 95% of Ras1p and Ras2p cosedimented with the insoluble membrane fraction (Fig. 3, *LeFTA*+*LeFTB*), as observed for *RAM1* cells (Fig. 3, RAM1).

Mating type a *ram1*Δ mutant yeast strains complemented with LeFTase produce a functional a-factor mating pheromone. *MAT***a***ram1*Δ yeast cells are unable to mate because the **a**-factor mating pheromone is not farnesylated and therefore is not exported (45). *MAT***a***ram1*Δ cells coexpressing *LeFTA* and *LeFTB* were able to export biologically active **a**-factor, as determined by their ability to mate with *MAT*α cells (Fig. 4A, *LeFTA LeFTB*) in a mating assay, and to inhibit growth of *MAT*α cells in pheromone diffusion (halo) assays (Fig. 4B, *LeFTA*+*LeFTB*). The growth inhibition halos formed around LeFTase-complemented *ram1*Δ cells were considerably smaller than those around *RAM1*-complemented *ram1*Δ cells (Fig. 4B, compare *LeFTA*+*LeFTB* and p*RAM1*), suggesting that the **a**-factor mating pheromone is prenylated less efficiently by the plant FTase. As expected, expression of *LeFTB* alone did not restore **a**-factor export (Fig. 4, *LeFTB*+*pURA3*), confirming that yeast FTase α and LeFTB cannot form an active FTase.

LeFTase produced in yeast or insect cells can farnesylate various CaaX-box-containing peptides. Protein extracts prepared from *ram1*Δ cells expressing LeFTA and LeFTB contained FTase activity capable of farnesylating peptides corresponding to **a**-factor mating pheromone (**a**-factor), Ras2p (Ras2), and ANJ1 (the plant homolog of the bacterial molecular chaperone DnaJ) (Fig. 5A and B, LeFTA/B; Fig. 5C, yeast cells, LeFTA/B). LeFTase was able to farnesylate Ras2L and **a**-factor L, peptides in which leucine was substituted for the C-terminal serine or alanine, respectively (Fig. 5A and B, **a**-factorL Ras2L, respectively). CaaX proteins with a C-terminal leucine are preferred substrates of GGTase-I (44). Neither RAS2L nor **a**-factor L was farnesylated by protein extracts prepared from *ram1*Δ yeast cells, indicating that labeling of peptides with [^{3}H]FPP is solely due to the activity of FTase and not to GGTase-I. Plant, yeast, and mammalian FTases have similar abilities to farnesylate CaaX substrates with C-terminal serine, glutamine, or cysteine residues, and each enzyme can farnesylate CaaX substrates terminating with leucine (CaaL) with low efficiency in vitro (31, 32, 51). Consistent with the genetic complementation of mating in *MAT***a***ram1*Δ cells, LeFTase could farnesylate **a**-factor (Fig. 5A), in contrast to mammalian FTase, which is inefficient at farnesylating **a**-factor (42). Coexpression of *LeFTA* and *LeFTB* in baculovirus-infected *Sf*9 cells (Fig. 5C, co-infected) or reconstitution of LeFTase with extracts from cells expressing either *LeFTA* or *LeFTB* (Fig. 5C, reconstituted) resulted in farnesylation of the ANJ1 peptide, confirming the ability of these subunits to form an active plant enzyme.

The occurrence of multiple products following farnesylation assays and TLC separations of the products that were carried out under exactly the same conditions were previously analyzed and reported (51). The multiple spots in Fig. 5A, B, and C are due to degradation of the farnesylated peptides by proteases originating from the protein extracts, which were tested for FTase activity. Since the shorter peptides are more hydrophobic, products with different hydrophobicities were produced, and these were consequently separated on the TLC plate.

Earlier studies demonstrated that mammalian FTase can bind GGPP but is unable to use it as a prenyl group donor in the prenylation reaction (39). More recent studies have demonstrated that recombinant human FTase can geranylgeranylate ras-CVIM substrate but is unable to geranylgeranylate other substrates, which contain different CaaX boxes (37). Although yeast FTase preferentially uses FPP, it can use GGPP as a prenyl donor (10, 51). In this study, under the conditions in which reactions were performed and with peptides that were tested, LeFTase could not utilize GGPP to prenylate proteins in vitro, including the ANJ1 peptide substrate (Fig. 5D, LeFTA/B). When the RAS2L peptide was used in the prenylation reaction together with [^{3}H]GGPP, weak spots were detected following long exposures (1 week) of the films to the TLC plates, even in the absence of LeFTase (data

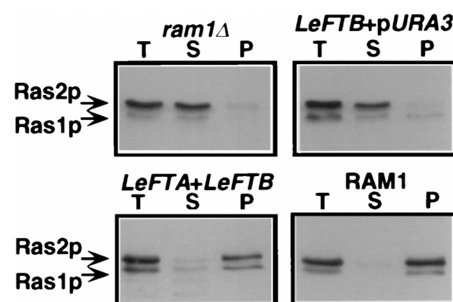

FIG. 3. Western blot analysis of intracellular localization of Ras1p and Ras2p in yeast cells. Yeast strains CTY716 (*ram1*Δ), PHY500 (RAM1), SYY101 (*LeFTB*+p*URA3*), and SYY105 (*LeFTA*+*LeFTB*) (see Fig. 2) were lysed (T) and then fractionated into soluble (S) and insoluble (P) fractions as described in Materials and Methods. Proteins in each fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (see Materials and Methods). Ras1p and Ras2p (arrows) were identified by Western blotting with anti-v-H-Ras monoclonal Ab (see Materials and Methods).

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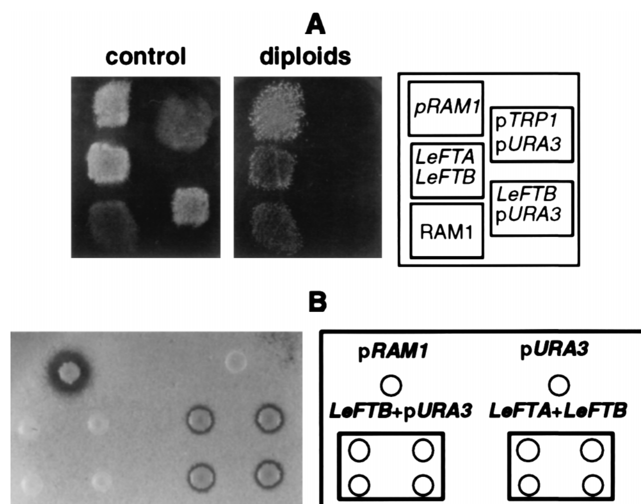

FIG. 4. Restoration of mating and of biologically active **a**-factor production in a yeast *ram1*Δ strain by LeFTase. (A) Mating assays. Strain JRY3387 (*MAT***a** *ade2 trp1 ura3 lys2 his4 ram1*Δ::*HIS3* [possibly *his3*]) was transformed with plasmids to form strains JRY3401 (p*RAM1*), JRY 3455 (p*TRP1* p*URA3*), SYY111 (*LeFTB* p*URA3*), and SYY115 (*LeFTA LeFTB*) (see Table 1). Strains were grown on minimal medium supplemented with His, Trp, Ade, and Lys and then were replicated onto a fully supplemented minimal-medium (YM) plate (control) and onto a YM plate that had been supplemented with His and Trp and had been spread with a lawn of JRY5269 (*MAT*α *his4 leu2 trp1 ura3*) (diploids). Only diploids are able to grow on the latter plate due to the absence of Ade, Lys, Leu, and Ura. JRY254 (*MAT***a***lys1*) (RAM1) was used as an additional positive control. (B) Pheromone diffusion (halo) assays. When the **a**-factor mating pheromone is processed and exported, it arrests growth of *MAT*α *sst2* cells, thereby producing a zone of growth inhibition (halo). The size of the halo is proportional to the amount of **a**-factor produced. Pheromone diffusion (halo) assays were carried out as described in Materials and Methods. JRY3387 cells (*MAT***a** *ade2 trp1 ura3 lys2 ram1*Δ::*HIS3*) were transformed with plasmids carrying *RAM1* and *URA3* (pJR856), *URA3* (pSEY8), *LeFTB* and *URA3* (pSYYFTB2 and pRH98-3, respectively), and *LeFTA* and *LeFTB* (pSYYFTA1 and pSYYFTB2, respectively) to form strains JRY3401 (p*RAM1*), JRY3399 (p*URA3*), SYY111 (*LeFTB*+ p*URA3*), and SYY115 (*LeFTA*+*LeFTB*), respectively (see Materials and Methods and Table 1).

not shown). The labeling of the peptide were likely due to the activity of the yeast GGTase-I. The reaction conditions, however, were unfavorable for GGTase-I, and therefore its activity is hardly detected.

LeFTB is unstable but becomes stabilized when it is coexpressed with LeFTA. The two subunits of FTase form very stable heterodimers; however, either subunit when expressed by itself is less stable (13, 24). To assess whether the same holds true for the plant FTase, the levels of LeFTB in protein extracts that were prepared from *Sf*9 either coexpressing LeFTA and LeFTB or expressing LeFTB alone were determined by immunoblot analysis with affinity-purified anti-LeFTB antibody (Fig. 6). Significantly higher levels of LeFTB were detected when it was coexpressed with LeFTA than when it was expressed by itself (Fig. 6; compare LeFTA LeFTB with LeFTB).

A mutated form of LeFTB missing the unique 66-amino-acid domain forms an active FTase with LeFTA in yeast. FTB subunit proteins from plants contain a novel 66-amino-acid domain which is absent from the yeast and mammalian FTase-β subunit proteins (Fig. 1B). An in-frame deletion mutant of this sequence domain was constructed to assess its function (Fig. 7A). Coexpression of LeFTBΔ66 with LeFTA in a yeast *ram1*Δ mutant strain resulted in complementation of the growth defect phenotype, allowing the cells to grow at 37°C and to grow at a higher rate than cells transformed with LeFTA or a vector control at 25°C (Fig. 7B; compare LeFTA

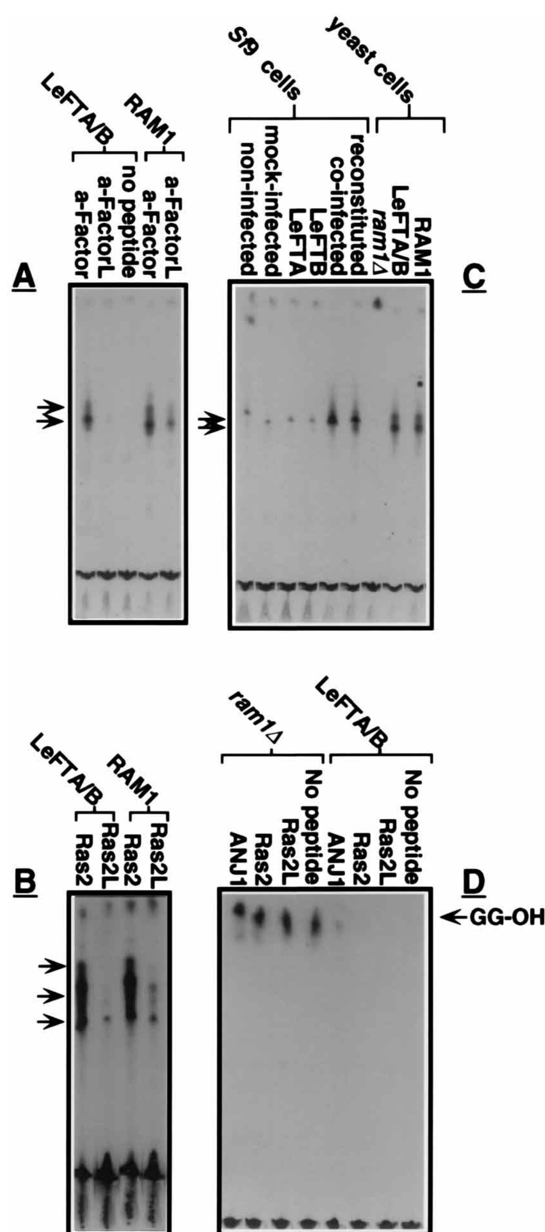

FIG. 5. Farnesylation of CaaX-box-containing peptides by LeFTA and LeFTB expressed in either yeast or baculovirus-infected insect cells. Protein extracts were prepared and farnesylation reactions were performed as described in Materials and Methods. (A) Reactions performed with **a**-factor, with **a**-factor L peptides (YIIKGVFWDPACVIA/L, respectively), and without peptide. (B) Reactions performed with Ras2 and Ras2L peptides (KKSGSGGSCIIS/L, respectively). (C) Reactions performed with ANJ1 peptide (KQRVQCAQQ), using protein extracts prepared from yeast strains CTY716 (*ram1*Δ), SYY105 (LeFTA/B), and PHY500 (RAM1). Using the same conditions, reactions were performed with protein extracts prepared from baculovirus-infected *Sf*9 cells (*Sf*9 cells). The locations of the labeled peptides are marked with arrows. The type of extract used in each individual reaction is marked. Co-infected, cells infected with both *LeFTA*- and *LeFTB*-containing viral stocks; reconstituted, extracts from *LeFTA* and *LeFTB* viral-stocks-infected cells were mixed at a 1:1 ratio. (D) Reactions performed with [^{3}H]GGPP. Reactions were performed with protein extracts prepared from either CTY716 (*ram1*Δ) or SYY105 (LeFTA/B), with the peptide substrates indicated on the figure. Reactions were performed under the same conditions described above except that [^{3}H]GGPP was used instead of [^{3}H]FPP.

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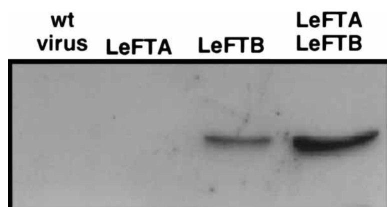

FIG. 6. Level of LeFTB expression in baculovirus-infected *Sf*9 cells as detected with affinity-purified α-LeFTB Ab. Protein extracts (see Materials and Methods) were prepared from cells infected with either wt virus or viral stocks containing either LeFTA or LeFTB or coinfected with LeFTA and LeFTB viral stocks. Ten micrograms of protein from each fraction was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29) and electrotransferred onto a nitrocellulose membrane. The membrane was stained with Ponceau S (Sigma) to make sure that the transfer of proteins was uniform. The membrane was probed with affinity-purified anti-LeFTB antibody (see Materials and Methods) at a dilution of 1:10,000 and then with HRP-conjugated goat anti-rabbit IgG as a secondary Ab. Blots were developed with an ECL kit (Amersham). Exposure time was a few seconds.

LeFTBΔ66 to LeFTA pTRP1). Complementation of the *ram1*Δ phenotype was lost when the cells lost the plasmid expressing LeFTBΔ66, confirming that it was dependent upon the presence of both LeFTA and LeFTBΔ66 in the cells (data not shown). Although the LeFTase formed with mutated LeFTBΔ66 was functional in yeast, it was either less active or less stable than wt LeFTase. This is apparent in the slower growth rate of cells transformed with the mutated LeFTase compared with that of cells transformed with the wt LeFTase. Expression of LeFTBΔ66, without LeFTA, in *ram1*Δ cells did not result in complementation of the growth phenotype of these strains, although the mutated plant protein more closely resembles the Ram1p protein of yeast (data not shown).

DISCUSSION

Sequence comparisons of FTase α and β subunits from plants, yeast, and mammals reveal that the plant and the yeast proteins are 33% identical and are somewhat more similar than the yeast and mammalian proteins. However, the plant and mammalian proteins are 45% identical and are much more closely related to each other than to the yeast FTase subunits. Interestingly, the degrees of conservation between the two plant FTase β subunit proteins and between the plant proteins and the mammalian FTase β subunit are similar. Moreover, the degree of conservation among the FTase α subunit proteins from plants, yeast, and mammals is similar to the degree of conservation found for the FTase β subunits. This implies that FTases have undergone concerted evolution of their amino acid sequences in evolutionarily divergent organisms. The structural differences between the yeast and plant FTase subunits are probably the reason for the inability of LeFTB to form an active heterodimeric FTase complex with the yeast Ram2 protein. At the same time, these apparent structural differences between the FTase subunit proteins should facilitate structure-function analysis to identify determinants necessary for the correct interaction of the two subunits to form an active enzyme. With the FTase sequences now available for three evolutionarily divergent eukaryotes, this analysis will be informative because crystallization of FTase has not yet been achieved. We have not addressed the functional conservation between the plant and mammalian FTases in the present study. Therefore, it was not possible to determine whether the conservation in the primary structure of these two proteins has a functional significance enabling the formation of an active FTase from the plant and mammalian subunits.

Based on the ability of the plant FTase to restore growth and mating to yeast strains lacking endogenous FTase, we can infer

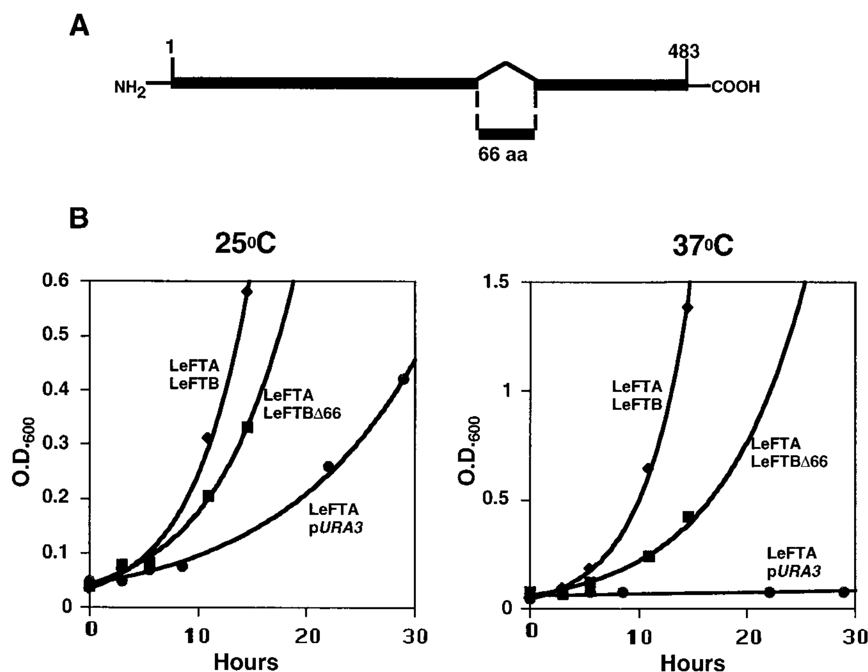

FIG. 7. A mutated form of LeFTB in which a unique 66-amino-acid domain was deleted restores growth in yeast *ram1*Δ mutant strains. (A) Schematic presentation of the deletion in LeFTB. (B) Growth curves. Duplicate cultures of cells expressing LeFTA alone (LeFTA p*URA3*), wt LeFTase (LeFTA LeFTB), or mutated LeFTase (LeFTA LeFTBΔ66) were grown as explained in Materials and Methods. At the indicated time points (0, 3, 5, 8.5, 11, 12, 14.5, 22, and 29 h), samples were withdrawn and their optical densities at 600 nm were measured. The results obtained with one culture are shown, and similar results were obtained for an independent culture.

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that the plant enzyme was able to farnesylate all essential yeast CaaX substrates (including Ras proteins and Ydj1p) and **a**-factor mating pheromone. The lower level of production of **a**-factor by a *ram1*Δ mutant yeast strain complemented with LeFTase (as apparent from the halo assays described in Fig. 4B) implies that the plant and yeast enzymes have somewhat different substrate specificities. Taken together, the data demonstrate that the yeast and plant FTases are functionally conserved although they are extremely divergent in amino acid sequence. This functionality suggests that the overall tertiary and quaternary structures of yeast and plant FTases are likely similar or that only a limited number of conserved amino acid residues in both the α and the β subunits determine substrate specificity. Most likely, the sequence divergence among the FTases also reflects differences in substrate specificity and in the biochemical regulation of the enzyme in mammals, yeast, and plants. This view is consistent with the observation that the novel 66-amino-acid sequence domain in plant FTB is not essential for the functional complementation of the *ram1*Δ growth defect.

LeFTase was unable to transfer GGPP to CIIS and CAQQ CaaX-box-containing peptides, which correspond to Ras2 and Anj1, respectively. LeFTase may utilize GGPP as a prenyl group donor if provided with a different CaaX-box peptide, as has been shown for the human FTase (37). In contrast, the yeast FTase can use GGPP as a prenyl group substrate in the prenylation reaction with a variety of peptide substrates, albeit inefficiently (10, 51). Thus, in multicellular eukaryotes, FTases appear to have evolved a higher degree of specificity with regard to the prenyl group acceptor substrate. Under in vitro conditions, FTases from yeast, plants, and mammals can farnesylate proteins with carboxy-terminal CaaL motifs, which are substrates of GGTase-I. These reactions are very inefficient, and their in vivo significance has not been determined (42, 51). It should be noted that in vitro studies of the yeast FTase did not detect geranylgeranylation of cysteine residues located within CaaL sequence motifs (10, 51).

LeFTase was still active following the deletion of the 66-amino-acid domain from LeFTB (Fig. 7). It can be concluded, therefore, that the mutant form of LeFTase was capable of prenylating all essential yeast proteins required for growth at 37°C, i.e., Ras1p, Ras2p, and Ydj1p. The 66-amino-acid domain, which is found only in FT β subunit proteins from plants, is not essential for FTase activity, indicating that it is likely not part of the active site and is not essential for protein-protein interactions between the α and the β subunits. The role of the 66-amino-acid domain in the β subunit of plant FTase is presently unknown. It is apparent that it is required for either full activity or stability of the holoenzyme since *ram1*Δ cells that were complemented with the mutated LeFTase had a slower growth rate than cells that were complemented with the wt enzyme. It is also possible that the novel 66-amino-acid domain in the plant FTase β subunit has a regulatory role for the enzyme in vivo, although it remains to be determined whether LeFTBΔ66 can form an active FTase in plants.

In this paper we report the complete sequence of the protein farnesyl transferase from plants and demonstrate the function of the enzyme both in vivo and in vitro. The cloning of the full-length *LeFTA* and *LeFTB* cDNAs, together with their ability to functionally complement the yeast *ram1*Δ mutant, allowed us to establish unequivocally that *LeFTA* and *LeFTB* encode the α and β subunits of plant FTase. During the preparation of our manuscript, a deletion mutant of the putative FTase β subunit of FTase from *Arabidopsis thaliana* (AtFTB) was reported (14). The *Arabidopsis* FTB mutant was first identified in a screen for mutants that are hypersensitive to the plant hormone abscisic acid and was later shown to have a delayed germination phenotype as well. The isolation of this mutant should greatly facilitate the study of protein the FTase in plants. It appears, however, that in the sequence reported for AtFTB a stretch of approximately 40 amino acids is missing in the amino-terminal region of the protein (14). Since this sequence domain is present in LeFTB and PsFTB (53), it is very likely that AtFTB also has this domain. Since the AtFTB cDNA was not used to determine if the protein can complement the abscisic acid and germination phenotypes, it is unknown at present whether the AtFTB cDNA encodes a functional FTase β subunit of AtFTase.

The molecular and biochemical tools described here will facilitate studies of protein farnesylation and its role in the regulation of growth in plants. In addition, since plant GGTase-I is likely to share a common α subunit with plant FTase, the FTase α subunit gene should aid the cloning of a plant GGTase-I β subunit gene, which will facilitate studies of the role and regulation of protein geranylgeranylation in plants.

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